The Influence of Temperature, Light and Pre-treatment on the Seed Germination of Critically Endangered Sikkim Himalayan Rhododendron (*R. niveum* Hook f.)

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Abstract: *R. niveum* Hook f. is a beautiful and endangered rhododendron that has limited distribution in the Sikkim Himalaya. In an effort to improve and promote the propagation of this over-exploited plant, the effect of temperature and light on the germination of seeds was investigated with various presoaking treatments of plant growth substances (GA_3, Kinetin and BAP) and nitrogenous compound (KNO_3). The combined effect of GA_3 with Kinetin or BAP (25 µM) was also examined. Seeds were given a presoaking treatment with GA_3, BAP or a combination of both to influence germination. A temperature of 21^{o}C was found optimum and showed 34.33% germination, with 21 days for onset and 50 days for final germination under 16 hr light condition. The seeds of *R. niveum* need light to trigger the germination and no germination was observed in darkness. Though the seed viability was 86% as determined by tetrazolium staining, maximum germination of 63.67 % was obtained only when the seed was soaked in GA_3 + BAP (25 µM each) solution for 24 h and incubated for germination at 21^{o}C, constant temperatures in 16 hr photoperiod. The other treatments were far less effective in promoting the germination of this endangered species. The present study indicates that constant 21^{o}C, temperature incubation and 16 hr photoperiod have a positive relationship with seed germination of *R. niveum* even under no pre-treatments. Seeds stored at low temperature (4 ^{o}C) could maintain viability for less than six month. Here, it is the first time we have described the seed germination requirements of *R. niveum*, which are under threat due to anthropogenic pressure [Journal of American Science 2010;6(8):172-177]. (ISSN: 1545-1003).

Keywords: *Rhododendron niveum*; seed germination; temperature; light; Sikkim Himalaya.

1. Introduction

The genus *Rhododendron* L. (family Ericaceae) includes a number of decorative species and cultivars that are often utilized in accent, garden and park architecture designs. In India, it is represented by about 80 species, of which 36 species are found in Sikkim (Pradhan and Lachungpa, 1990; Singh et al., 2003; Bhattacharyya and Sanjappa, 2008). Among these, *R. niveum* is considered to be the most beautiful with lilac-purple flowers and dull green foliage having creamish white undersurface and was declared as the State Tree of Sikkim. Over exploitation of this species has caused a serious threat. The regeneration status in the form of available seedlings/saplings is very poor due to the above situation for many of the rhododendrons (Semwal and Purohit 1980; Singh et al., 2008a; Singh et al., 2008b; Singh 2009). So far no attempt has been made to develop suitable germination protocols and large scale production of rhododendron species which are under the threat of extinction. Germination is a complex process that is controlled by several biological (species, seed viability, seed dormancy, seed size) and environmental (moisture availability, temperature, relative humidity, light intensity and duration) factors. Since plant species vary in their response to these factors, it is important to determine the optimum conditions and seed treatments for germination and seedling establishment under the prevailing climatic conditions. Though studies have been conducted on different aspects of *R. niveum* (Singh et al., 2008a; Singh et al., 2009), no information is available on seed germination and seed storage aspects of this species.

The aim of the present study was therefore to investigate the effects of growth regulators and nitrogenous compound on the germination of *R. niveum* seeds under different temperature and conditions both in light and darkness.

2. Material and methods

2.1 Brief description of plant material: *R. niveum* Hook. f. [Snow-leaved Rhododendron, Nepali: Hiun-pate Gurans)]; First report by Hooker (1849). More or less localized within 3500-4500 m elevations of the Sikkim Hills, *R. niveum* is scarce in the Darjeeling hills, almost a rare element. Within the rhododendron community of the region, *R. niveum* is a comparatively large tree, attaining heights of over 3 m. It is quite a distinctive species as far as its general habit and flowers are concerned. Flowers of deep magenta or lilac with darker nectar pouches appear in

April and May. The leaves are hairy underneath. Under our recent field exploration it has been found out that the *R. niveum,* endemic to Sikkim *(*also the state tree of Sikkim) is limited to a microniche at a place called Yakchey in northern Sikkim, and individual count shows less than 45 plants. Flowering-April; Fruiting-July. As the species is much less in number and at the verge of extinction it needs high-priority conservation measures.

2.2 Seed Collection: Seed lots of *R. niveum* Hook. f. 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). Seeds were collected in small cotton bags. Immediately after collection, capsule were dried at room temperature for 1 week, then stored in small plastic bags at 4^{o}C.

2.3 Seed viability assessment: To ensure that the seeds used for the experiment were viable and of high quality, the sample lots were subjected to viability test using the tetrazolium technique (Peters, 2000). Seed viability was observed immediately at the time of collection and after 2, 4, 6, 10 and 12 months of storage at 4^{o}C.

2.4 Effects of temperature and light: To determine the effect of different temperatures, the seeds were incubated at 7 ^{o}C, 10 ^{o}C, 13 ^{o}C, 17 ^{o}C, 21 ^{o}C, 25 ^{o}C and 30 ^{o}C constant temperatures under either a 16 h photoperiod or total darkness. To determine the effect of different photoperiod, the seeds were incubated at 21, and 25^{o}C constant temperatures. These treatments were conducted in 0 (total darkness), 2, 4, 8, 12, 16, 20 or 24 hr photoperiod conditions for 30 consecutive days in the seed germinator. Daily photoperiod treatments were regulated by removal and placement of the petri dishes into black carbon paper. Seed germinators were equipped with cool-white fluorescent lamps that provided a photosynthetic photon flux (400-700 nm) of approximately 40 $\mu mol.m^{-2}\cdot s^{-1}$.

2.5 Effects of pre-treatment: Various seed treatments were attempted for improving germination percentage. To test germination, seeds were disinfected with 0.05% aqueous solution of $HgCl_2$ for 3 min, washed thoroughly with double distilled water, and dipped in various pre-treatment solutions, viz. gibberellic acid (GA_3); (25, 100, 250 µM), 6-benzylaminopurine (BAP); (25, 100, 250 µM), Kinetin (Kin); (25, 100, 250 µM), GA_3 + BAP (25 µM each), GA_3 + BAP (250 µM each), GA_3 + Kin (25 µM each), GA_3 + Kin (250 µM each), potassium nitrate (KNO_3); (50, 100, 150 mM) for 24 h. Control was maintained using double distilled water. After treatment, seeds were washed with distilled three times and placed in glass petri dishes (90 mm) on a single layer of Whatman No. 1 filter paper with about 5 ml of distilled water. A set of seeds without any treatment was used as control. The moisture levels of filter paper were maintained by adding distilled water as required. For prevent of infection and evaporation of solution, all of plates were closed with parafilm. All operations were done under laminar flow. Germination was monitored daily from the date of seed sowing. Seeds showing signs of decay were removed immediately from the Petri plates. Seeds were considered germinated when the radicals reached ≥1 mm in length. Germination percentage was recorded every day until no further germination was found. Observations on germination of seeds kept in dark were taken in dull green light. Percent germination was calculated as a mean of four replications per treatment.

2.6 Statistical analysis: Every treatment has three replicates of 25 seeds each because of the limited availability of seeds. 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).

3. Results and discussion

3.1 Seed viability: In view of the low germination rates, a tetrazolium test was carried out, which showed that 86% of the seeds were viable at the time of collection. It was noted that after two months of storage at 4^{o}C, seed viability decreased significantly (P = 0.05) and the end of twelve months, it was only 8% (Figure 1). Loss of moisture content and very low reserves of nutrients are the chief cause of deterioration of seeds under storage condition. 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).

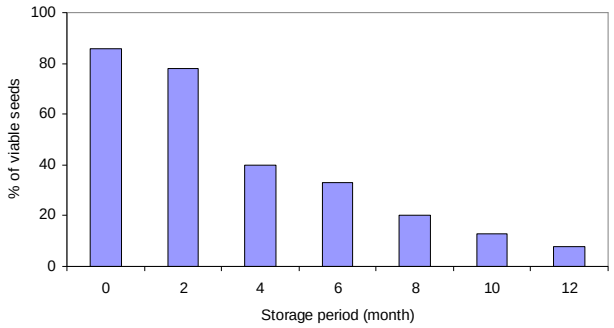

Figure 1. Changes in seed viability of *R. niveum* during storage at 4ºC.

3.2 Effects of temperature and light: In this study, effects of temperatures and light treatments on germination rate of *R. niveum* are presented in Table 1. Since the threshold temperature for germination was 13°C, the presentation of the 7 and 10°C variants were omitted in the Table 1. The rate of germination increased with increasing temperature, and then decreased at temperatures above 25 °C. At 13 °C, the percentage germination was significantly lower than those at other temperature (Table 1). Furthermore, variation germinability at different temperatures was significant (F = 4.36; P = 0.05). The optimum temperatures for germination of the seeds of these species are 17-21 °C and no difference in germination was observed between seeds incubated at 21-25 °C, but at 7 °C the seed was not germinated. Seedlings germinated at 25 °C and 30 °C under light condition were not seems to be healthy and most of them will die after producing from radicles. High temperature is well known to prevent radicle and shoot

Table 1. Seed germination at different temperatures under 16 h light and dark conditions

Temperature/ light or dark	Days required for onset of germination	Days required for completion of germination	Germination percentage
13°C, Light	30	55	14.33 ± 1.86
17°C, Light	26	54	25.00 ± 1.73
21°C, Light	21	50	34.33 ± 0.88
25°C, Light	25	48	35.00 ± 0.58
30°C, Light	36	49	33.33 ±1.76
Alternate temperature (21°C in light and 10°C in dark for 12 h)	21	50	32.66 ± 3.38

F = 4.36 (significant at P = 0.05).
Values are the mean ± SE of four replicates and the experiment was repeated twice.
At 13°C, 17°C, 21°C, 25°C, 30°C temperature under dark condition no germination took place.

elongation by inhibiting synthesis of protein and nucleic acid (Hegarty and Ross, 1979; Sivaramakrishnan et al., 1990). Germination at alternate temperature (21°C in light for 12 h and 10°C in dark for another 12 h) was also not very successful (Table 1). Seeds of *R. niveum* required light for germination regardless of temperature. This effect is illustrated by comparing germination percentages between 21 °C and 25 °C at different photoperiods (Figure 2). Among different photoperiods, 16 hr duration was found optimum for germination. At 21 °C, increasing photoperiods increased germination with 22.67 % and 34.33 % germination occurring by day 30 for the 12- and 16 hr photoperiods, respectively.

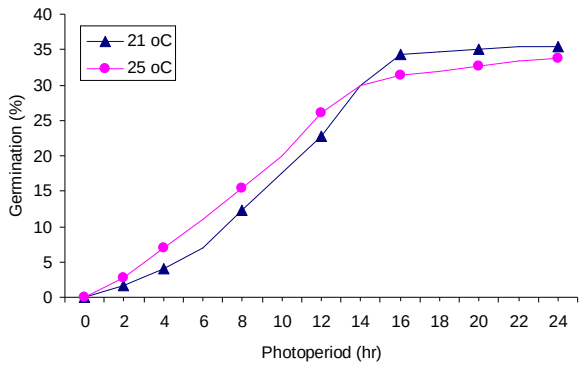

Figure 2. Cumulative (30-day) seed germination of *R. niveum* as influenced by photoperiod and temperature. The effect of photoperiod on germination percentage was significant (F = 4.68; P = 0.05) for both temperatures.

Seeds of *R. niveum* in dark did not germinate at stipulated experimental time. It has been conclude that most of the Himalayan species do not show intrinsic dormancy and that germination in most of

these is better in light than in darkness (Semwal and Purohit, 1980). Our results indicate that light is a necessary factor for germination of *R. niveum* seeds. The analysis of variance proved that increasing photoperiods have had significant effects ($F = 4.68$; $P = 0.05$) on percent germination at both temperatures of 21°C and 25°C. Generally, 8-12 hr day light is necessary for maximum germination and growth in forest trees (Kozlowski, 1971), whereas in the present studies a 16 hr photoperiod was found optimum for seed germination and seedling growth. A similar effect has been reported for other species of rhododendron (Blazich et al., 1991; Blazich et al., 1993; Tort 1996; Arocha et al., 1999; Kumar et al., 2004; Faravani and Bakar 2007). In general, absence of light has a negative effect on germination in several Rhododendron species (Juntilla, 1972; Singh, 2008).

3.3 Effects of pre-treatment: The effects of different pre-treatments on *R. niveum* seed germination are shown in Table 2. In the control treatment, *R. niveum* showed the lowest germination percentage and longest time to germination. Of the growth regulator pretreatments alone, GA_3 and BAP shortened the time required for germination by alleviating its germination-delaying effect. On the other hand, double combinations of these regulators much more successfully shortened the period of germination. Seeds treated with GA_3 began germinating sooner and germination was completed earlier than that of untreated seeds at low temperature (21ºC). Germination of untreated seeds also began after 21 days. They germinated slowly, but after 30 days, the rate began to accelerate. All concentrations of GA_3 had higher germination and increased seedling vigour over control, where its highest concentration (250 µM) was most effective. More than two fold improvement in germination was recorded in seeds treated with GA_3 + BAP (25 µM each). The other treatments were far less effective in promoting the germination of this endangered species. The present study indicates that constant 21°C, temperature incubation and 16 hr/8 hr (light/dark) photoperiod have a positive relationship with seed germination of *R. niveum* even under no pre-treatments. However, on the basis of ANOVA, significant variation ($F = 4.48$; $P = 0.05$) was found in germination due to these hormonal treatments. However, this study demonstrates that exposure of seeds of *R. niveum* to the GA_3 + BAP (25 µM each) for 24 h and incubation at 21°C in 16 h light photoperiod conditions can result in almost 63.67% germination and should provide a method to assist in the *ex situ* management of *R. niveum.* Several studies from recent years have shown that gibberellin is an effective germination stimulator (Thompson, 1969; Juntilla, 1972). According to the magnification of stimulatory effects of GA_3 and BAP in this study, conclusion can be drawn that GA_3 are permitted to reach their active sites through the modifying influence

Table 2. Effects of various pretreatments on seed germination of *R. niveum* at 21ºC and 16 h photoperiod.

Treatments	Days required for onset of germination	Days required for completion of germination	Germination percentage
Control	21	50	32.00 ±1.15
GA_3 (25 µM)	18	27	34.67 ± 0.88
GA_3 (100 µM)	17	24	46.33 ±0.88
GA_3 (250 µM)	18	23	51.00 ±1.52
Kinetin (25 µM)	22	47	35.00 ± 0.58
Kinetin (100 µM)	23	47	36.33 ± 0.33
Kinetin (250 µM)	20	48	33.67 ± 0.77
BAP (25 µM)	18	31	34.67 ± 0.67
BAP (100 µM)	17	32	35.67 ± 1.45
BAP (250 µM)	18	30	39.00 ± 0.49

GA_3 + Kinetin (25 µM each)	25	44	36.00 ± 0.58
GA_3 + Kinetin (250 µM each)	21	44	29.67 ± 0.88
GA_3 + BAP (25 µM each)	15	22	63.67 ± 1.45
GA_3 + BAP (250 µM each)	14	22	56.00 ± 0.58
KNO_3 (50 mM)	22	46	34.33 ±1.20
KNO_3 (100 mM)	21	45	33.67 ± 0.67
KNO_3 (150 mM)	21	45	32.67 ± 1.45

F = 4.48 (significant at *P* = 0.05).
Values are the mean ± SE of four replicates and the experiment was repeated twice.

of cytokinins on transport across membranes and are thus able to initiate the biochemical processes necessary for germination to occur (Thomas et al., 1975). The cytokinin probably penetrates the testa and neutralizes the inhibitors present in the embryo, thus enabling the embryo to rupture the seed coats (Khan, 1971).

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