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Research Article

IN VITRO PROPAGATION OF *RHODODENDRON NIVEUM* HOOK F (STATE TREE OF SIKKIM) AN ENDANGERED RHODODENDRON SPECIES OF SIKKIM HIMALAYA

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ABSTRACT
An efficient protocol for plant regeneration through multiple shoots induction from shoot tips of *Rhododendron niveum* was established. The highest percentage (68±1.15) of multiple shoot induction and number of shoots (8.66±0.57) per explants were found on AM supplemented with 5.0 mg/l 2-isopentyladenine (2iP). Then the 2ip (5.0 mg/l) combined with 0.1 mg/l IAA (Indole acetic acid) was found to be more suitable for getting more number of shootlets (9.00±0.33). The induced shoots were excised and inoculated on to liquid AM containing different concentrations of NAA or IBA on Filter Paper Bridge for rooting. The highest percentage (70) of root induction and the highest number of roots per shoot (6.67 ± 0.33) was found on liquid AM having 0.2 mg/l IBA. Well rooted plantlets were transferred to small polythene bags containing peat moss and soil (1:3) and maintained with a high humidity for acclimation. This is the first report for in vitro regeneration of *R. niveum* where large number of plant have been successfully produced and maintained in polyhouse/misthouse. 70% of the plants survived and all were morphologically normal.

Key Words: *Micropropagation, Shoot Formation, Rooting, Acclimatization, Rhododendron Niveum, Sikkim Himalaya.*

INTRODUCTION
Rhododendrons are amongst the dominant species along the temperate, subalpine and alpine zones in the Sikkim Himalaya. *Rhododendron* L. is one of the largest genus of the Ericaceae family, occurring in the higher altitudes with great ecological significance and economic importance, in addition to their splendiferous flowers. It has aesthetic, sacred, aromatic, medicinal and fuel wood values. The genus *Rhododendron* (ca. 1000 sp. worldwide) has the greatest diversity record in the Sino-Himalayan Mountains. This rhododendron habitat spreads within a short arc covering the highlands of Nepal, India and China (east of Yunnan and Sichuan). The genus *Rhododendron* is represented by about 85 species in India mainly distributed in the Himalayan region (one species, *R. nilagiricum* in South India). Out of this, a total of 36 species with 45 different forms, including subspecies and varieties, occur in Sikkim alone (Gamble, 1936; Cowan and Cowan, 1938; Sastry and Hazra, 1983; Pradhan and Lachungpa, 1990; Kumar *et al.*, 2004; Bhattacharyya and Sanjappa, 2008). Steady decline in the number of species and drastic changes in their natural habitat, as well, as in some cases, complete population annihilation in the wild has become a strong issue of concern, and a compelling reason to start work on the conservation of rhododendrons in the region. The rise in human population with demand on land for farming, increased animal husbandry practices, construction of roadways, hydel power projects and allied works, and of late the tourist influx have collectively resulted in building up of considerable pressure on the very survival of *Rhododendron* species. Various reports are available on the *Rhododendrons* research and development internationally (Rotherham, 1983; Krishna *et al.*, 2002), however so far much more information has been accumulated till now over this genera (mostly Azalea group) in regards to it's growing condition, reproductive methods, taxonomy, breeding etc. (Hsia and Korban, 1997), but actual study on conservation

issue in the Sikkim Himalaya is almost non existent. *Rhododendron niveum* Hook f. (Ericaceae) locally known as Hiupatae gurans (snow leaf), endemic to Sikkim (also the state tree of Sikkim) more or less localized within 3500-4500 m elevations limited to a microniche at a place called Yakchey in northern Sikkim. The species is much less in number and at the verge of extinction it needs high-priority conservation measures (Singh, 2009). The regeneration status in the form of available seedlings/saplings is very poor for many of the *Rhododendrons* (Krishna *et al*., 2002; Singh *et al*., 2003; Singh, 2008; Kumar *et al.*, 2005; Paul *et al.*, 2005; Singh *et al.*, 2008; Singh *et al*., 2008a). Tissue culture is the only method to maintain and propagate the genetically identical clone rapidly in large numbers and in long term culture. Hence, *in vitro* conservation seems to be an appropriate option for the protection of *R. niveum* out of its natural habitat.In this communication, the authors report a high frequency regeneration system for the first time from cotyledonary node explant of *R. niveum* using 2ip alone or in combination with IAA and successful establishment of micropropagated plant to field conditions.

MATERIALS AND METHODS

Seed vigour test

Seed vigour was tested with the tetrazolium test (Agrawal *et al.*, 1973). Halved seeds were treated in tetrazolium solution (TTC, 0.1%) for 2 h at room temperature and red-staining embryos were evaluated as alive.

Plant material

Seeds were collected in October 2008 in Yakchey in North Sikkim (Longitude 27^o 43' North, and Latitude 88^o 45' East with an elevation 3500 m amsl). Immediately after collection, capsule were dried at room temperature for 1 week and then stored in small plastic bags at 4^{o}C.

Seed sterilization and culture conditions

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 in a laminar flow hood and implanted on hormone free MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.8% (w/v) agar in culture tubes for germination. The cultures were kept at 24^{o}C ± 1 ^{o}C and 16/8 light/dark cycle with irradiance of 60 μmol m^{-2} s^{-1} provided by cool white fluorescent tube. *R. niveum* seeds germinated within 15-20 days of inoculation on hormone free MS medium (Figure 1A).

Multiple shoot formation

The nodal segment explants (1.0-1.5 cm in length) from aseptic seedlings of *R. niveum* were cultured on the AM medium (Anderson, 1984) supplemented with different Cytokinins, 6-benzyladenine (BA); 2-isopentyladenine (2iP) or Kinetin (KIN) at different concentrations (0.5, 1.0, 2.5, 5.0, 7.0, 10.0 mg/l) along with additives (100 mg/l PVP, 100 mg/l ascorbic acid and 10 mg/l citric acid). The medium was gelled with 0.3 % phytagel adjusted to pH 5.8 using 1N NaOH before autoclaving at 121^{o}C at 1.06 $kgcm^{-2}$ for 15 min. All the culture maintained at 17±1 ^{o}C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 μmol m^{-2} s^{-1} provided by cool white fluorescent lamp (Philips, India Ltd.) and 60% relative humidity.

In addition, the effects of these, the optimal concentration of Cytokinin (2iP) was used in combination with 0.1 mg/l Indole-3-acetic acid (IAA). Seven shoots per glass flask (250 mL) were cultured in 60mL of medium. In all treatments, the frequency of shoot induction, number of shoots and mean shoot length and callus formation were recorded after 8 weeks of culture. All cultures were subcultured to a fresh medium after every 4 week intervals.

In vitro rooting and acclimatization

The elongated shoots (4-5 cm) were excised individually and transferred to liquid AM supplemented with NAA or IBA (0.05, 0.1, 0.2, 0.3, 0.5, 1.0 mg/l) on filter paper bridge for rooting. The results of the rooting experiments were expressed as percentages. In cultures, where the shoots were inoculated on

auxins free AM medium, no root formation was observed. Data on percentage of rooting and mean number of roots per shoot were recorded after 4 weeks of transfer. Plantlets with good developed leaves and root were washed with sterile distilled water and dipped with systemic fungicides (Bavistin, 0.15%, w/v, 20 mins) to minimise pathogen attack and planted in plastic pots containing fresh peat moss and soil (1:3). Plantlets were initially maintained under culture room (17±1 °C) conditions for five weeks. After some growth these plantlets were transferred to plastic bags containing sterile normal garden soil and placed for hardening under relative humidity (80%) in the green house at 24 ± 2 °C with a 16:8 photoperiod.

Experimental design and data analysis

In all experiments, each treatment consisted of 10 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). All plant growth regulators (PGRs) used was from Sigma Chem. Company, USA.

RESULTS AND DISCUSSION

In view of the low germination rates, a tetrazolium test was carried out, which showed that 70% of the seeds collected in September and 80% of the seeds collected in October were viable at the time of collection. This obvious difference between survival percentages may have resulted from the time of seed collection. The seeds collected in October, on the other hand, completed their developmental phases and therefore responded positively to the tetrazolium test at a rate of 80%. This reveals that the best time to collect *R. niveum* seeds is October. Seeds from species of the genus Rhododendron tend to be relatively short-lived compared with species from other families and/or genera (Troup, 1981; Hay, 2006).

For successful in vitro propagation, shoot tips are the preferred explants as they possess pre - existing meristem that is easily developed into shoots while maintaining clonal fidelity. The sterilized seeds of *R. niveum* were found to germinate within 3 weeks of inoculation on hormone-free MS medium (Figure 1A).

Shoot tip explants excised from 15-day-old seedlings of *R. niveum* were cultured on AM medium supplemented with different Cytokinins, 6-benzyladenine (BA); 2-isopentyladenine (2iP) or Kinetin (KIN) at different concentrations (1.0, 2.5, 5.0, 7.0, 10 mg/l) at to induce multiple shoots. Regular subculturing was done every 3-4 weeks on to fresh medium. 2ip proved a more effective cytokinin than BA, kinetin for multiple shoot induction. The highest percentage (68±1.15) of multiple shoot induction and Maximum number of multiple shoots per explants (8.66±0.57) was on AM media supplemented with 5 mg/l 2ip (Table 1, Fig.1B,C). On the other hand, the lowest percentage of shoot (23±1.52) and the lowest number of shoot (1.66 ± 0.33) per explant were found on AM containing of 0.5 mg/l Kinetin.

The effect of auxin and cytokinin combination was also evaluated on multiple shoot induction from nodal segments (Table 2). 2ip with IAA was found more effective combination for multiple shoot regeneration. The addition of 0.1 mg/l IAA with optimal concentration of 2ip significantly increase the frequency of shoot formation compared to 2ip alone. At this concentration, all the explatns shows a high proliferation multiple shoots after 8 weeks. The highest number of shoots/explants (9.00± 0.33) was recorded on AM medium supplemented with 5 mg/l 2iP and 0.1 mg/l IAA (Table 2). Although BA has often been reported to stimulate regeneration and proliferation in some rhododendron species (Cantos, 2007; McCown and Lloyd, 1982). After 8 to 9 weeks, when regenerated shoots reached a length of more than 5 cm, they were separated and placed on liquid AM medium containing different concentrations of NAA or IBA, where root formation was observed after two weeks of inoculation. Various concentrations of IBA or NAA in AM medium had significant effect on rooting frequency and number of roots per explant in *R. niveum.* In cultures, where the shoots were inoculated on auxins free AM medium, no root formation was observed. In general, increase in the concentration of IBA resulted in corresponding increase in the

number of roots per explant, with maximum rooting frequency (70%) at 0.2 mg/l of IBA (Table 3, Figure 1D,E). Contrarily NAA was not so promising and resulted in sharp reduced frequency of rooting on any

Table 1: Effect of different concentrations of various plant growth regulators on shoot regeneration

Plant Growth Regulators (mg/l)			Frequency (%) of explants producing shoots	Mean number of shoots/explant	Mean shoot length (cm)
BA	Kin	2-iP			
0.5			20±0.67	3.00 ± 0.57	0.70 ± 0.06
1.0			25±1.73	5.00 ± 1.15	1.33 ± 0.09
2.5			30±0.67	5.00 ± 0.57	2.10 ± 0.15
5.0			34±1.15	4.76 ± 0.33	1.57 ± 0.31
7.0			29±3.78	4.33 ± 0.33	1.00 ± 0.12
10.0			20±0.67	2.66 ± 1.20	0.50 ± 0.15
	0.5		23±1.52	1.66 ± 0.33	0.70 ± 0.06
	1.0		30±1.67	2.00 ± 0.57	0.73 ± 0.09
	2.5		35±1.00	4.00 ± 0.57	1.66 ± 0.09
	5.0		37±0.88	4.06 ± 0.57	2.13 ± 0.19
	7.0		32±1.45	4.00 ± 0.57	2.00 ± 0.06
	10.0		23±0.88	4.00 ± 0.57	1.33 ± 0.12
		0.5	26±2.40	3.33 ± 0.88	0.90 ± 2.38
		1.0	43±3.60	5.00 ± 1.15	1.26 ± 0.23
		2.5	55±2.03	8.33 ± 0.42	4.60 ± 0.66
		5.0	68±1.15	8.66 ± 0.57	7.90 ± 0.23
		7.0	64±1.52	8.33 ± 0.33	4.26 ± 0.67
		10.0	46±2.40	5.33 ± 0.88	3.06 ± 0.12
LSD at the 5% level			17.25	4.30	2.60

Values represent means ±SE. Each treatment consisted of 10 replicates and the experiment was repeated twice.

concentration of NAA. In the treatments of NAA, the induced roots were short and thick. On the other hand, in the treatments of IBA, the induced roots were long and thin According to Jutta (2000) transport velocity of IBA was markedly slower as compared to other growth regulators.

Table 2: Effect of different concentrations of various plant growth regulators on shoot regeneration

Regeneration (%)	Mean number of shoots/explants	Mean shoot length (cm)
BA (5 mg/l)	3.00 ± 0.33	4.60 ± 0.25
BA (5 mg/l) +IAA (0.1 mg/l)	4.00 ± 0.33	5.60 ± 0.35
KIN (5mg/l)	4.75 ± 0.33	3.76 ± 0.36
KIN (5mg/l) +IAA (0.1 mg/l)	5.50± 0.57	3.90 ± 0.06
2-iP (5mg/l)	8.75 ± 0.57	7.10 ± 0.58
2-iP (5mg/l) +IAA (0.1 mg/l)	9.00± 0.33	7.73± 0.23
LSD at the 5% level	3.5	5.27

Values represent means ±SE. Each treatment consisted of 10 replicates and the experiment was repeated twice. Data were recorded after 8 weeks of culture.

Higher concentration of auxin lowered the rooting percentage as well as root number. Data on percentage of rooting and mean number of roots per shoot were recorded after 6 weeks of transfer. Plantlets with good developed leaves and root were washed with sterile distilled water and dipped with systemic fungicides (Bavistin, 0.15%, w/v, 20 mins) to minimise pathogen attack and planted in plastic pots containing fresh peat moss and soil (1:3). Plantlets were initially maintained under culture room (17±1 °C) conditions for five weeks (Figure 1F,G). The micropropagated plants, following hardening and establishment in the greenhouse were transferred to the field at Arboretum of the Institute (Figure H). 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. 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.

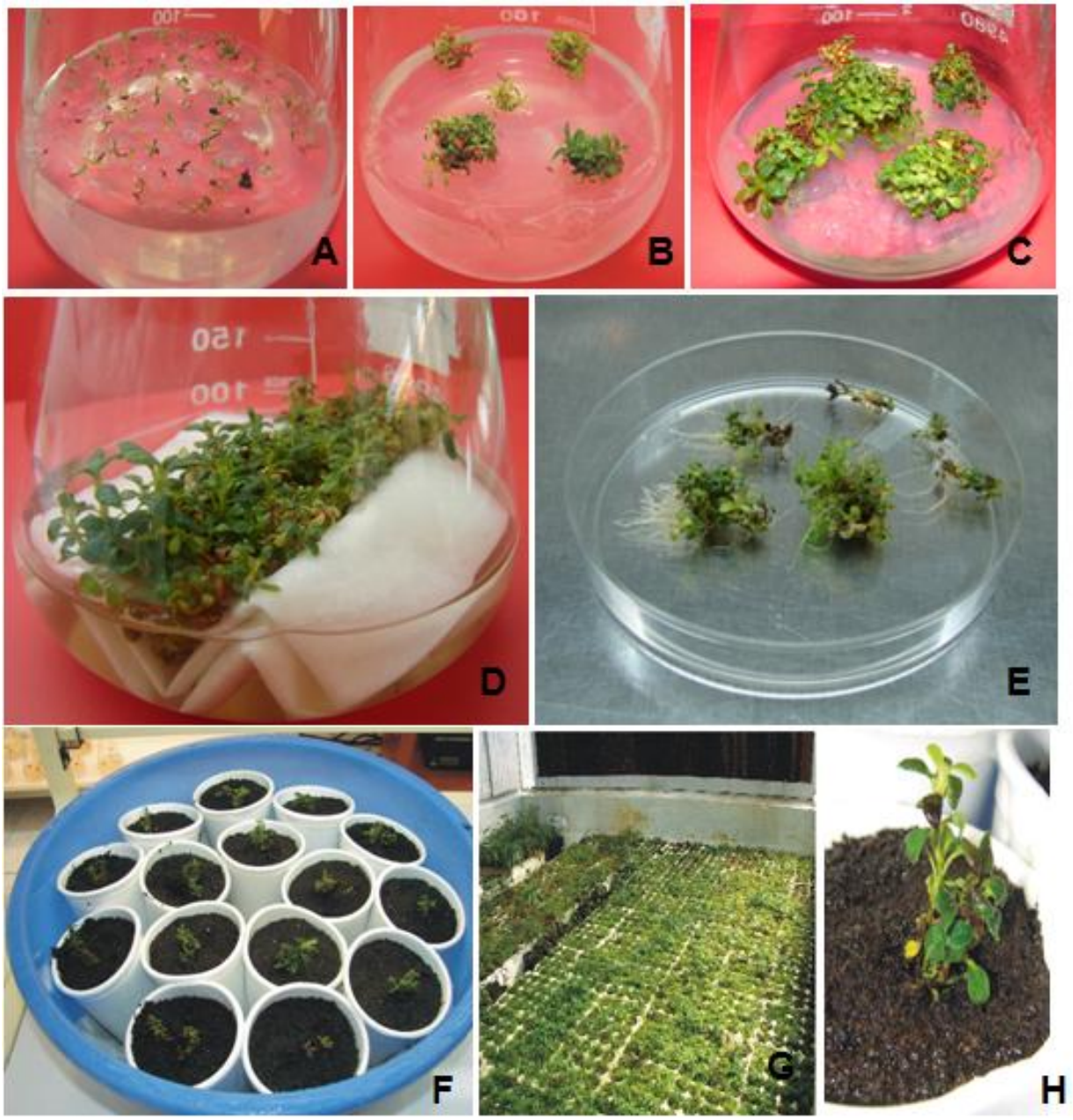

Figure 1: ***In vitro*** **propagation of** ***R. niveum***: (A) germinating seeds in AM medium, (B) established shoots derived from nodal segment grown on AM medium supplemented with GA_3 and (C) 2iP, (D) root induction from *in vitro* regenerated shoot on liquid AM medium with IBA (0.2 mg/l), (E) Rooted microshoots, (F, G) Plantlets established on fresh peat moss and soil (1:3), (H) Hardened plants in the greenhouse.

Table 3: Effect of auxins on root induction from *in vitro* raised microshoots of *R. niveum* in liquid Anderson medium

Auxins (mg/l)	Number of roots per explants	Frequency of root regeneration	Callusing
Control	-	-	-
NAA			
0.05	-	-	-
0.10	3.33 ± 0.33	22	C++
0.20	5.33 ± 0.33	28	C+
0.30	4.00 ± 0.58	52	C++
0.50	2.33 ± 0.57	36	C+++
1.00	1.67± 0.33	22	
IBA			
0.05	3.00± 0.57	28	C++
0.10	3.67 ± 0.67	38	C+
0.20	6.67 ± 0.33	70	C++
0.30	4.67 ± 0.88	28	C+++
0.50	3.00± 0.41	30	C+++
1.00	3.00± 0.58	20	
LSD at the 5% level	2.30		

Values represent means ±SE. Data were recorded 6 weeks after transfer to liquid AM; each treatment consisted of 10 replicates and the experiment was repeated twice. C+: indicates less callusing, C++: indicates more callusing, C+++: indicates profuse callusing.

Conclusion

In the present investigation, a high frequency of multiple shoot induction was achieved in *R. niveum* through nodal explants with 2ip (5 mg/l). Based on the data of the present study we can conclude that *in vitro* technique may help in the conservation of the species in Sikkim Himalaya. This achievement can be applied to mass production and provides a uniform product quality and contribute to the conservation of the valuable biodiversity of the region.

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