Eco-Physiology of the Rhododedron anthopogon D. Don a dwarf aromatic shrub of the Indian Himalaya

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Abstract

Rhododendrons are the Himalayan ecological, cultural, aesthetic, and economic entity; some of its varieties are edible and medicinal. *Rhododendron anthopogon* D. Don is an alpine *Rhododendron* that grows above tree lines in the Himalaya. This is a dwarf *Rhododendron* with fragrant leaves that are employed in numerous traditional Himalayan medical systems. This plant's essential oil is pale and contains antibacterial, antioxidant, and insecticidal effects. This manuscript comprises an eco-physiological investigation of *R. anthopogon* on an altitudinal and seasonal basis in the Tungnath Himalaya of Uttarakhand, India. We observed that the altitudinal gradient shapes the phyto-sociology of *R. anthopogon*, which is one of the dominant species in its habitat. Leaf morphology and phytochemicals (pigments, total soluble sugar, starch and protein, total phenolic content, Malondialdehyde content, Superoxide dismutase and Peroxidase enzyme activities) of the *R. anthopogon* leaves showed altitudinal and seasonal variations, indicating their importance in thriving in the harsh alpine conditions. The seed germination experiment in *R. anthopogon* was also carried and observed that the synergistic effect of cold stratification and GA_3 reduced the mean germination time while increasing the final germination percentage.

Introduction

The Himalayas were home to a plethora of exceptional, endemic, and ecologically valuable plants that benefited both humans and nature. There is a wealth of literature on Himalayan flora that examines distribution, survival, eco-physiology, and other elements of life cycles. Rhododendrons provide ecological services as well as cultural and aesthetic value to the community; they are sacred species and have a significant role in the traditional wisdom of many Sino-Himalayans ethnic groups (Bhattacharyya 2011). Rhododendron is a member of the Ericaceae family and is found mostly in India's Himalayan area. However, seven *Rhododendron* species are found in Uttarakhand (Western Himalaya) *viz. R. arboretum* Sm., *R. lapidotum* D. Don, *R. anthopogon* D. Don, *R. barbatum* Wall. ex G. Don, *R. campanulatum* D. Don, and *R. nivale* Hook. The *Rhododendron anthopogon* D. Don is distributed in the Himalayan region's alpine zone between 3200 and 5200 m asl (Bhattacharyya, 2011). It is found in India, Pakistan, Nepal, Bhutan, and Tibet (Trivedi et al. 2017). It is distributed in alpine pastures above tree lines in Uttarakhand, Jammu & Kashmir, Himachal Pradesh, Arunachal Pradesh and Sikkim, in India. On steep or boulder-strewn terrain, the plant frequently produces dense gregarious patches, mainly on the slope's northern side. It is one of the smallest *Rhododendron* species (2–3 feet), with dark, green, oval leaflets that are aromatic. Flowers are white or yellow with a reddish tinge that blooms in bunches from May to July. Fruit capsules, tea of leaves, and flowers have long been used to cure cough and the common cold in native places. The essential oil of *R. anthopogon* stimulates the neurological system and is used to treat aching muscles and gouty rheumatic diseases (Siwakoti 2008). It is a well-known medicinal plant and used in skin care, relieve liver disorder diseases treatment, as Himalayan healers use the leaves and fresh flowers of this dwarf shrub to make tea and drink to promote digestive heat, treat throat and counteract water-earth illness, cure cold, remove headaches and back pain, bone disease, blood disorders, vomiting, relieve liver disorders and stimulate appetite and the essential oil is known for its calming and relaxing effects (Siwakoti 2008, Kumar et al. 2009, Popescu and Kopp 2013, Scherrer 2019).

Over the last few decades, scientists have taken keen interest in analysing the distribution and fitness of natural communities across environmental gradients in order to better understand community assembly norms, which are a key aspect in evaluating the influence of global change on biodiversity (Cadotte et al., 2013). Previous studies examined the processes underpinning species assemblages by analysing taxonomic diversity, presuming that all species are equally distinct and contribute equally to ecosystem functioning. Plant species, on the other hand, show great functional heterogeneity, and various taxa are expected to play distinct roles in ecosystem functioning (Bricca et al. 2020). Functional traits are morpho-physio-phenological attributes that influence species fitness, which includes growth, reproduction, competition, and survival in a particular environment (Violle et al. 2007). Alpine plants are subjected to challenging conditions at high mountain altitudes, such as extreme cold, dryness, low oxygen concentration, strong ultraviolet (UV) radiation, and powerful winds. Different adaptations in alpine plants have been documented, including changes in leaf shape and physiological characteristics. Structure and functional plasticity, as well as adaptation methods, become crucial for plant growth and persistence at higher altitudes (Korner 2003). Such kinds of structural diversities enables plants to improve their fitness for successful establishment in high-elevation habitats typified by harsh temperatures, a short growth season, limited nutrient availability and snowfall (Korner 2012). In high altitudes, height of the plant, diameter of its stem, area of a leaf and its specific leaf area (SLA) all tend to be decreased as elevation increased (Oberhuber 2004, Korner 2013). Leaves having lower SLA can thrive in resource-constrained, lower-soil-moisture and lower-temperature situations. Furthermore, dwarf plants are more likely to survive low temperatures, snowfall, and high winds (Korner 2012). Plants at higher elevations are predicted to be smaller with thick and narrow leaves as a result of the stressful environment, whereas plants at lower elevations generate larger and thinner leaves than plants at higher elevations (Zhang et al. 2012, Liu et al. 2020). The keys to alpine plant survival in such harsh conditions are structural changes, stomatal control, dwarfism with a low leaf index, pigment accumulation to protect them from UV radiation exposure, solute amounts such as total protein, total phenolic content, total soluble sugar, and enzymatic adjustment.

R. anthopogon is the least concerned *Rhododendron* species, according to IUCN red list categories (Gibbs et al. 2011), yet changing temperature and other environmental harmful actions are rendering this plant species vulnerable. When IUCN criterion B2 is applied to *R.*

anthopogon in India, it is found in endangered category in Sikkim Himalaya (Singh and Rai 2010). Climate change is well-known for causing changes and contractions in high altitude floras (Tingly et al. 2012). As the climate warmed, species moved upslope in search of suitable habitats, but those at the top had nowhere to go and became extinct. *R. anthopogon* is a *Rhododendron* species that is typically found on higher mountain peaks, particularly in the western Himalaya, and has a higher risk of becoming a victim of climate change. Seed germination and seedling establishment are the stages in plant reproduction which are most precarious (Baskin and Baskin 2001) and playing a pivotal role in conservation practises. There have been several researches on *Rhododendron* sp. seed germination and other criteria, including *R. protistum* (Shen et al. 2015), R. *catawbiense* (Rowe et al. 1994), R. *niveum* (Singh et al. 2010), and others. However, there is no or very little literature indicating the germination behaviour of *R. anthopogon*.

The goal of this study was to examine *R. anthopogon* eco-physiological studies along an altitudinal gradient in Tungnath, Western Himalaya. Keeping the foregoing facts in mind, and in order to fill a research gaps (1) Ecological studies include phyto-sociological studies of *R. anthopogon* along an altitudinal gradient (2) To study the morphological and physiological-biochemical variation of *R. anthopogon* along an altitudinal gradient in Tungnath, Western Himalaya and (3) To determine *R. anthopogon* seed germination behaviour. This study may aid in predicting the future composition of forests and *R. anthopogon* adaption techniques in this region of the Western Himalaya.

Material And Methods

Study area and sample collection

The current study was conducted in the alpines of Tungnath Himalaya of Western Himalaya, India, (30°29'19.3"N 79°13'04.1"E). The sampling and study sites in this study were established at three elevations, along the altitudinal transect (Site I- 3370 m, Site II- 3470 m, and Site III- 3570 m asl) (Fig. 1). Leaves sampling was conducted in May 2021 (summer) and October 2021 (autumns) from the north facing slope of the mountain from the same tagged plants in the both months. The seeds were collected in October 2021 after maturation. The collected samples were brought to the High Altitude Plant Physiology Research Centre (HAPPRC, HNBGU) laboratory in Srinagar, Uttarkahnd, India for further study, where they were washed and pat dry before each experiment.

Phyto-sociological Analyses

Between June and October 2021, when the majority of species are in flowering stage, selected places were visited, and geographical data (elevation, slope, aspect, latitude, and longitude) were recorded using a portable global positioning system (GPS). A stand of 50 m x 50 m was identified at each of the specified locations in the study area for quantitative and qualitative studies of the community sustaining the target species, and random sampling was conducted by laying twenty quadrates (11m) in each of the stands for herbaceous species and four 5 m x5 m (25 m2) for shrubs species (Fig. 2). The raw data collected from sampling in each stand was aggregated and analysed for quantitative variables such as density (D plants m^{-2}), frequency (F%), and abundance according to the techniques given by Curtis and Mc -Intosh (1950) and Mishra (1968).

Morphological Parameters

Ten mature plants were chosen from each group to assess the following morphological parameters: leaf length, width and thickness, petiole length and diameter, leaf dry matter content (LDMC), leaf area (LA) and specific leaf area (SLA). In total, 60 fresh leaves (20 from each site) were collected from the various altitudes of the study area. Several metrics were measured in each case after sampling. Leaf length, width, and petiole length were measured using a measuring scale, while digital Vernier calliper used for measuring leaf thickness. Leaf area was determined using the leaf area meter (Li- Cor, Inc, and Lincoln, Nebraska, USA) and leaf weight was measured using analytical balance.

Physio-biochemical Estimation

Pigment content

For pigment estimate, the Holm (1965) approach was employed. 50 mg of the plant's leaves were homogenised in the dark with 5 ml of acetone before being centrifuged at 3000 rpm in a chilled centrifuge for 10 min (eltek RC 4100F, Hyderabad, India). Supernatant was collected for pigment analysis, and absorbance was determined at three different wavelengths (662, 644, and 440.5 nm) and reported as mg $g^{-1}FW^{-1}$.

Total Soluble Sugar Content

The method of MC Cready et al. (1950) used to determine the carbohydrate content. 50 mg of plant leaves with 5 ml of ethanol were homogenised before being centrifuged at 3000 rpm in a chilled centrifuge for 10 min (Eltek RC 4100F, Hyderabad, India). The obtained supernatant was used to estimate total soluble sugar and the pallet for total soluble starch, which was digested in 52% perchloric acid and centrifuged for 15 minutes at 3500 rpm. After diluting the supernatant with distilled water, Anthrone reagent (4 ml) was gently poured through the test tube walls. The test tubes were then well mixed with a cyclomotor for 1–2 min before being placed in a boiling water bath (7–8 min) and cooled it (20–30 min) at room temperature. Absorbance of the solutions was measured at 620 nm (Systronic AU-2701) using a UV-Vis spectrophotometer and sucrose was used as the standard for total soluble sugar and dextrose for total soluble starch to generate the standard curve.

Total Soluble Protein Content

The Bradford (1976) method was used to measure total soluble protein. To accomplish this, 50 mg of plant leaves were homogenized in Triss buffer (5 ml, pH 7.5), then centrifuged for 10 min in a chilled centrifuge at 10,000 rpm (Eltek RC 4100F, Hyderabad, India). The plant sample, distilled water, and 4.9 ml of Bradford reagents were then used to make dilutions. After that, samples were cyclo mixed, and absorbance was measured in a UV-Vis spectrophotometer at 595 nm (Systronic AU-2701). The standard curve was generated by using the BSA solution as the standard.

Lipid Peroxidation Or Malondialdehyde (Mda) Content

Heath and Packer (1965) method was used to determine the MDA content, 250 mg of fresh leaves homogenised with 10% (*w/v*) trichloroacetic acid (TCA, 8 ml). 2ml of the supernatant was then added to a test tube containing 2 ml of thiobarbituric acid (0.6% in 10% TCA) after centrifuging the homogenate for 20 min at 4°C and 12000 rpm. The mixture was heated for 15 min in water bath at 100°C before being chilled in an ice bath. After cooling, the mixture was again centrifuged at 12000 rpm for 20 min and absorbances were measured at 450, 532, and 600 nm. The MDA content was calculated using the formula: MDA (mol/L) = [6.45 (A532 A600)] (0.56 A450), where A denotes absorbance and expressed as n mol g^{-1} FW^{-1} of leaf tissue.

Enzymatic Antioxidant Protection

Enzyme Extraction

0.5 g leaves were homogenised in 5 ml phosphate buffer (ice-cold, pH 7, 0.1 M) containing EDTA using a pestle and mortar (pre-chilled), as described by Esfandiari et al (2007). In a centrifuge, homogenates were centrifuged for 15 min at 15,000 rpm (Eltek RC 4100F, Hyderabad, India). The resulting supernatant was utilized to evaluate the enzyme activity.

Enzyme Activity

The method of the Sengupta et al. (1993) was utilized to assess the SOD activity. 0.1 ml of enzyme (Supernatant) was added to the assay mixture to generate 3 ml final volume. The assay combination contained 13 mM methionine, 75 mM Nitro Blue Tetrazolium chloride, 0.1 mM EDTA, 50 mM phosphate buffer, and 50 mM sodium bicarbonate. 0.1 ml of 2 M riboflavin was added to start the reaction. Two sets of the test tubes were made for each sample: one in the dark (non-irradiated) and the other in 15W fluorescent light for 15–20 min. By shutting off the light and covering the sample test tubes in black, the reaction was halted. The control tube (without enzyme) became blue. The whole sample mixture that had not been irradiated served as a control for the irradiated sample mixture. At 560 nm, absorbance was measured using a UV-Vis spectrophotometer. 1 Unit of enzyme activity was the enzyme required (amount) of to reduce the absorbance reading by 50% compared to the control and enzyme activity was calculated as Ug^{-1} FW^{-1}.

POD enzyme activity was assessed using Pyrogallol as the substrate with a slight modification (Trivedi et al. 2020). 3 ml reaction mixture comprising 100 mM potassium phosphate buffer (0.32 ml, pH 7.0), 0.027% *(v/v)* peroxide solution (0.16 ml) was taken in a test tube. The addition of 0.1 ml of enzyme extract started the reaction. The increase in absorbance was immediately detected at 420 nm for three minutes on a UV-Vis spectrophotometer (Systronics, Gujarat, India) and enzyme activity was calculated as Ug^{-1} FW^{-1}.

Total Phenolic Content

Method described by Aniswarth and Gillespei (2007) used to determine the total phenolic content. To accomplish this, 100 mg of plant leaves were extracted and homogenised in 5ml of 40% ethanol. It was then refrigerated centrifuged for 15 minutes at 5000 rpm (eltek RC 4100F,

Hyderabad, India). For the phenolic measurement, different quantities of supernatant were obtained, and then 5 ml Folin ciocalteu reagent, and 4 ml of l 7.5% sodium carbonate were added. After 1 hour in the dark, the absorbance at 765 nm UV-Vis spectrophotometer was measured (Systronic AU-2701). Total phenolic content was expressed as mg GAE g^{-1} FW^{-1}.

Seed germination behaviour of R. anthopogon

R. anthopogon mature capsules were air dried; separated seeds were prepared for germination studies and kept in airtight containers at 4°C until further use. *R. anthopogon* minuscule, yellow-brown seeds were divided into two lots, one of which was stratified with double-distilled water (dw) at 4°C for a month while the other served as the control (P1). The cold stratification seed lot was stored in 100-ml airtight plastic vials for a month at 4°C on a double layer of filter paper (Whatman filter paper) moistened with dw. One portion of the cold- stratified seeds was used for a germination experiment (P2), and the other portion was divided into two parts. One portion of the cold-stratified seeds was used for a germination experiment (P2), and the other portion was treated with GA_3 (250 ppm) (P3). The pre-treated *R. anthopogon* seeds were planted on three different substrata: filter paper (T1), soil (T2), and Murashige and Skoog (MS) medium (T3). Some of the P1, P2, and P3 seeds (treated) were placed on moistened filter paper (Whatman No. 1) in Petri dishes after being surface sterilised with 0.5% w/v Bavistin for 30 min (90 mm diameter). The *R. anthopogon* growing regions' soils were collected, transported to the lab, and kept there at 4°C until needed. P1, P2, and P3 treated seeds were inoculated into the soil and placed in glass petri dishes. Pre-treated seeds (P1, P2, and P3) were properly sterilised once more in a laminar hood for 20 minutes with a solution of 0.1% Tween-20, rinsed three times with dw, and then rinsed once more with autoclaved double distilled water (dw) to remove any remaining disinfectant. Seeds were then dipped in 70% (v/v) ethanol for 70 seconds inside the laminar air hood before being rinsed with dw. To remove any residues, seeds were further immersed in a 0.01% (w/v) mercuric chloride (HgCl2) solution for 3 min. before being rinsed with dw three to four times. After being surface sterilised, seeds were put in a 100 ml conical flask with 40 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962). The incubation conditions for each treatment were 25°C, 16 hours of light, and 75% humidity.

The glassware used was purchased from Borosil India, and all chemicals were purchased from HiMedia India.

Statistical analysis

Twenty replicates for the leaf morphological studies and three replicates for physio-biochemical studies of each variable were used to calculate the mean standard deviation in excel. One-way ANOVA was performed to evaluate morphological traits between elevations, and it was followed by a sample t-test ($p \leq 0.05$) test. To assess the impact of seasonal changes and the altitudinal gradient on the variables, a two-way ANOVA was applied on the data. The Tukey HSD post hoc test was then used to determine the values that differed significantly from the mean. At $p \leq 0.05$, differences in the values were considered as significant. The software programme IBM SPSS (version 28.0.1.1) was used for all analyses. Germination attributes *i.e.* mean germination time (MGT), first day of germination (FDG), 20th day germination percentage (TDGP) and final day germination percentage (FGP) were calculated. Two way analysis of variance (ANOVA) was analyzed to estimate the effect of substratum and treatments on germination attributes of *R. anthopogon*. Duncan multiple range tests were applied to find out the significant difference among the treatments. The data is shown as mean ± standard error of mean and numbers of replicates for each treatment were also three for the seed germination.

Results And Discussion

Community composition and phyto-sociological study of R. anthopogon

In the study area, 30 species belonging to 27 genera and 20 families were identified as being associated with *R. anthopogon*. Rosaceae was the most dominant associated family with *R. anthopogon*, followed by Asteraceae and Poaceae. *R. anthopogon* was the dominant shrub species, with the highest density (stem/25 m^2) and importance value index (IVI) in the entire study area. Only two shrub species (*R. anthopogon* and *R. campanulatum)* are found at Site I which comprised overall 24 species, Site II consisted total 29 species with three shrub species (*R. anthopogon, R. campanulatum* and *Juniperus communis*) and Site III consisted highest number of species (30 species). On Site I, *Tenaxia cachemyriana* (Jaub. & Spach) N.P.Barker & H.P.Linder was the dominant herbaceous species with the highest density (39.2 plants m^{-2} and IVI value 52.72), followed by *Trachydium roylei* Lindl. (Density 33.86 plants m^{-2} and IVI value 33.37), *Persicaria wallichii* Greuter & Burdet (density 7.36 plants m^{-2} and IVI 33.17), and *Geum elatum* Wall. ex Hook.f. (Density 12.8 plants m^{-2} and IVI 28.82) (Supplementary material 1). *Trachydium roylei* co-dominated with *R. anthopogon* at Site II and had the highest density (39.55 plants m^{-2} and IVI 35.35), followed by *Tenaxia cachemyriana* (density 33.7 plants m^{-2} and IVI 34.36), *Carex setosa* Boott (density 22.75 plants m^{-2} and IVI 26.48), and *Anaphalis royleana* DC. (density 10.95 plants m^{-2} and *Tenaxia cachemyriana* was found to be the most dominant species at Site III, with the highest density (45.2 plants m^{-2} and IVI 51.24), followed by *Trachydium roylei* (density 24.15 plants m^{-2} and IVI 29.03), *Acomastylis elata* (Wall. ex G.Don) F. Bolle (density 8.65 plants m^{-2} and IVI 18.85), and *Plantago himalaica* Pilg. (density 6.4 plants m^{-2} and IVI 17.41 *R.*

campanulatum was a co-dominant shrub species of *R. anthopogon* and recorded in all of the study areas. Diversity indices of *R. anthopogon* also showed variations along the altitudinal gradients, however, the value of Shanon diversity indices ranged between 2.28 to 2.5 and it was observed maximum at Site III while the minimum at Site I. On the other hand, the values of Simpson index of dominance ranged from 0.86 to 0.95. Similar to Shanon diversity indices, the maximum value of Simpson index of dominance was recorded at Site III whiles the minimum at Site I. The values of Simpson index of dominance near to 1 indicate the dominant nature of only few species in the entire study area.

Morphological characteristics of R. anthopogon **along an altitudinal gradient**

The leaf morphological characteristics of *R. anthopogon* varied with altitude; however the variations were non-significant for most of the leaf characteristics excluding the petiole diameter ($P < 0.01$), SLA ($P < 0.01$) and LDMC ($P < 0.01$) which showed significant variations along the altitudes. Leaf length and leaf area were highest at Site II, whereas leaf width, petiole length, specific leaf area and LDMC were highest in Site I leaves. Leaf thickness, fresh weight and dry weight were highest in leaves of the Site III (Table 1).

Table 1
Morphological Characteristics of the *R. anthopogon* along the altitudinal gradients

Study Sites	Length (cm)	Width(cm)	Thickness (mm)	Petiole length (cm)	Petiole Diameter (mm)	Fresh Weight (g)	Dry weight (g)	LA (cm^2)	SLA	LDMC
Site I	2.48 ± 1.22	1.44 ± 2.32	0.25 ± 1.87	0.67 ± 2.14	0.713 ± 3.22	0.08 ± 1.32	0.04 ± 1.27	2.72 ± 2.56	64.60 ± 3.33	0.49 ± 2.98
Site II	2.44 ± 1.45	1.21 ± 0.99	0.30 ± 2.54	0.66 ± 2.54	0.72 ± 1.24	0.07 ± 1.88	0.04 ± 1.66	2.46 ± 1.65	60.96 ± 4.22	0.46 ± 2.22
Site III	2.71 ± 0.43	1.29 ± 0.86	0.28 ± 2.21	0.53 ± 1.88	0.72 ± 2.12	0.09 ± 1.35	0.05 ± 2.65	2.83 ± 54.7	56.082.24	0.46 ± 2.15
F value	0.166	1.41	0.621	0.692	9.14	0.595	1.176	0.122	13.001	8.89
P value	NS	NS	NS	NS	P < 0.01	NS	NS	NS	P < 0.01	P < 0.01
T test	2.69*	2.56**	6.8***5	5.72***	5.14**	7.67***	7.87***	2.97**	32.19***	6.29***

* p < 0.05, ** p < 0.01, *** p < 0.001, ns = non significant, LA- leaf area, SLA- specific leaf area, LDMC- leaf dry matter content.

Physio-biochemcal properties of R. anthopogon

In both seasons, all physio-biochemical characteristics assessed in *R. anthopogon* leaves revealed altitudinal differences. Along the altitudinal gradients, Chl a exhibited substantial ($p < 0.001$) variation and Chl b showed non-significant variation, with the highest content at Site I and the lowest at Site III. Carotenoid content increased along altitudinal gradients ($p < 0.05$) and varied significantly across altitudes in both seasons ($p < 0.01$). Since there is a significant relationship between the changing seasons and the mean Chl a content of each altitude, it follows that the content from each altitude changes significantly with the season ($p < 0.01$). The chl a content in *R. anthopogon* leaves showed a non-significant correlation ($r = -0.657$) with altitude and positive correlation ($r = 0.379$) with season. On a seasonal basis, an increase in the content of Chl a, b, and carotenoids was found in all attitudes. Chl a increased by 24.41% at Site I, 45.54% at Site II, and 39.06% at Site III. Chl b increased by 811.11% at Site I, 770.50% at Site II and 218.18% at Site III. The chlorophyll b content in *R. anthopogon* leaves showed a non-significant correlation ($r = -0.152$) with altitude and positive correlation ($r = 0.031$) with season. Carotenoids increased by 100%, 37.04%, and 7.89% in Sites I, II, and III, respectively. Chl a and b ratio declined along the altitude gradient in May but increased along the gradient in October, and total Chl carotenoids ratio declined along the gradient in both months. In both study months, total chlorophyll concentration declined as altitude increased. Since there is a significant relationship between the changing seasons and the mean carotenoid content of each altitude, it follows that the content from each altitude changes significantly with the season ($p < 0.01$). The carotenoid content in *R. anthopogon* leaves showed a significant correlation ($r = 0.078$, $p < 0.05$) with altitude and negative correlation ($r= -0.604$) with season.Total soluble sugar revealed considerable differences ($p < 0.001$) across altitude gradients and seasons. In May, it was highest at Site III and lowest at site I, but in October, the order reversed, with highest at Site I and lowest at Site III. It increased by 59.68%, 26.30%, and 0.60% in *R. anthopogon* from Site I to III accordingly (Table 2, 3, 4).

Table 2
Seasonal and altitudinal variation in biochemical properties of *R. anthopogon*

Elevation	Chl a	Chl b	Carotenoid	Soluble sugar	Non soluble sugar	MDA	SOD	POD	TPC
	(mg $g^{-1}FW^{-1}$)	(mg $g^{-1}FW^{-1}$)	(mg $g^{-1}FW^{-1}$)	(mg $g^{-1}FW^{-1}$)	(mg $g^{-1}FW^{-1}$)	n mol $g^{-1}FW^{-1}$	$Ug^{-1}FW^{-1}$	$Ug^{-1}FW^{-1}$	mg GAE $g^{-1}FW^{-1}$
May									
Site I	1.27 ± 1.24 [c]	0.18 ± 1.93 [b]	0.15 ± 0.79 [a]	19.42 ± 1.76 [a]	28.76 ± 0.23 [a]	27.97 ± 1.87 [c]	21.98 ± 1.99 [a]	2.27 ± 2.01[a]	61.75 ± 1.23 [a]
Site II	1.01 ± 2.34 [b]	0.17 ± 2.45 [b]	0.27 ± 0.97 [b]	23.80 ± 0.867 [b]	32.96 ± 0.54 [b]	21.32 ± 0.88 [b]	25.22 ± 1.32 [ab]	2.96 ± 1.45 [ab]	74.69 ± 1.28 [ab]
Site III	0.64 ± 1.22[a]	0.11 ± 2.22 [a]	0.38 ± 1.34 [c]	26.25 ± 0.78 [c]	36.98 ± 1.09 [c]	14.86 ± 0.76 [a]	28.85 ± 1.23 [b]	3.84 ± 0.98[b]	80.47 ± 1.56 [b]
October									
Site I	1.58 ± 0.89 [b]	1.64 ± 2.01 [b]	0.3 ± 1.93 [a]	31.01 ± 1.56 [b]	37.12 ± 1.01 [a]	16.94 ± 3.54 [b]	8.42 ± 1.65 [a]	0.92 ± 1.98 [a]	85.24 ± 1.76 [a]
Site II	1.47 ± 1.77 [ab]	1.48 ± 1.88 [ab]	0.37 ± 1.45 [ab]	30.06 ± 2.56 [b]	38.09 ± 2.45 [a]	12.83 ± 1.4 [ab]	12.36 ± 2.54 [ab]	1.37 ± 2.65 [ab]	89.66 ± 1.87 [b]
Site III	0.89 ± 1.01 [a]	0.35 ± 0.91 [a]	0.41 ± 1.98 [b]	26.41 ± 2.45 [a]	41.35 ± 1.56 [b]	10.30 ± 2.34 [a]	16.06 ± 2.45 [b]	1.79 ± 1.97 [a]	93.52 ± 1.34 [c]

Chl a- Chlorophyl a, Chl b – chlorophyll b, MDA- Malondialdehyde, SOD- superoxide dismutase, POD- peroxidase, TPC- Total phenolic content

Table 3
Summary of two-way ANOVA showing the effects of altitude, season and their interactions on physiological and biochemical traits

Parameters	Elevation		Season		Elevation*Season	
	F value	P value	F value	P value	F value	P value
Chl_a	66.1	***	27.03	***	8.35	**
Chl_b	0.495	ns	0.018	ns	2.64	ns
Carotenoid	4.31	*	28.2	**	14.18	**
TPC	31.4	***	36.34	***	2.9	**
Protein	3.56	**	63.09	**	0.52	*
Carbohydrate	44.32	***	45.43	***	78.34	***
Starch	26.94	***	23.54	***	88.56	***
SOD	30.3	***	76.32	***	0.3	ns
POD	13.4	**	12.09	***	0.44	ns
MDA	4.66	*	48.82	**	0.75	ns

Table 4
Correlation between different parameters of *R. anthopogon*

	Altitude	Season	Chl a	Chl b	Car	TPC	Soluble Protein	Soluble Sugar	Soluble Starch	SOD	POD	MDA
Altitude	1											
Season		1										
Chl_a	-0.657	0.379	1									
Chl_b	-0.152	0.031	0.36	1								
Car	0.078*	-0.604	.475*	-0.342	1							
TPC	.577*	.726**	-0.173	0.089	-0.544	1						
Protein	0.42	.726**	-0.029	-0.011	0.413	.774**	1					
Carbohydrate	0.466	.719**	0.171	-0.217	.710**	.792**	.601**	1				
Starch	0.173	.765**	0.284	-0.245	.805**	.784**	.563*	.807**	1			
SOD	-0.414	− .883**	.562*	0.11	-0.458	-0.409	-0.415	-0.423	− .628**	1		
POD	− .568*	− .716**	.658**	0.037	-0.436	-0.216	-0.357	-0.236	-0.401	.836**	1	
MDA	-0.494	− .629**	0.154	0.127	− .519*	− .770**	− .523*	− .758**	− .635**	0.331	0.109	1

Since there is a significant relationship between the changing seasons and the mean soluble sugar of each altitude, it follows that the soluble sugar from each altitude changes significantly with the season ($p < 0.001$). The soluble sugar content in *R. anthopogon* leaves showed a non-significant correlation ($r = 0.466$) with altitude and significant correlation ($r = 0.719$, $p < 0.01$) with season. Non soluble starch showed considerable fluctuation ($p < 0.001$) along altitudinal gradients in two different seasons, with its content increasing up the gradient in both. It increased by 29.07%, 15.56%, and 11.82% in *R. anthopogoan* leaves at Sites I, II, and III, respectively. Since there is a significant relationship between the changing seasons and the mean non-soluble sugar each altitude, it follows that the content from each altitude changes significantly with the season ($p < 0.001$). On the other hand, non-soluble sugar in *R. anthopogon* leaves showed a non-significant correlation ($r = 0.173$) with altitude and significant correlation ($r = 0.765$, $p < 0.01$) with season (Table 2, 3, 4).

Total soluble protein concentration increased significantly along with altitudinal gradients in both seasons ($p < 0.01$). When comparing the two seasons, total soluble protein increased by 116.67%, 84.60%, and 96.99% on the leaves of *R. anthopogon* from Sites I, II, and III, respectively. The total soluble protein content in *R. anthopogon* leaves showed a non-significant correlation ($r = 0.42$) with altitude and significant correlation ($r = 0.726$, $p < 0.01$) with season. Since there is a significant relationship between the changing seasons and the protein content of each altitude, it follows that the content from each altitude changes significantly with the season ($p < 0.05$) (Table 2, 3, 4).

MDA concentration varied significantly with elevation ($p < 0.05$) and season ($p < 0.01$), with decreasing concentration with altitude and season. It was highest at Site I and lowest at Site III. With the changing seasons, the MDA content in *R. anthopogon* at Site I, II and III decreased by 39.44%, 39.82%, and 30.69%, respectively. The MDA content in *R. anthopogon* leaves showed a non-significant correlation ($r = -0.494$) with altitude and negative correlation ($r= -0.628$) with season. SOD enzyme activity varied significantly depending on altitude and season ($p < 0.001$). SOD enzyme activity increased in *R. anthopogon* leaves as altitude increased in both seasons, with the maximum SOD enzyme activity at Site III. From April to October, SOD enzyme activity fell by 61.69%, 50.99%, and 44.33% in *R. anthopogon* leaves at Site I, Site II, and Site III, respectively. The SOD activity in *R. anthopogon* leaves showed a non-significant correlation ($r = -0.414$) with altitude and significant negative correlation ($r=- 0.883$, $p < 0.01$) with season. POD enzyme activity followed a similar pattern, with highest POD activity in *R. anthopogon* leaves from Site III and lowest from Site I ($p < 0.01$) in both seasons with significant variation ($p < 0.001$). From April to October, the POD enzyme activity in the leaves of *R. anthopogon* declined by 59.47%, 53.71%, and 53.39%, respectively, at Sites I, II, and III. The POD activity in *R. anthopogon* leaves showed a significant correlation ($r = -0.568$, $p < 0.05$) with altitude and significant positive correlation ($r= -0.716$, $p < 0.01$) with season. Total Phenolic Content (TPC) varied significantly across altitudinal gradients and seasons, with maximum TPC at Site III and lowest at Site I in both seasons ($p < 0.001$). It increases by 38.04%, 20.04%, and 16.22% in samples from Sites I, II, and III, respectively. The TPC content in *R. anthopogon* leaves showed a significant correlation ($r = 0.577$, $P < 0.01$) with altitude and significant correlation ($r = 0.726$, $p < 0.05$) with season. Since there is a significant relationship between the changing seasons and the mean TPC of each altitude, it follows that the content from each altitude changes significantly with the season ($p < 0.01$) (Table 2, 3, 4).

Seed Germination

R. anthpogon seeds are exceedingly small (Fig. 3) and delicate, especially during surface sterilizing. The pre-germination treatment had a significant effect on all germination attributes of *R. anthopogon*, but the individual and interactive effect of substratum on all germination attributes was non-significant, with only TDGP significantly affected by substratum (p = 0.002) and interaction (p = 0.007) between substratum and treatment (substratum treatment; Table 3). Without cold stratification, seeds were unable to germinate in all substratums. When stratified seeds were subsequently treated with GA_3, the effect of cold stratification treatment was improved, resulting in the highest final germination percentages (FGP). We discovered the highest FGP in soil followed by MS media in all three substratums, with soil having the highest FGP (76.67%) in seeds treated with P3. The substratum T1 containing seeds treated with P2 had the lowest FGP value (50.33%). Cold stratified seeds treated with GA_3 germinate faster; it reduced MGT by 4 days in filter paper, 3 days in MS media, and 2 days in soil when compared to seeds treated just with cold stratification (Table 2). It was lowered to 22.78 days in T1 x P3 treatment and 26.01 days in TI x P2 treatment (without GA_3 treatment); this pattern was found in all types of substratum germination tests. Cold stratified seeds treated with GA3 take longer to attain peak germination percentage; first day germination percentage in all substratums was lower in these seeds than in cold stratified seeds. Although the subsequent effect of GA_3 was significant and helpful, as the 20th day germination percentage (TDGP) was increased by 16 to 21% when compared to cold stratified seeds alone (Table 5, 6).

Table 5
Effect of different treatments on germination attributes of *R. anthopogon* grown in different substratum

	Treatment	Filter paper	Soil	MS Media
FGP (%)	Without cold treatment (P1)	NF	NF	NF
	With cold treatment (P2)	50.33 ± 1.20[a]	57.00 ± 3.21[a]	50.33 ± 1.21
	Cold + GA3 (P3)	73.00 ± 3.05[b]	76.67 ± 2.33[b]	74.67 ± 2.19[b]
FDG (days)	Without cold treatment (P1)	NF	NF	NF
	With cold treatment (P2)	15.67 ± 0.33[a]	16.33 ± 0.34[a]	16.32 ± 0.36[a]
	Cold + GA3 (P3)	11.33 ± 0.33b	12.32 ± 0.35[b]	11.67 ± 0.31[b]
TDGP (%)	Without cold treatment (P1)	NF	NF	NF
	With cold treatment (P2)	12.00 ± 1.73[a]	6.67 ± 0.88[a]	7.33 ± 0.68[a]
	Cold + GA3 (P3)	28.33 ± 0.33[b]	25.66 ± 0.91[b]	28.67 ± 0.42[b]
MGT (days)	Without cold treatment (P1)	NF	NF	NF
	With cold treatment (P2)	26.01 ± 1.66[a]	25.79 ± 0.75[a]	26.30 ± 0.67[a]
	Cold + GA3 (P3)	22.78 ± 0.33[a]	23.77 ± 0.59[b]	23.48 ± 0.69[b]

NF- not found, FGP- Final germination percentage, FDG- First day of the germination, TDGP- 20th day germination percentage, MGT- Mean germination time. Similar alphabets (Derived from DMRT post hoc test) in the column showed non-significant difference among the treatments.

Table 6
Effect of substratum and treatments (Two way ANOVA) on germination attributes of *R. anthopogon*.

	Substartum		Treatment		Substartum × Treatment	
	F	p	F	p	F	p
FGP	2.82	0.086	1214.67	< 0.001	1.08	0.392
FDG	3.17	0.066	2815.17	< 0.001	1.17	0.358
TDGP	9.21	0.002	1026.47	< 0.001	5.00	0.007
MGT	0.17	0.842	1189.04	< 0.001	0.23	0.919

NF- not found, FGP- Final germination percentage, FDG- First day of the germination, TDGP- 20th day germination percentage, MGT- Mean germination time.

Discussion

The alpines of the high altitude mountains are known for their hostile environment, which include fluctuations in air temperature, total atmospheric pressure, heat and UV radiation, and plants growing there encounter interacting stresses such as dehydration and low temperature (Körner 2007, Shepherd & Griffiths 2006). Altitudinal gradients in high altitude mountain ecosystem further influenced plant growth and reproduction, as well as the resource availability of such as water, heat and nutrients (Körner 2003). High altitudes mountains ecosystems' biological diversities have long been investigated by ecologists, which are most fragile habitats and are also rich depositories of the species (Kumar and Sharma 2016). *R. anthopogon* is especially interesting due to its altitudinal range and capacity to tolerate the year's major environmental fluctuations. As a result, it is anticipated to have adequate systems to deal with the extreme circumstances of high altitudes. Hence, we investigated how an altitudinal gradient influenced the eco-physiological and biochemical characteristics of this evergreen dwarf shrub. *R. anthopogon* grows on the highest tops of the mountains in the study area, which is why three study sites were chosen. Site I is the starting location for the *R. anthopogon* occurrence, Site III is the peak of the mountain summit, the top resort of the *R. anthopogon* in the study region, and Site II is the intermediate site between the two.

Community composition and phyto-sociological study of R. anthopogon

Quantitative data of the phyto-sociological parameters are the major players in determining the status of a plant community and its ecosystem patterns (Bhat et al. 2020). The phyto-sociological study of *R. anthopogon* showed variation with altitude. In this study we found that the several herbaceous plant species are associated with *R. anthopogon* and the maximum number of herbaceous species associated with this species was reported at higher elevation. The vegetation analysis shown that *R. anthopogon* was associated with 30 species (falling into 27 genera and 20 families). The dominant herbaceous species associated with *R. anthopogon* in the study sites encompassed *Tenaxia cachemyriana, Acomastylis elata, Viola biflora, Trachydium roylei*, shrub species *Rhododendron campanulatum* and *Juniperus communis*. These species were stated earlier also as the dominant plant species in sub-alpine and alpine region of Garhwal Himalaya (Chandra et al. 2018, Jamloki et al. 2021) and Nepal Himalaya (Sharma et al. 2020). Altitude is a key physiographic attribute that extremely impacts the distribution, structure of plant species and growth forms, and slight modification in altitude sharply changes the topographic and climatic conditions (Kharakwal et al. 2005). The density of *R. anthopogon* was found maximum at higher elevation (3570 m asl) while minimum at lower elevation (3370 m asl).The importance value index (IVI) of this species showed its dominant nature at higher elevation where it forms pure dense patches and support the favourable habitat for the growth and development of other herbaceous plant species (Sharma et al. 2020). The variance in species diversity between the communities at different altitudes usually arises from variation in the quality of the site (Denslow, 1980). In this study, we observed that the different diversity indices such as Shanon- Wiener diversity index and Simpson index of dominance showed a variation with altitude. Shannon Wiener diversity values parallel to present study reported by the many investigators in the Himalayan range. However, the present study diversity values (2.28 to 2.50) and Simpson diversity values (0.86 to 0.95) suggest a dominant nature of only few species at higher altitudes. The value of Simpson diversity values in the study sites indicated the dominance of one or few species i.e., *Trachydium roylei, Tenaxia cachemyriana, Carex setosa, Sibbaldia cuneata*, (Jamloki et al. 2021).

Morphological characteristics of R. anthopogon **along an altitudinal gradient**

Leaf size is the most significant factor in acclimatisation and adaptation to harsh environmental conditions, and reduction in leaf size is the key feature in most stresses in alpine plants, whether trees, herbs, or shrubs (Paudel et al. 2019, Liu et al. 2020). Low temperature, high light intensity, and wind speed are important factors influencing leaf form and size in alpine plants, and leaf trait diversity along altitude is an adaptive strategy to deal with environmental problems (Liu et al. 2020, Li et al. 2020). The leaf length and area of *R. anthopogon* decreased with increasing elevation in this study as well. Low temperatures, other climatic extremes including smaller growing season impede plant development and growth at high altitudes (Körner 2012). Plant organ size reduction with increasing elevations is helpful in regions with chilling temperature, high wind speed and heavy snowfall, since it helps the plant to reduce damage of the tissues while retaining strong aerodynamic and thermal resistance (Körner 2003, 2012). We found considerable variation and trends in leaf area (LA), leaf thickness, specific leaf area (SLA), and leaf dry matter content (LDMC) over the elevation gradient in our study. However, Rathore et al. (2018) found no differences in SLA and LDMC along altitudinal gradients in the *R. anthopogon* population in Himachal, India. Reductions in leaf area and SLA at higher elevations were too the adaptations to lower temperature, higher light intensities, and other associated stress factors, as detected in some alpine evergreens *viz. Nothofagus menzisii* (Körner et al. 1986) and *Metrosideros polymorpha* (Tang and Oshawa 1999). We observed that the leaf thickness of *R. anthopogon* increased with elevation in our study. This demonstrates that smaller and thicker leaves are in general at higher elevations; increases in leaf thickness also account for enhanced mechanical strength to endure stressful circumstances such as cold temperature and heavy wind at higher altitude (Lütz 2010). Leaf thickness rises together with altitudinal gradients, which is consistent with previous research (Körner 2003, Zhang et al. 2014, Liu et al. 2020). Thicker leaves allow better leaf adaptation in harsh environments; the thicker the leaves, the better the buffer between inner and outside leaf temperatures, which contributes to sustaining normal physiological activity in plants at higher altitudes when temperatures are low (Liu et al. 2020). Thicker leaves at high altitudes can help protect against the damage caused by high-level ultraviolet irradiation (Ma et al. 2012) and allow for increased water storage (Guo et al., 2017). Petiole length decreased with elevation gradients in *R. anthopogon* in our study; however petiole diameter did not decrease as much as length, indicating that

petiole became thicker and shorter as altitude increased. It could be an adaptation for plants in more exposed alpine beds, because shorter petioles allow plants to arrange their leaves more compactly and provide better temperature homeostasis.

Physio-Biochemcal properties of R. anthopogon **in the study area**

Temperature extremes caused by seasonal and often diurnal freeze-thaw cycles with hot days and cold nights, water desiccation, particularly observed by perennials and evergreens in the alpine highlands during winters, can kill plants if they are not properly acclimated (Körner 2007). Plants must develop cold hardiness and resistance in order to acclimate or adapt in such hostile environments, which requires morphological as well as biochemical and physiological adjustments to the various plant biochemical and physiological processes. Soluble sugar and soluble protein are important in plant osmoregulation (Cui et al. 2018), and their accumulation in cells increases cellular fluidity, stabilises cell membranes, and decreases osmotic potential (Basu et al. 2007). In this investigation, soluble carbohydrates and soluble protein appear to play an essential role in cold acclimation in *R. anthopogon*. We found that the soluble starch and protein content of *R. anthopogon* increased along with the altitudinal gradient in both seasons studied, which was consistent with previous research in alpine floras (Rathore et al. 2018, Cui et al. 2018), but that soluble sugar positively correlated with altitude in summer but decreased with altitude in winter. However, as elevation increased, the chlorophyll content decreased while the carotenoids increased. Higher elevations floras do have higher concentration of carotenoids, which protects plants from UV radiation and oxidative stress. Carotenoids are also lipophilic antioxidants that may detoxify many kinds of reactive oxygen species (ROS).

Alpine ecosystems present extreme challenges to its inhabitants, resulting in the development of reactive oxygen species (ROS) diurnal and seasonal freeze-thaw cycles, high light intensities, increased UV radiation exposure, desiccation, and other factors all contribute to cellular damage and ROS generation in alpine plants (Trivedi and Nautiyal 2020). Although alpine flora protected very well from the ROS by enzymes, pigments like carotenoids and their higher level of the secondary metabolites (Germino 2014). The plant cell's lipid peroxidation by ROS can be assessed by detecting the Malondialdehyde (MDA) concentration, which is an indication of lipid peroxidation and oxidative stress (Hashim et al. 2020). The environment becomes harsher in alpines as altitude increases, and more oxidative stress is expected, yet *R. anthopogon* appears to be well buffered from ROS due to its lower lipid peroxidation or MDA activity as altitude increases. Lower MDA content as elevation increases indicate that plants have a high anti-oxidative ability to avoid lipid peroxidation, indicating greater stress resistance (Campos et al., 2003). Presence of the lower MDA concentration in plants from higher elevations also shown their stronger cold resistance capacities, as it is commonly considered that plants with higher lipid peroxidation are susceptible to cold (Rathore et al. 2018) This appears to be clear in the case of *R. anthoogon* in this study since as altitude increased, important antioxidants such as phenolics, carotenoids, and enzyme activity of the SOD and POD increased. Plants such as *Leymus secalinus*, an alpine plant of the Tibetan Plateau, similarly demonstrated a drop in MDA concentration as altitude increased (Cui et al. 2018). Several alpine flora showed the very high ROS protection such as *Dryas octopetala, Rhododendron ferrugineum*, and *Vaccinium myrtillus* with high phenolic content (Lefebvre et al. 2016) a study in *Gaultheria trichophylla* showed positive correlations between the altitudes and the phenolic content (Bahukhandi et al. 2017) such like the our study. The present study was observed that the several enzymatic activity of *R. anthopogon* such as SOD and POD increased with increasing elevation which favours their growth in such types of adverse climatic conditions. Antioxidant protection was proved to play important role in *Rhododendron chrysanthum* (Zhou et al. 2017) adaptation in alpine habitat in the same way higher SOD and POD activities are must for *R. anthoogon* existence in higher altitudes. The phenolic content of *R. anthopogon* was highest at higher elevations and lowest at lower elevations. A variety of plant species have shown increases in phenolic content after UV-B exposure (Chaves et al. 1997, Turunen et al. 1999).

The most notable finding in this study was the seasonal change in biochemical components, which was particularly noticeable for the lower altitude or Site II grown *R. anthopogon* plants. Plants of the same species demonstrated stronger freezing tolerance than their lower altitude counterparts in numerous alpine plant species investigations. Neuner et al. (2020) shown convincingly that elevation has a substantial effect on freezing tolerance in numerous alpine plant species. Sit III of the study area is present in near the peak of the mountain summit and hence exposed to more climatic extremes in the whole time of a year, to cope up those extremes plants are already well acclimatized. Lower altitude plants, on the other hand, are often less exposed to climate extremes in summer, but before autumn and winter, they require physio-biochemical adjustment to achieve freezing resistance (Sierra-Almeida et al. 2009, Neuner et al. 2020). In October, the pigments, total soluble proteins, total soluble sugar, total soluble starch, and total phenolic content were higher than in May. In the research area, the end of September and early October mark the beginning of winter and the period for evergreen plants to develop more freezing tolerance. The accumulation of osmolytes for osmotic adjustment is the most common low temperature adaptation of plants (Magaña Ugarte et al. 2019), and an increase in total soluble sugar is found in many alpine plants as winter approaches and other abiotic stress conditions. In their investigation of four *Rhododendron* species, Li et al. (2022) found the highest total soluble sugar levels in fall and winter in *R. aganniphum, R. nyingchiense, R. wardii*, and *R. triflorum*. In October, there was a higher accumulation of total soluble protein, and many research revealed that proteins have a role in the formation of freezing tolerance in plants. Physiological, biochemical, and proteomic investigation of the alpine plant *Potentilla saundersiana* indicates that proteins promoted abiotic acclimatisation to high-altitude conditions in plants and that protein-driven acclimation was altitude dependent (Ma et al. 2015). In our investigation, all pigments showed an increase in the onset of winter in *R. anthopogon*, with Chl

b showing the greatest increase. High altitude plants are characterised by a higher ratio of Chl a/b and a lower ratio of Chl/Car (Germino 2014) due to higher xanthophylls and other carotenoids content that also provides oxidative protection from non-radiative dissipation of excitation energy and showed altitudinal and seasonal variations (Gonzalez et al. 2007). Higher pigment and carbohydrate content in *R. anthopogon* leaves at the start of winter indicated that the plant might be physiologically active and continue out photosynthesis until late winter. Some evergreen alpine plants, such as *Euonymus kiautschovicus* and *Mahonia repens*, showed no winter photosynthetic down-regulation (Adams et al. 2002). The initial study period is May, which represents the beginning of summer or the beginning of the active growth period in the northern hemisphere's alpine eco-system, and October represents the end of the active growth period, which could be one reason for the higher pigments and primary metabolites at the end of the growing season so that the plant can gain as many benefits as possible The activity of most enzymes decreases over the winter due to the obvious temperature kinetics of the enzymes, which could explain why SOD and POD enzyme activities decreased in *R. anthopogon* in this study. Because of the decrease in overall physio-biochemical activity during the winter, ROS production is also reduced, resulting in lower MDA content. In winter, low antioxidant enzyme protection may be compensated by increasing total phenolic content to defend against potentially hazardous ROS and radiations in *R. anthopogon*.

Seed Germination

The seeds of *R. anthopgon* needed cold stratification to germinate, and hormonal treatment with GA_3 improved germination even more. The strict requirement for cold stratification for germination in *R. anthopogon* seeds revealed that the species has physiological dormancy (PD) and that cold temperatures below 4 Ċ are required for breaking seed dormancy and increasing seed germination percentage by GA_3, indicating that the PD in *R. anthopogon* seeds is intermediate to non-deep PD (Baskin and Baskin 2004). According to Baskin and Baskin (2004), PD dormancy is present in more than 70% of arctic-alpine floras, and cold stratification is the most efficient technique to overcome such dormancies. Cold stratification has also been demonstrated to serve a substantial influence in reducing the higher temperature requirements for seed germination by some Tundra species (Baskin and Baskin 2001). In various studies, Rhododendrons showed enhanced germination after cold stratification, such as in *Rhododendron arboreum* Smith, where germination increased significantly on stratification but declined as the stratification period progressed (Vipasha et al. 2017), and *Rhododendron aureum*, where germination percentage was also higher in moist chilled treated seeds (Shimono and Kudo 2005). The cold stratification requirement in nature in alpine areas is fulfilled by their snow fed beds, but due to the current climate change scenario, increasing soil temperature leads to earlier snow layer thinning, so the stratifying requirement of the seeds may not be completed, resulting in low propagation via seeds in some alpine flora in the near future (Briceño et al. 2015). As a result of the investigation, the failure of *R. anthopogon* to germinate without stratification may be a dangerous circumstance for the species.

GA_3 pre-treatment has been shown to promote total seed germination in several floras with dormant or non-dormant seeds (Herranz et al. 2010), as well as to increase seed germination percentage and speed. In our study, GA_3 was able to increase germination percentage by 20–25% in *R. anthopogon* and also accelerate germination pace with lower MGT, greater germination percentage on the 20th day, and earlier germination beginning. Other *Rhododendron* species, including *R. niveum* (Singh et al. 2010), R. *purdomii* (Zhao 2014), and *R. aureum* (Shimono and Kudo 2005), showed improved germination after GA3 treatment. Even low temperatures improved germination by increasing endogenous GA_3 levels; GA_3 improves seed germination by lowering endosperm resistance and encouraging radical development during germination (Baskin and Baskin 2001). On the basis of final seed germination percentages, the soil substratum was shown to be the most effective for *R. antopogon* seed germination; nevertheless, the difference between the substratums for seed germination features is not as pronounced. When cold pre-treated *R. anthopogon* seeds are treated with GA_3 prior to germination, any substrate, such as filter paper, native soil, or MS media, can be used. As a result, the applicability and suitability of a specific substratum can be crucial on a necessity basis. For example, MS media is the best substratum for preparing disease-free sterile seedlings, while soil substratum is the best for growing plants in the field.

Conclusion

R. anthopogon is an important but understudied plant of the Western Himalaya with high bio-prospecting potential and a dominant shrub species in its habitats. The current study shows that the altitudinal gradient influences the phytosociology, morphology, and physio-biochemical components of *R. anthopogon*. According to the findings of the current study, *R. anthopogon* is one of the most dominant shrub species at higher elevations, where it provides a safe and warm environment for the growth and survival of many herbaceous species. The study demonstrated the importance of *R. anthopogon* leaf morphological traits in its widespread distribution and successful adaptation to harsh alpine conditions. At higher elevations, the leaves of *R. anthopogon* become smaller and thicker with lower SLA, allowing them to adapt with severe environmental conditions. The physio-biochemical components of the leaves, on the other hand, showed significant alteration from summer to winter. Winter was indicated by increased pigment, soluble carbohydrates, soluble proteins, and total phenolic content, while lipid peroxidation and antioxidant enzyme were decreased, indicating the acquisition of perfect freezing resistance prior to the harsh winter. Our research on seed germination in *R. anthopogon* concluded that suitable cold stratification treatment is required for *R. anthopogon* seed

germination. The increasing occurrence of global warming, which causes soil warming and limited snow cover in alpine places, is a concerning situation for sexual reproduction in alpine flora, including *R. anthopogon*, whose seeds require prolonged cooling for germination. GA_3 treatments can significantly boost the germination of *R. anthopogon* seeds, which may lead to increased seedling performance and is the topic of further research.

Declarations

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Competing Interest

Authors declare no competing interests

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Author Contributions Statement

"Trivedi VL conceptualized the idea, Maurya SJ, Sati P, Trivedi VL and Jamloki A performed the experiments, Trivedi VL and Jamloki A wrote the main manuscript text and prepared figures. Sati P and Chandra S had done the calculations and the Statistical Analysis. Nautiyal MC reviewed the manuscript."

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Figures

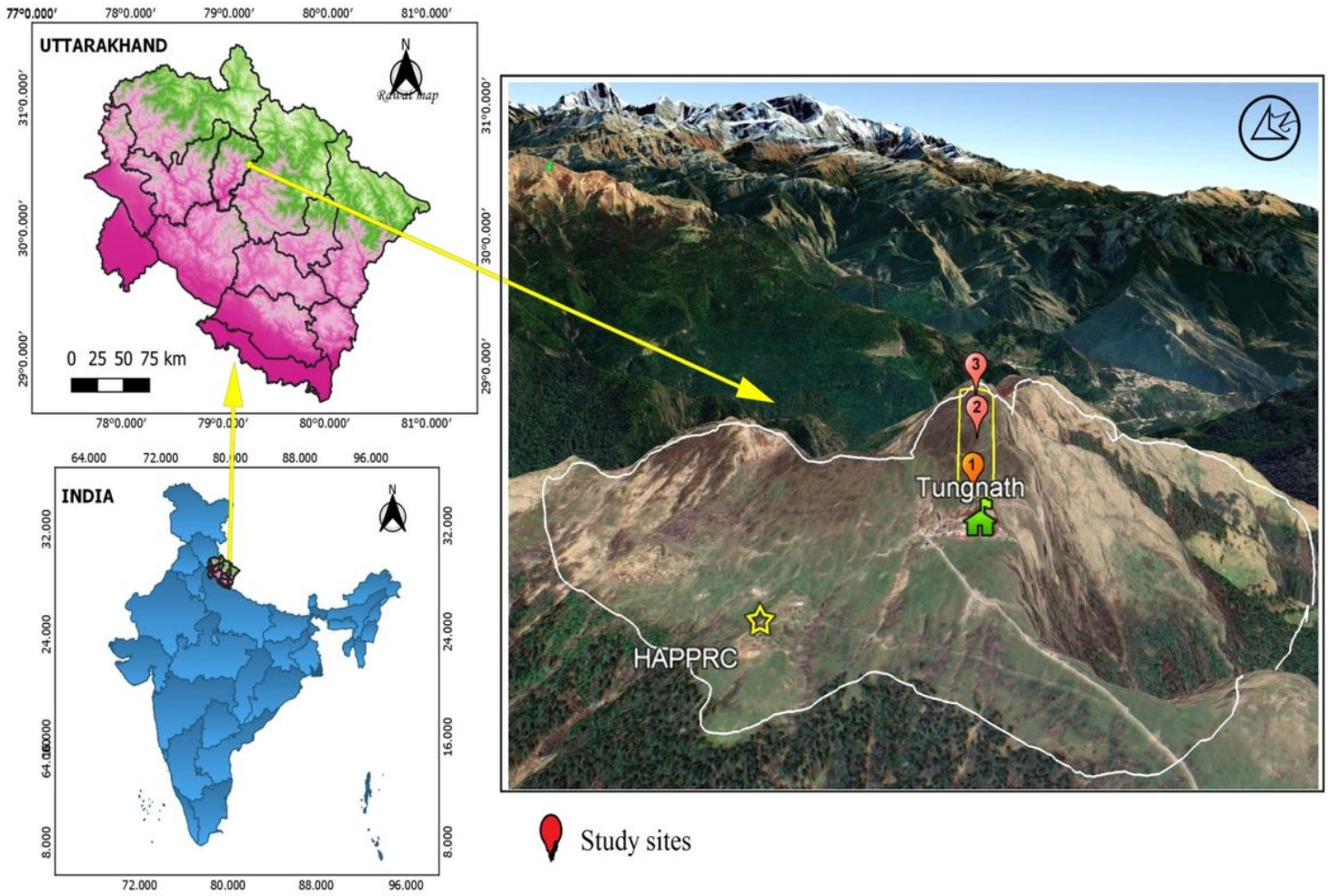

Figure 1

Location of the study area

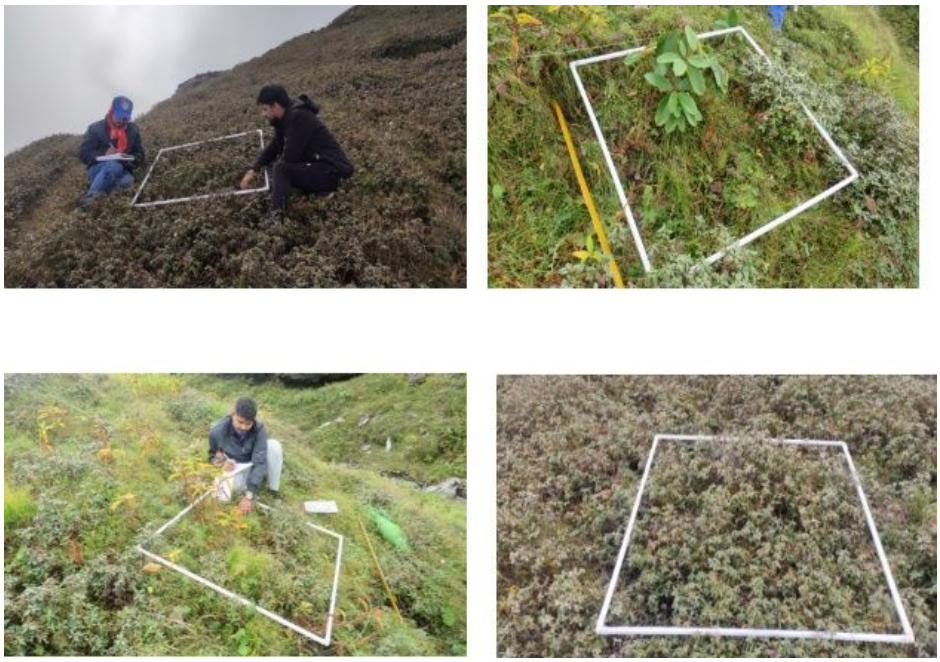

Figure 2

(a-d) Photograph during field survey in Tungnath region with *R. anthopogon*

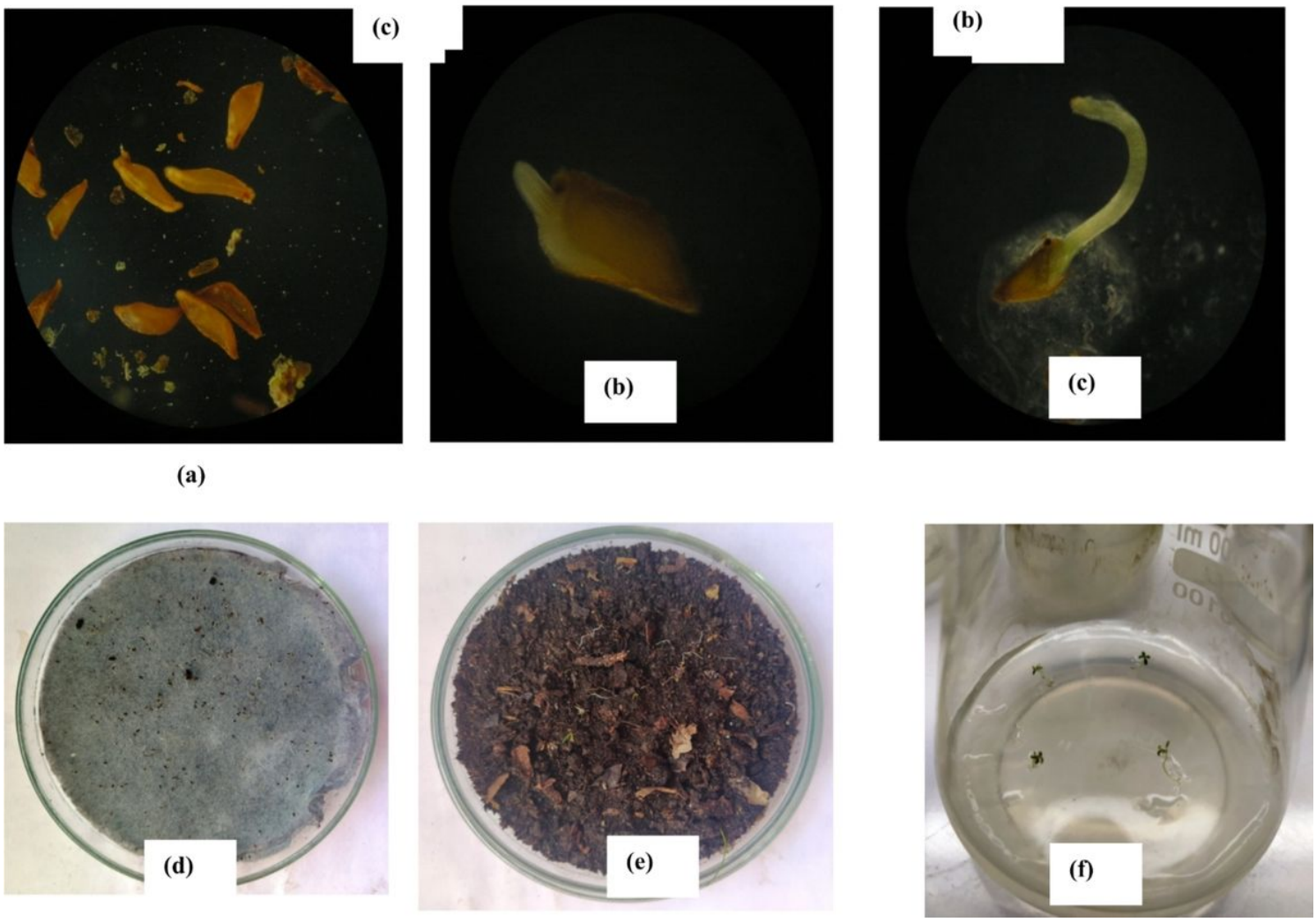

Figure 3

Seed germination in *R. anthopogon*- a. Mature Plant in flowering stage, b. Seed under microscope (10 X), c. Emergence of the radicle under microscope (10 X), d. Enlargement of the radicle and emergence of the cotyledons under microscope (10 X), e. Seedling under microscope (10 X), f. Seed germination treatment in filter paper, g. Seed germination treatment in soil, Seed germination treatment in MS media.

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