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In Vitro Propagation of *R. maddeni* Hook. F. an Endangered Rhododendron Species of Sikkim Himalaya

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Abstract

A protocol is described for rapid and large scale propagation of an endangered, important Sikkim Himalayan rhododendron (*R. maddeni* Hook. f.) by *in vitro* culture of cotyledonary nodes from 15 days old seedlings. Several cytokinin types were evaluated for their effect on shoot multiplication from cotyledonary nodes. Maximum numbers of shoot (12.00 ±0.58) were observed on the AM containing 7 mg/l 2iP, 0.1 mg/l IAA after eight weeks of culture. Incorporation of 0.1 mg/l IAA in the medium during the first subculture after establishment and initiation of shoot buds significantly improved the shoot elongation. Regenerated shoots were separated and rooted on same strength AM medium supplemented with 0.2 mg/l of IBA alone for three weeks. Well-developed complete plantlets were transferred on to specially made plastic cup containing soilrite. The rooted plantlets were hardened and successfully established in greenhouse, the plants were transferred to field site at Pangthang arboretum of the Institute and the 'Rare & Threatened Plant Conservation Park' of Zoological Park, Gangtok, Sikkim.

Keywords: multiple shoot, *in vitro* propagation, *Rhododendron maddeni*, Anderson medium, isopentenyladenine, indole-3-acetic acid, indole-3-butyric acid, naphthalene acetic acid

Introduction

Rhododendron is one of the largest genus of the Ericaceae family, occurring in the higher altitudes having ecological significance and economic importance in addition to its graceful flowers. Rhododendron covers a vast section of southeastern Asia between the northwestern Himalaya through Nepal, Sikkim, eastern Tibet, Bhutan, Arunachal Pradesh, and upper Burma, western and central China. The genus Rhododendron, having about 80 species in India, mainly distributed in the Himalaya region (one species R. nilagiricum in South India), out of which a total of 36 species with 45 different forms including subspecies and varieties occur in Sikkim alone (Pradhan and Lachungpa, 1990; Singh *et al.*, 2003; Bhattacharyya and Sanjappa, 2008). Rhododendron maddeni, the only rhododendron in Sikkim Himalaya with fragrant flowers, is facing danger of extinction. However, *R. maddeni* generally occur in regions under intensive environmental disturbance, which leads to habitat destruction and genetic erosion (Singh, 2008). Urgent conservation measures are required to maintain the availability of these species.

There are sufficient reports available about the protocols on *in vitro* micropropagation of many rhododendron species, mostly Azalea group (Fordham *et al.*, 1982; Hsia and Korban, 1997) but the *in vitro* protocol for *R. maddeni* has not been elucidated so far. The rhododendron species are propagated by vegetative means as well as through seeds (Singh *et al.*, 2008a, b). The rate of vegetative propagation is very slow in many rhododendron species and seed germination in nature is also very poor. Micropropagation is an ideal technique for cloning the elite germplasm and conservation of rare and threatened rhododendron species of the region. Tissue culture is the only method to maintain and propagate the genetically identical clone rapidly in large numbers and in long term culture.

The present paper has a great relevance from the conservation point of view and we highlight an efficient and reproducible procedure for the large scale propagation of *R. maddeni* through tissue culture techniques has been described. The procedure will not only ensure mass scale availability of planting material of an endangered *R. maddeni* Hook.f. a scented flower, which are in great demand, but will also help in conservation of important genetic resources.

Materials and methods

The seeds of R. maddeni were obtained from Rate-chu, East Sikkim (Longitude 27o5'47" to 28o8'50" North, and Latitude 88o56'27" to 88o56'25" East with an elevation 2500 m amsl) between, October-November. Seeds were

thoroughly washed with a detergent (Tween -80; 1.0%, v/v; 20 min), and surface sterilized in 0.15%; w/v mercuric chloride ($HgCl_2$) for 3 min, subsequently washed 5 times in sterile distilled water and implanted on hormone free MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.8% (w/v) agar in culture tubes (Borosil Glass Works Ltd., Mumbai, India) for germination. Cotyledonary node and shoot tip obtained from in vitro derived seedlings were used in this experiment.

Culture medium

Modified AM (Anderson, 1984) medium for shoot induction and multiplication was used. In the experiments, five concentrations 1, 2, 5, 7, 10 mg/l 2iP were used in the induction and multiplication medium, either separately or in combination with IAA in the concentration 0.1 mg/l (Tab. 1).

Multiple shoot formation

To screen for an optimal shoot multiplication medium (Tab. 1), nodal segment explants (1.0-1.5 cm in length) from 15 - day-old aseptic seedlings of *R. maddeni* were cultured on the AM medium (Anderson, 1984) containing various concentrations of of 2iP along with additives (100 mg/l PVP, 100 mg/l ascorbic acid and 10 mg/l citric acid) in the primary set of experiments. In the second set, the effect of 2iP (1 – 10 mg/l) and IAA (0.1 mg/l) in different combinations and concentrations was tested. The pH was adjusted to 5.6 and gelled with phytagel (0.3%) before autoclave at 121°C for 15 minutes at 1.05 kg/cm^2 pressure. The cultures were maintained at 17 ±1 °C temperature and 60% relative humidity under 16 hr/8 hr (light/ dark) photoperiod and a light intensity of 60 μmol m^{-2} s^{-1} photon flux provided by cool-white fluorescent lamps. Regenerated shoots were cultured onto AM medium supplemented with 7 mg/l 2iP along with additives (100 mg/l PVP, 100 mg/l ascorbic acid and 10 mg/l citric acid). The culture condition was the same as described above. Sub culturing was carried out at regular intervals for the formation of large number of shoots from single culture.

Rooting and hardening

The shoots of 4-6 cm length were isolated from the shoot clump and kept for rooting in AM. Anderson medium containing 0.2 mg/l IBA (optimum concentration) and activated charcoal (500 mg/l) were used for filter paper bridge technique using liquid medium. The *in vitro* grown plantlets were then transferred to greenhouse under 70% shade and planted in thermocole cups containing a mixture of autoclaved fresh peat moss and soil (1:3) enriched with Anderson nutrient salts and placed for hardening under high relative humidity (80%) in the mist chamber of greenhouse (25°C) at the Pangthang (2047m amsl of the Institute).

Data analysis

All experiments were repeated two times and 10 replicates were employed for each treatment. 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).

Results and discussion

The sterilized seeds of *R. maddeni* were found to germinate within 3 weeks of inoculation on hormone-free MS medium. Nodal explants excised from 15-day-old seedlings of *R. maddeni* were cultured on AM medium with five different concentrations (1, 2, 5, 7, 10 mg/l) of 2iP to induce multiple shoots. 2iP proved a more effective cytokinin than BA, kinetin for multiple shoot induction (data not shown). Although BA has often been reported to stimulate regeneration and proliferation in some rhododendron species (Cantos, 2007; McCown and Lloyd, 1982). A varying degree of multiple shoot formation was observed at lower concentrations of 2iP whereas a higher concentration (10 mg/l) led to the formation of callus. Out of various concentrations of 2iP tested, AM medium containing 7 mg/l 2iP and 0.1 mg/l IAA proved the best with a maximum of 12.00 ±0.58 shoots per nodal explant (Tab. 2; Fig. 1A, B). Incorporation of IAA (0.1 mg/l) in the medium during the subculture after establishment and initiation of shoot buds significantly improved the shoot elongation. A regular subculture in every 4 weeks increased the multiplication rate which became maximum after three to four subculture cycles. After 8 to 9 weeks, when regenerated shoots reached a length of more than 5 cm, they were separated and placed on AM medium containing IBA and activated charcoal.

In cultures, where the shoots were inoculated on auxins free AM medium, no root formation was observed. Whereas root primordial emerged from the shoot base on second week of culture on auxin-supplemented medium. In the presence of auxins the shoots produced root in all four concentrations, but IBA was found more suiTab. for root induction than IAA and NAA. Maximum number roots (6.3 ±0.33) were produced from IBA at 0.2 mg/l (Tab. 3; Fig. 1C, D). According to Jutta (2000) transport velocity of IBA was markedly slower as compared to other growth regulators. Higher concentration of auxin lowered the rooting percentage as well as root number. Plantlets with fully expanded leaves and well developed roots were washed with sterile distilled water then dipped in systemic fungicide (bavestin, 0.15%, w/v; 20 min) and planted 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

Fig. 1. *In vitro* propagation of Rhododendron maddeni: (A) germinating seeds in MS medium; (B) multiplication of shoots on AM medium with 2iP (7 mg/l); (C) and (D) root induction from in vitro regenerated shoot on liquid AM medium with IBA (0.2 mg/l)

greenhouse (25°C). After one month these were planted in polythene bag containing normal garden soil (Figu 2A, B). The regenerated plants showed 96% survival during hardening and acclimatization and there were no observable variations between the parent plants and *in vitro* raised plants. The micropropagated plants, following hardening and establishment in the greenhouse were transferred to the field at Arboretum of the Institute (Fig. 2C) and unique kind of Rare and Threatened Plant Conservation Park of Himalayan Zoological Park, Bulbulay-Gangtok, Sikkim (Singh *et al.*, 2008b). The transplanted plantlets established well in pots and in the field. The *in vitro* regeneration of shoots, rooting and soil establishment protocol in this study suggests that there is possibility of adopting tissue culture techniques for mass propagation of rhododendron spp. This achievement can be applied to commercial mass production and provides a uniform product quality. Considering the status of this species in terms of its distribution, threats and conservation values in the study area, its *in vitro* propagation will compliment and strengthen the large-scale plantation activities towards the conservation or restoration of rhododendron forests.

Tab. 1 Composition of modified AM Medium

Medium compounds	(Mg/l)
Macroelements	
NH_4NO_3	400
KNO_3	480
$CaCl_2.6H_2O$	440
$NaH_2PO_4.2H_2O$	380
$MgSO_4.7H_2O$	180
Microelements	
Na_2EDTA	74.5
$FeSO_4.7H_2O$	55.7
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
KI	0.30
$MnSO_4.4H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
Vitamins	
Myo-Inositol	20
Thiamine	200
Nicotinic acid	100
Pyridoxine	100
Plant growth regulators and other components	
Isopentenyladenine	1, 2, 5, 7, 10
Indole-3-acetic acid	0.1
Polyvinylpyrrolidone	150
Ascorbic acid	100
Citric acid	10
Charcoal	500
Sucrose (%)	3
Phytagel Sigma (%)	0.3
pH	5.6

Conclusions

In vitro propagation of plantlets presented in this paper could open a new way of making large-scale production of *R. maadeni.* The success of the system lies in the development of suiTab. regeneration procedure of such important and difficult to propagate elite species. This *in vitro* regenerated of shoots, rooting and soil establishment protocol in this study suggests that there is possibility of adapting tissue culture technique to mass propagation of endangered fragrant *R. maadeni* plant. Several plantlets could be regenerated and the numbers would increase with repeated subcultures. This achievement can be applied to commercial mass production, germplasm conservation and provides a uniform product quality.

Tab. 2. Effects of 2iP combined with IAA on shoots proliferation from cotyledonary node of *R. maddeni* on AM medium.

Plant growth regulators (mg/l)		Frequency (%) of explants producing shoots	Mean no. of shoots	Mean shoots length (cm)
2iP	IAA			
1	-	39 ±1.00	7.33 ±0.33	1.20 ±0.07
1	0.1	45 ±1.73	8.00 ±0.58	2.80 ±0.15
2	-	47 ±2.86	8.33 ±0.67	1.80 ±0.10
2	0.1	50 ±1.73	9.00 ±0.58	2.90 ±0.06
5	-	52 ±1.54	8.67 ±0.33	2.72 ±0.27
5	0.1	56 ±1.15	10.00 ±1.73	3.14 ±0.26
7	-	65 ±1.00	9.33 ±0.33	3.70 ±0.57
7	0.1	70 ±1.15	12.00 ±0.58	4.64 ±0.25
10	-	54 ±2.30	7.00 ±1.00	3.80 ±0.26
10	0.1	55 ±0.33	8.00 ±1.54	3.75 ±0.20
LSD at the 5% level		52.27	7.15	2.68

Each treatment consisted of 10 replicates and the experiment was repeated twice. Values are the mean ± SE of two independent experiments.
Data were recorded 12 weeks after transfer to MS medium.

Fig. 2. (A) Plantlets established on fresh peat moss and soil (1:3), under in vitro condition; (B) Acclimatized plants in a greenhouse; (C) Plantation of hardened plants in the Rare & Threatened Plant Conservation Park' of Zoological Park, Gangtok, Sikkim

Tab. 3. Effect of different concentration of auxins incorporated in AM medium on rooting of regenerated shoots of *R. maddeni.*

Auxins (mg/l)			Rooting (%)	Mean no. of roots/ shoot	Mean root Length (cm)
IAA	AA	IBA			
0.1	-	-	40	3.7 ±0.33	0.60 ±0.05
0.2	-	-	38	4.0 ±0.57	0.80 ±0.57
0.5	-	-	55	3.3 ±0.88	0.46 ±0.07
1.0	-	-	40	2.7 ±0.33	0.70 ±0.06
-	0.1	-	42	2.7 ±0.33	0.83 ±0.12
-	0.2	-	60	3.0 ±1.15	1.35 ±0.05
-	0.5	-	78	3.3 ±0.33	1.28 ±0.05
-	1.0	-	30	2.3 ±0.67	1.35 ±0.06
-	-	0.1	45	5.3 ±0.33	1.40 ±0.04
-	-	0.2	93	6.3 ±0.33	1.68 ±0.04
-	-	0.5	80	5.3 ±0.67	1.30 ±0.8
-	-	1.0	47	4.7 ±0.33	1.32 ±0.16
LSD at the 5% level				2.30	1.24

Each treatment consisted of 10 replicates and the experiment was repeated twice. Values are the mean ± SE of two independent experiments. Data were recorded 9 weeks after transfer to liquid-AM medium.

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