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In vitro Propagation of *Rhododendron griffithianum* Wt.: An Endangered Rhododendron Species of Sikkim Himalaya

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ABSTRACT

A reproducible protocol for *in vitro* propagation of *R. griffithianum* has been established. Multiple shoot proliferation from shoot-tip explants was occurred in the presence of 2-isopentenyl adenine (2-iP) alone or in combination with indole 3-butyric acid (IBA) in Anderson's medium. The best treatment for shoot regeneration was Anderson's medium supplemented with 5 µM 2-iP and 1.0 µM IBA, which promoted shoot proliferation in more than 75% culture with an average of 11.00 shoots per explants after twelve weeks. These shoots were successfully rooted on Anderson's liquid medium supplemented with 1.5 µM IBA. The rooted plantlets were hardened in greenhouse with 84% survival rate.

1. INTRODUCTION

R. griffithianum (Wt.) Lord Auckland's Rhododendron is commonly known as "seto chimal" by the local people of Sikkim. It is a tree of 3-8m height growing at an altitude of 2000 - 3200m and possesses the largest flower of the genus. Though it is very common in Central Bhutan, it seems to have vanished from its type habitat in Chungthang and is rare in the Lachung valley of north Sikkim [1]. The major threats to Rhododendrons in this region are direct, such as its use as fuel wood substitute and incense, as well as indirect, such as habitat disturbances brought about by forest clearances, construction works (built-up area) and tourist influx. These interferences later pool up to bring about more nuisance to the Rhododendron growing area in the form of avalanche, unchecked rains and surface flow, flashfloods, etc. Scarce population of this species is also found in Barsey, West Sikkim. Despite of its high diminishing rate, so far very little work has been done for their conservation. Till date whatever work has been done in Rhododendron is regarding its growing condition, reproductive methods, taxonomy, breeding etc [2-4], but actual study on conservation of this high value tree in the Sikkim Himalaya is almost missing. Conventionally Rhododendron species are propagated by seeds and cutting which is found to be difficult or ineffective [5-7]. Therefore, there is an urgent need of an alternative method by which large scale propagation of this plant can be done. In this respect, *in vitro* technologies are very promising which has been used in the present study to develop propagation protocol for *R. griffithianum*. Mass propagation using tissue culture protocol developed in the present study and plantation in natural habitats and arboretum will improve conservation status of this plant.

2. MATERIALS AND METHODS

2.1 Plant material, sterilization and culture conditions

Seeds were collected from healthy plants from Barsey Rhododendron Sanctuary, West Sikkim in the first week of September, 2013. They were dried at the institute until the capsules released the seeds from inside. The seeds were then stored in glass vials at 4 °C. The seeds were used within few days of storage. They were wrapped in a muslin cloth and washed 30 min in running tap water followed by detergent (Tween-80; 1.0%, v/v; 10 min). Further surface sterilization was performed with freshly prepared aqueous solution of mercuric chloride (0.05%; w/v; 3 min). Each treatment was followed by repeated washing (4 times) with sterile distilled water under aseptic conditions. The seeds were placed aseptically in test tubes (10 seeds per culture test tube, 25 mm

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diameter, 15 ml medium) onto Anderson's medium [9], supplemented with antioxidants (150 mgl^{-1} Polyvinylpyrolidone (PVP) + 100 mgl^{-1} L-ascorbic acid + 10 mgl^{-1} citric acid) containing 5µM gibberellic acid (GA_3) and 0.8% (w/v) agar. The medium was adjusted to pH 5.6 before autoclaving at 121°C for 15 minutes at 1.05 kg/cm^2 pressure. All the culture were maintained at 23 ± 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. The aseptic shoot tips obtained from 2-week old *in vitro* seedlings were used as explants for the multiple shoot induction.

2.2 Planting Medium and shoot induction from shoot-tip explants

In order to induce *in vitro* shoot proliferation, following culture media were tested: full strength of Murashige and Skoog's (MS) (1962), half-strength of MS (half amount of inorganic constituents, full amounts of organic and other constituents) [8] and Anderson's Medium (AM) (1975) [9]. The medium was gelled with phytagel (0.3%) and supplemented with 2-isopentenyl adenine (2-iP; 1, 5, 15 µM), indole 3-butyric acid (IBA) and indole 3-acetic acid (IAA) along with additives (100 mg l^{-1} PVP, 100 mg l^{-1} ascorbic acid and 10 mg l^{-1} citric acid) and 3% sucrose.

2.3 *In vitro* root induction

For rooting, well developed *in vitro* shoots were transferred to Anderson's-liquid medium containing different concentrations of IBA (0.5, 1.0, 1.5 and 2.5 µM) on filter paper bridge (Table 3). After 5 weeks of culture, percentage of rooted shoots, mean number of roots per shoots and root length were recorded.

2.4 Hardening and acclimatization

Rooted plants were washed with sterile distilled water then dipped with systemic fungicides (bavestin, 0.15% , w/v; 20 min) and then planted in plastic pots (125 ml) containing autoclaved fresh peat moss and soil (1:3).

The plants were hardened under high relative humidity (80%) in the mist chamber of greenhouse (25 °C) at the Pangthang, East Sikkim (Longitude 27°4'46" to 28°7'48" North, and Latitude 88°55' to 88°55'25" East with an elevation 2087 m amsl) of the Institute.

Well developed surviving plants were transferred to pots containing normal garden soil and maintained in greenhouse. Later, the plants were established in the field site (arboretum, Pangthang of the Institute).

2.5 Statistical analysis

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 [10].

Table 1: Effects of different media/substrates supplemented with 5 µM 2-iP on shoot proliferation from shoot tip explants of *R. griffithianum.*

Media/substrate	Percent of explants producing shoots	Mean number of shoots/explant	Shoots length(cm)
½ MS	33.00 ±2.08	4.33 ±0.33	1.87 ±0.15
MS	67.00 ±3.52	7.00 ±0.58	2.73 ±0.88
AM	70.44 ±1.07	8.00 ±0.27	2.82 ± 0.04
LSD at the 5% level	37.8	5.10	1.77

Nodal segments cultured on four different medium without growth regulators did not show any response. Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 12 weeks after transfer to culture medium.

Table 2: Effects of 2-iP combined with auxins (IBA and IAA) on shoots proliferation from shoot tip explants of *R. griffithianum* on Anderson's medium.

Plant growth regulators (µM)		Percent of explants producing shoots	Mean number of shoots/explant	Shoots length (cm)
2-iP	IAA			
1	-	52.00 ±1.15	4.33 ± 0.33	0.53 ± 0.08
1	1.0	55.00 ±2.08	5.33 ± 0.33	1.00 ± 0.06
5	-	70.44 ±1.07	8.00 ±0.27	2.82 ± 0.04
5	1.0	70.00 ±0.88	8.00 ± 0.58	2.61 ±0.02
15	-	66.00 ±0.88	7.60 ± 0.88	2.10 ±0.09
15	1.0	65.00 ±0.58	8.66 ±0.33	2.20 ±0.10
2-iP	IBA			
1	-	53.00 ±1.20	7.00 ± 0.58	0.63 ± 0.03
1	1.0	55.00 ±2.08	7.67 ± 0.33	1.67 ± 0.45
5	-	70.44 ±1.07	8.00 ±0.27	2.82 ± 0.04
5	1.0	75.00 ±1.73	11.00 ± 0.58	3.60 ±0.12
15	-	65.00 ±1.45	8.33 ± 0.33	2.03 ±0.03
15	1.0	67.00 ±0.88	9.66 ±0.67	2.20 ±0.20
LSD at the 5% level		57.38	5.94	1.98

Values represent means ± standard error. Each treatment consisted of 20 replicates and the experiment was repeated twice. Data were recorded 12 weeks after transfer to culture medium.

Table 3: Effect of different concentration of IBA incorporated in Anderson's liquid medium on rooting from regenerated shoots of *R. griffithianum*.

IBA (µM)	Rooting (%)	Mean number of roots per shoot ± SE	Mean root length (cm) ± SE
0.5	40	3.33 ±1.20	1.00 ±0.12
1.0	67	4.00 ±0.58	1.13 ±0.07
1.5	72	4.66 ±0.33	1.53 ±0.03
2.5	48	4.00 ±1.54	1.01 ±0.06
LSD (P=0.05)		1.65	1.12

Values represent means ± standard error. Data were recorded 5 weeks after transfer to Anderson's liquid medium.

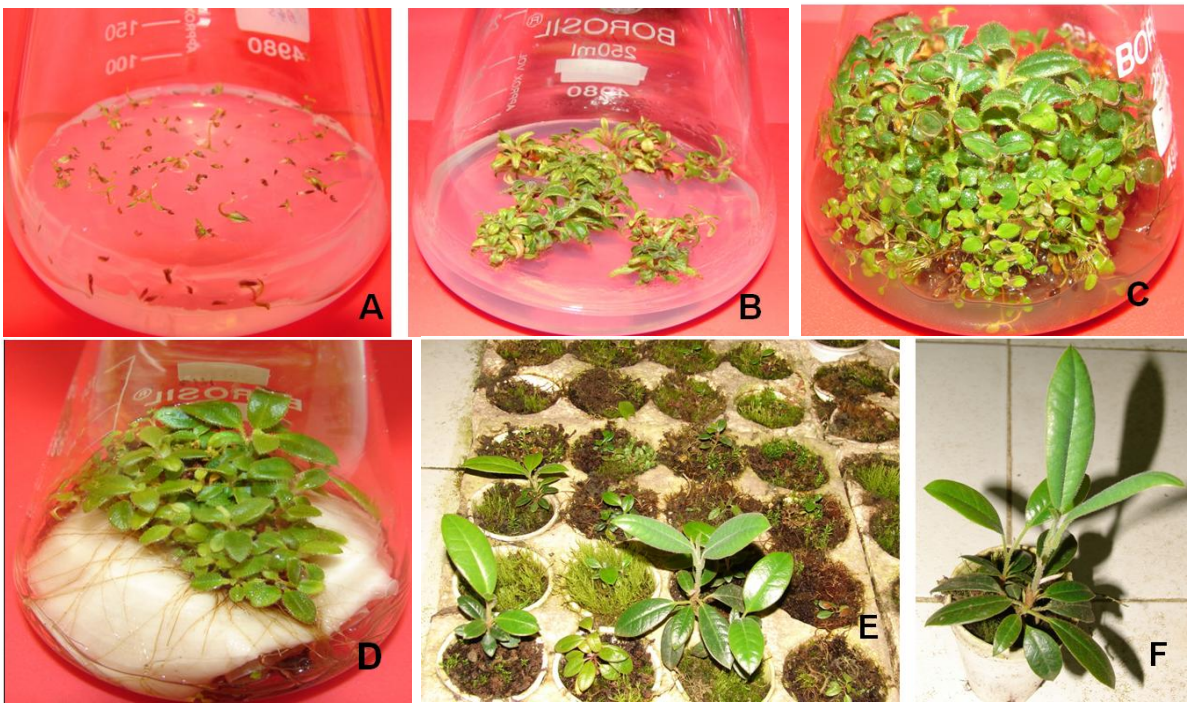

Fig. 1: *In vitro* propagation of *R. griffithianum*. (A) Germinating seeds in Anderson's medium+5 µM GA_3. (B) Multiplication of shoots on Anderson's medium with 5 µM 2-iP. (C) Established shoots derived from shoot tips grown on Anderson's medium supplemented with 5 µM 2-iP and 1.0 µM IBA. (D) Root induction from *in vitro* regenerated shoot on Anderson's liquid medium with 1.5 µM IBA. (E) Hardened *in vitro* raised plants after transfer to fresh peat moss and soil in green house. (F) Plant ready for field transfer after hardening.

3. RESULTS AND DISCUSSION

R. griffithianum seeds were found to germinate within 15-20 days of inoculation on the MS medium supplemented with 5µM of GA_3 with 31% of seeds showing germination (Fig. 1A). For shoot multiplication, shoot tips from 2-week old *in vitro* grown seedlings were excised and cultured on MS, ½ MS and Anderson's media, each supplemented with 5 µM 2-iP. Of the three different media tested, the frequency of multiple shoot was highest (70.44 %) in Anderson's medium and the lowest (33 %) was in ½ MS medium (Table 1). In AM + 2-iP (5 µM) maximum mean number of shoots (8.00 ±0.27) and mean shoot length (2.82 ± 0.04) was observed (Fig. 1B). The number of multiple shoots was decreased with further increase in 2-iP concentration. The superiority of 2-iP over other growth regulators for multiple shoot induction has been reported in many other Rhododendron species [11-12] and related species such as azalea [13]. Kumar *et al.* (2004) [16] had also proved that 2-iP was effective for shoot multiplication of *Rhododendron maddeni*. Shoot tips cultured on different medium without any growth regulator did not show any response. To further improve shoot multiplication response, 2-iP was tested with IBA and IAA. Incorporation of 1.0 µM IBA in the 2-iP supplemented medium during the first subculture significantly improved the shoot multiplication response. Maximum percent of multiple shoot (75 %) and maximum mean number of shoots (11.00 ± 0.58) were recorded in Anderson's medium supplemented with 5 µM 2-iP and 1.0 µM IBA (Table 2; Fig. 1C). The effect of auxins and cytokinin on enhancing shoot regeneration has been reported in several other species, such as *Solanum viarum* [14], *Catalpa ovate* [15] and *Rhododendron maddeni* [16].

For rooting *in vitro* grown individual shoots were separated from the shoots of the primary culture and placed in Anderson's liquid medium containing different concentrations of IBA. A maximum frequency of root formation (72%) and the highest number of roots (4.66 ±0.33) with maximum root length (1.53 ±0.03) was achieved on Anderson's medium with 1.5 µM IBA after 5 weeks (Table 3; Fig. 1D). At higher concentration (2.5 µM IBA), a small amount of callus formed at the base. Control

shoots that did not receive auxin treatment did not root. Similar observations were made in other plants [17-19]. The roots were thick and healthy and new shoots continued to regenerate from the rooted basal portion of the plant on prolonged culture. Lower concentration of IBA found to be ineffective for rooting and higher concentration of auxins inhibited root growth and induced callusing from the base of shoot. This might be due to the fact that supplied IBA may have increased endogenous auxin levels or level of other root-inducing substances. Alternatively, it may have reduced the relative concentration of inhibitory substances of cytokinins [20-21]. Plantlets produced using standard protocol were subjected to hardening and acclimatization in field conditions with 84% survival rate (Fig. 1E & F).

4. CONCLUSION

The findings of the present study are of considerable significance, since it has described a regeneration system for *R. griffithianum* which has not previously been reported. *In vitro* plants appeared morphologically uniform with normal leaf form, shape and growth pattern. *In vitro* propagation for regeneration and rapid multiplication seems viable approach to conserve this important Rhododendron species of Himalaya to restore the Himalayan ecosystem.

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