

Intra-Annual Variation in Leaf Anatomical Traits of an Overwintering Shrub of High Elevations of Himalaya

Nikita Rathore, Dinesh Thakur, Nang Elennie Hopak
and Amit Chawla

Abstract

Trait variability in response to seasonal variations can be hypothesised as an advantageous strategy for overwintering shrubs. This hypothesis was tested by elucidating patterns of trait variation in an evergreen alpine shrub, *Rhododendron anthopogon* D. Don. The study site was established at Rohtang (3990 m a.s.l.) in western Himalaya. Its leaves were sampled at 10 time points spanning a period of 1 year (beginning from 22-August-2017 to 14-August-2018) for estimating anatomical traits using light and scanning electron microscopy. The data were analysed using one-way analysis of variance, and the trait-temperature relationships were analysed using linear regression. The results indicated a lower variability in the anatomical traits. A few traits (e.g. cuticle thickness and epidermal scales) were found to be significantly correlated with temperature ($p < 0.05$). Our analysis revealed increase in cuticle thickness and a decrease in epidermal scales (size) during low-temperature conditions. The lesser variability found in anatomical traits of overwintering shrub could be explained as 'evolutionary gained adaptive traits'.

Keywords: acclimatory responses, broadleaved evergreen shrub, *in situ* analysis, glandular scales, seasonal variations, temperature regime

1. Introduction

Temperature, as one of the major abiotic factors in high altitudes limiting plant growth and distribution, varies on seasonal basis to a greater extent [1]. Therefore, the plants growing in such environments must be able to respond to these changes by actively acclimating their biology [2, 3]. Most exposed in this regard are the overwintering woody perennials, which get subjected to substantial variations in temperature in the course of a year, from temperatures reaching as high as up to 30°C the during summers to severe freezing conditions (dropping below –30°C) in the winters [4]. Because the overwintering evergreen woody perennials are exposed to seasonal shifts in temperature (favourable to harsh), they are expected to have evolved better to exhibit transitory acclimatory responses [5, 6]. Therefore, these represent an excellent system to study plant persistence strategies in harsh environments.

At high altitudes, plants must develop their ‘defences’ (during harsh conditions) necessary to survive in a highly variable environment [7]. It has been proposed that persistence in such environments is likely to be facilitated by plasticity in ‘anatomical traits’ of plants [3, 8]. For instance, the changes in leaf anatomical traits (e.g. increased leaf thickness along altitudinal gradient) have been reported to be linked to the plant’s adaptation to changes in environmental conditions (e.g. decrease in temperature, etc.) [9, 10]. However, so far little is known about differential anatomical responses of overwintering evergreen shrubs to seasonal variability. An understanding of the patterns of variation in these traits, while they experience seasonal shifts, will provide insights regarding adaptive strategies in varying environmental conditions (from favourable to harsh).

Rhododendron anthopogon is a dominant evergreen woody shrub occurring in the alpine regions in Himalaya (**Figure 1**) [11]. It is also among those shrub species which are reported to occur at the highest elevations in Himalaya. The species has a broad niche width [12]. In its natural environment, this shrub often gets subjected to variations in environmental conditions along spatio-temporal gradients. All these characteristics indicate well adaptability of this species to grow in the high-altitude environment.

Studying adaptive responses of species growing in their natural habitat could provide invaluable information about how plants prepare themselves to persist under changing environmental conditions. The common approach to investigate plant responses to environmental variability has been to resample the same plant population(s) and directly make comparison between them [13]. *R. anthopogon* with overwintering foliage is appropriate for studying intra-annual adaptive responses, as its leaves have a life span of more than 1 year, thus enabling repeated measurements on a given leaf type to be conducted across multiple time points. This evergreen species provides an opportunity to study the cold acclimation physiology in overwintering foliage without any interference of any endo-dormancy transitions that are reported to occur in other perennials [14]. So, this alpine dwarf shrub could be utilised to understand plant adaptation strategies in harsh environmental conditions of high elevations.

In the present study, the aim was to understand the extent of variations in leaf anatomical traits that enable plant survival in the harsh climate of high elevations. This study will shed light on strategies of plants to persist in high-elevation natural ecosystems. Specifically, it was hypothesised that in response to seasonal variability of high-elevation environments, (i) the evergreen species will show conspicuous changes in anatomical traits (towards optimal values for a given temperature) if their foliage has to last throughout the year including the overwintering phase, (ii) changes



Figure 1.
R. anthopogon in flowering stage.

in trait values will show reverse trends once the conditions shifts towards growth optima. Specifically, the objectives of the study were: (i) to elucidate the patterns of variability in leaf anatomical traits along the seasonal gradient and (ii) to test for a relationship between traits and temperature.

2. Plant sampling

The study was conducted at a site near Rohtang Pass which lies in the east of Pir Panjal Range of western Himalaya, India. This place is characterised by severe cold and long winters with plenty of snowfall. The study site was established at an elevation of 3990 m a.s.l. (32°37'41" N latitude and 77°25'65" E longitude). At the study site, the vegetation remains under snow cover from mid-November to the end of May, and thus, has a short growing season.

To have accurate estimation of trait variability, the study necessitated repetitive trait measurements to be conducted on leaves developed in the same year. As observed in the field, the newly developed leaves of *R. anthopogon* become fully expanded during mid-August. At this stage, the abaxial surface of these leaves is yellowish-green in colour. Thereafter, a transition to characteristic brownish colour of abaxial surface could be observed towards the onset of winters. Afterwards, the brownish colour remains as such, as the plants progress through overwintering phase till senescence in the following year. Likewise, the leaves developed in the same year could be identified by looking at the position and colour pattern on abaxial surface of leaves. If the leaves developed in two consecutive years are compared during August, leaves developed in first year will occupy the lower position and will have brownish colour on the abaxial surface, whereas leaves developed in the following year will occupy the uppermost whorl position and will be completely yellowish-green (**Figure 2**).

Considering all these facts, the sampling was started in the third week of August, 2017, and continued till November in the same year. Further measurements were not possible as the plants got covered under a thick layer of snow for approximately 240 days. Sampling was again resumed after the snow-melt in mid-June which was continued till second week of August, 2018, to complete a full annual cycle. Sampling was done multiple times in a successive manner with an interval of 2–3 weeks, depending on availability of clear sunny days. Thus, the



Figure 2.
Leaves of R. anthopogon in the month of August [leaves developed in previous year occupy the lower position (brownish colour of the abaxial surface; blue arrow), whereas leaves developed in the present year occupy the uppermost whorl position and are yellowish-green (red arrow)].

sampling was accomplished for a total of 10 different time points: 22-August-2017, 12-September-2017, 29-September-2017, 11-October-2017, 23-October-2017, 4-November-2017, 15-June-2018, 28-June-2018, 14-July-2018 and 14-August-2018. At every sampling time point, leaves were cut under deionised water and fixed immediately in FAA [comprising Formaldehyde: Glacial acetic acid: Absolute ethanol in 1:1:18 ratio [15]] to measure the anatomical traits, such as total leaf thickness and thickness of cuticle, epidermis (both adaxial and abaxial), palisade and spongy parenchyma and total mesophyll. Also, sun-exposed, healthy, fully expanded leaves of *R. anthopogon* were collected to perform scanning electron microscopy (SEM) to observe the adaxial and abaxial surfaces of a leaf.

Temperatures (for both air and soil) were recorded at the study site during the entire study period, i.e. from August-2017 to August-2018 using temperature data loggers (M-Log5W, GEO Precision, Germany) [16]. Further, to extract the temperature values for each sampling time point, data values of 3 days (sampling day and the 2 days preceding this day) were used as suggested by Lee et al. [17]. The extracted temperature values were used to calculate the mean, minimum and maximum for a particular 'sampling time-point' for use in regression analysis.

3. Leaf anatomical measurements

Leaf sample preparations for light microscopy and SEM analysis were performed following the method of Tripp and Fatimah [15]. For light microscopy, rectangular pieces of leaves fixed in FAA were cut transversely avoiding the midrib. The samples (transverse sections) were passed through tertiary butyl alcohol (TBA) series (50–100%) and infiltrated with paraffin wax (58–60°C). The sections were embedded in small blocks of paraffin wax, and leaf samples of 12- μ m size were obtained with a rotary ultra-microtome (Shandon™ Finesse™ 325, Thermo Scientific). The samples were progressively dehydrated in an ethanol series (30–100%), followed by double staining with 1% aqueous safranin and 0.5% fast green. The sections were permanently mounted on to slides with di-butyl phthalate polystyrene xylene (DPX). Micrographs were taken with camera (Nikon Digital Camera, D5300, Nikon Inc., Japan) mounted on light microscope (Nikon Eclipse E200, Nikon Inc., Tokyo, Japan), focussed at 40 $\times$. Total leaf thickness along with thickness of cuticle, epidermis (both adaxial and abaxial surface), palisade and spongy parenchyma and mesophyll tissue were measured (in μ m) in randomly selected microscopic fields using ImageJ software. Maximum of three values were taken from three microscopic fields, respectively. These were later averaged for a given trait making one replicate. A total of five such biological replicates were estimated. ImageJ was calibrated with an image of ocular micrometre scale (taken at 40 $\times$).

For SEM, five leaves from samples collected at every sampling time point were used to determine the size of epidermal scales. Single leaf tissue was cut into two small rectangular pieces (about 4 $\times$ 4 mm) from either side of midrib in order to have representations of both the surfaces (adaxial as well as abaxial) of leaf, making it a replica. Both the surfaces of a leaf were mounted immediately on a single aluminium stub and then coated with a thin film (~30 nm) of gold-palladium for 3 minutes (15 kV, 20 mA) in a sputter coater (Hitachi coating unit E1010). The images were taken using a scanning electron microscope (Hitachi S3400N) at scales of 400 and 200 μ m. SEM micrographs taken at 200 μ m were used to determine the size of abaxial epidermal scales using image J software. Ten scales per micrograph were selected to calculate the diameter (twice), followed by their area estimation (assuming scale to be circular).

4. Statistical analysis

The mean $\pm$ standard deviation was calculated from five independent replicates for all the variables considered for the samples collected at each sampling time point. Linear model assumptions of normality and homoscedasticity were tested using Shapiro-Wilk test and modified Levene's test, respectively. After the data met basic requirements of analysis of variance, one-way ANOVA was performed, and the means were compared to understand the variations in anatomical leaf traits across the 10 time points. This was followed by Tukey's *post hoc* test for identifying the significant pairwise differences ($p < 0.05$). All the analyses were performed with R 3.6.1 statistical software [18]. Linear regression was performed to test the dependence of all the traits on temperature using 'ggscatter' in 'ggpubr' package [19] of R.

5. Anatomical trait variability and temperature relationships

The overall range of leaf anatomical traits of *R. anthopogon* was estimated across the different time points of the growing season (**Table 1**). The total leaf thickness was estimated to be between 24.45 and 30.36 μm during the whole study period. A thick layer of cuticle (0.69–0.84 μm) was present above the adaxial epidermis. Thickness of adaxial and abaxial epidermis varied from 0.93 to 1.16 μm and from 1.12 to 1.26 μm , respectively. The mesophyll tissue (21.14–27.45 μm) comprised of elongated palisade (12.74–16.17 μm) and isodiametric spongy parenchyma cells (8.56–11.03 μm). The transverse section (T.S) of a leaf of *R. anthopogon* is shown in **Figure 3**.

Further, the change in mean values of some of the leaf anatomical traits of *R. anthopogon* across different sampling time points was found to be statistically significant ($p \leq 0.05$), as revealed through one-way ANOVA (**Table 2**). In particular, there were significant differences in total leaf thickness as well as thickness of mesophyll and palisade parenchyma [However, no specific patterns were observed in these anatomical traits along the seasonal gradient (**Figure 4**)]. The cuticle thickness was found to be the highest during onset of winter season (4-November-2017) and the lowest in August (22-August-2017 and 14-August-2018) (**Figure 4**), which is the peak growing season. No significant differences in adaxial and abaxial epidermal thickness were observed for leaves sampled during different time points (**Figure 4**).

The adaxial and abaxial surfaces of leaf showed the presence of glandular scales (**Figures 3 and 5**). These were typically distributed throughout the abaxial surface of leaves during whole of the study period. However, these were mainly observed on the adaxial surface during August (22-August-2017 and 14-August-2018). The size of these glandular scales was estimated to be in the range from 0.015 to 0.025 mm^2 . Variations in the size of abaxial epidermal scales due to changes in temperature regime were significantly more pronounced in comparison to other studied anatomical traits. Their size decreased during the early winter time points (i.e. 23-October-2017 and 4-November-2017) (**Figure 6**).

It was found that the air and soil temperatures were found to be positively correlated with each other. Moreover, similar correlation was observed between majority of the studied traits and temperature (both air and soil) ($p < 0.05$); therefore, the results for air temperature only are presented here (**Figure 7**). A positive correlation with temperature was observed for total thickness, mesophyll thickness and spongy parenchyma thickness. However, the thickness of cuticle increased with decreasing temperature (**Figure 7**). Thickness of epidermis (adaxial and abaxial), palisade

Time-point	Total thickness	Cuticle thickness	Adaxial Ep. thickness	Abaxial Ep. thickness	Mesophyll thickness	Palisade thickness	Spongy thickness	Scales size (mm ²)
22-August-2017	26.44 ± 1.058	0.712 ± 0.051	0.927 ± 0.043	1.215 ± 0.107	23.50 ± 1.430	13.12 ± 1.308	9.989 ± 1.198	0.025 ± 0.001
12-September-2017	25.53 ± 2.216	0.750 ± 0.016	1.028 ± 0.150	1.172 ± 0.089	22.19 ± 2.256	13.08 ± 1.272	8.560 ± 1.121	0.023 ± 0.007
29-September-2017	24.45 ± 1.686	0.743 ± 0.059	0.927 ± 0.136	1.146 ± 0.208	21.14 ± 2.215	13.29 ± 1.525	8.727 ± 1.118	0.023 ± 0.003
11-October-2017	25.39 ± 0.728	0.765 ± 0.042	1.075 ± 0.130	1.161 ± 0.070	21.22 ± 1.430	12.74 ± 1.379	9.066 ± 0.731	0.022 ± 0.003
23-October-2017	26.32 ± 2.139	0.774 ± 0.057	0.941 ± 0.047	1.233 ± 0.076	23.44 ± 2.543	13.92 ± 2.906	8.966 ± 1.540	0.018 ± 0.005
04-November-2017	26.98 ± 3.391	0.838 ± 0.075	0.977 ± 0.174	1.234 ± 0.117	23.63 ± 3.775	14.21 ± 2.468	8.891 ± 1.693	0.017 ± 0.002
15-June-2018	29.99 ± 5.396	0.841 ± 0.041	1.045 ± 0.098	1.244 ± 0.168	27.08 ± 6.036	14.82 ± 1.514	10.18 ± 2.268	0.015 ± 0.003
28-June-2018	28.87 ± 1.689	0.789 ± 0.069	1.043 ± 0.154	1.263 ± 0.246	25.47 ± 1.484	14.77 ± 0.426	10.16 ± 1.611	0.015 ± 0.002
14-July-2018	30.21 ± 4.268	0.778 ± 0.090	1.000 ± 0.088	1.146 ± 0.072	27.45 ± 5.181	15.90 ± 2.215	11.03 ± 2.527	0.017 ± 0.003
14-August-2018	30.36 ± 3.295	0.695 ± 0.050	1.159 ± 0.105	1.122 ± 0.099	27.20 ± 3.583	16.17 ± 1.828	10.69 ± 1.827	0.021 ± 0.004
Values are given as mean ± standard deviation. Ep., epidermis.								

Table 1.
Variability in anatomical traits [thickness in µm (n = 5)] of *R. anthopogon* during the study period.

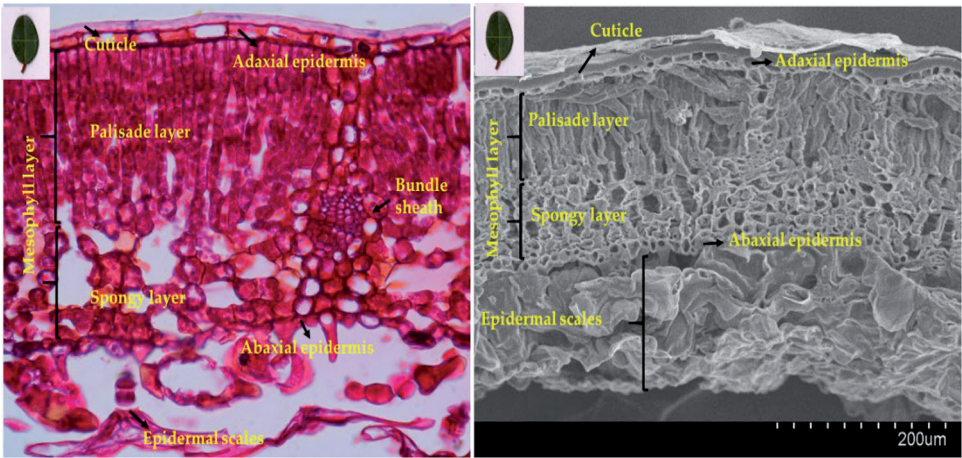


Figure 3.
View of transverse section of leaves of *R. anthopogon* using light microscopy (left) and scanning electron microscopy (right).

Parameter	Df	TSSq	MSSq	F-value	P value
Total thickness (µm)	9	220.0	24.45	2.825	0.011
Cuticle thickness (µm)	9	0.101	0.011	3.332	<0.001
Adaxial epidermis thickness (µm)	9	0.243	0.027	1.869	0.085
Abaxial epidermis thickness (µm)	9	0.111	0.012	0.645	0.752
Mesophyll thickness (µm)	9	265.7	29.52	2.610	0.018
Palisade parenchyma thickness (µm)	9	64.72	7.190	2.190	0.040
Spongy parenchyma thickness (µm)	9	35.26	3.917	1.442	0.204
Abaxial Epidermal scales (mm ²)	9	0.006	0.0007	27.18	<0.001

Df, degree of freedom; TSSq, total sum of squares; MSSq, mean sum of squares.

Table 2.
Summary of one-way ANOVA results showing the effect of ‘seasonal gradient’ factor on anatomical traits of *R. anthopogon*.

parenchyma and size of abaxial epidermal scales did not show any significant correlation with variation in temperature across the study period.

6. Anatomical traits can be explained more as ‘evolutionary gained adaptive traits’

Plants often exhibit considerable variations in their anatomical traits enabling them to adapt to changing environments [20]. Therefore, the analysis of anatomical traits is crucial for understanding of plant functioning and survival at high elevations. It has been suggested that leaf anatomical structures are associated with physiological functionality in *Rhododendron* spp. and provide more competitiveness in variable environments [21]. In this present study, anatomical traits were not found to be with much variability. Some of the anatomical traits (e.g. thickness of adaxial and abaxial epidermis and spongy parenchyma) did not show any significant variations across different sampling time points during the study period. However, statistically significant differences were found for other traits such as total leaf

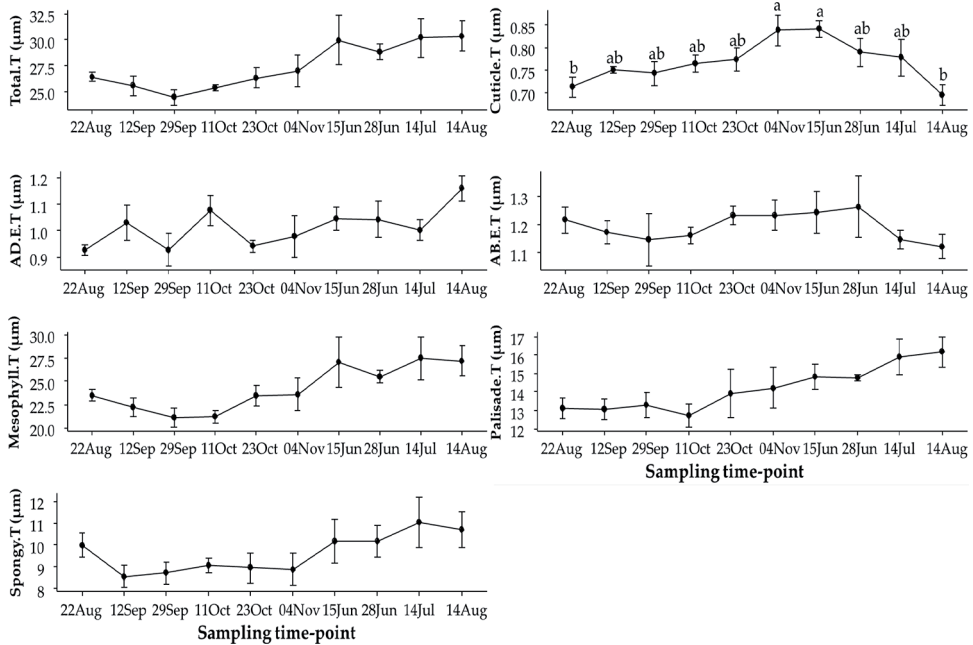


Figure 4. Variability in leaf anatomical traits of *R. anthopogon* during the study period. Vertical bars indicate standard error around the mean. Different alphabets (a, b) represent statistically significant values ($p \leq 0.05$) as determined by Tukey's post-hoc test. Legend on x-axis, i.e. from 22-August to 04-November are the sampling time points for the year 2017, whereas from 15-June to 14-August represent the sampling time points of year 2018. Total.T, total thickness; Cuticle.T, cuticle thickness; AD.E.T, adaxial epidermal thickness; AB.E.T, abaxial epidermal thickness; Mesophyll.T, mesophyll thickness; Palisade.T, palisade parenchyma thickness; Spongy.T, spongy parenchyma thickness.

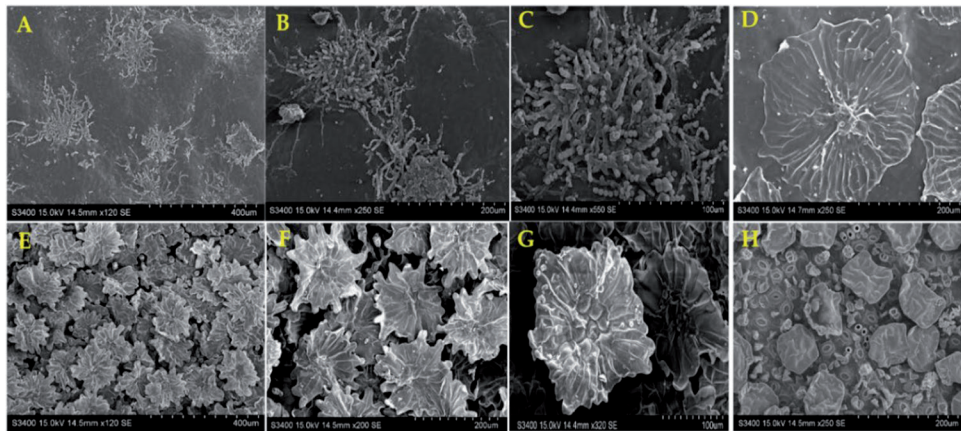


Figure 5. SEM images showing epidermal scales on the adaxial (A–D; images take at a resolution of 400 μm, 200 μm and 100 μm, respectively) and abaxial surface (E–G; images take at a resolution of 400 μm, 200 μm and 100 μm, respectively) of leaves of *Rhododendron anthopogon*. The stomata on the abaxial surface of a leaf (taken at a resolution of 200 μm) can also be observed (H).

thickness, cuticle thickness and thickness of mesophyll and palisade parenchyma. An increasing trend for cuticle thickness of leaves was observed with decreasing temperature. The cuticle thickness was found to be maximum during the onset of winter, which thereafter remained constant till snowmelt in the following year (i.e. 15-June-2018). Higher cuticle thickness in leaves helps reduce the transpiration

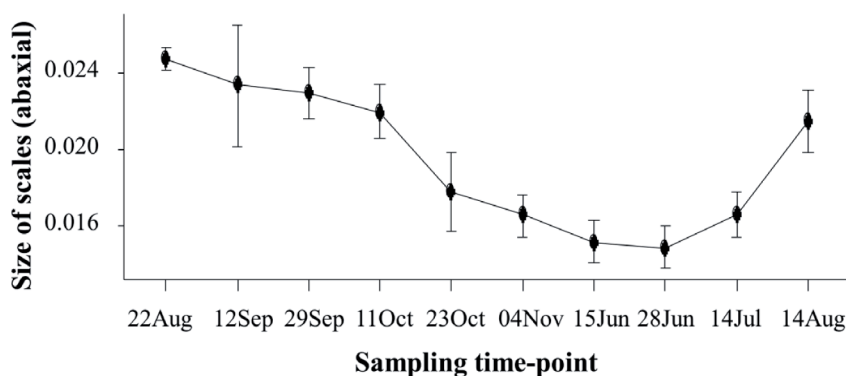


Figure 6. Variability in the size of abaxial scales (mm^2) of *R. anthopogon* during the study period. Vertical bars indicate standard error around the mean. Legends on x-axis, i.e. from 22-August to 04-November, are the sampling time points of year 2017, whereas from 15-June to 14-August represent the sampling time points of year 2018.

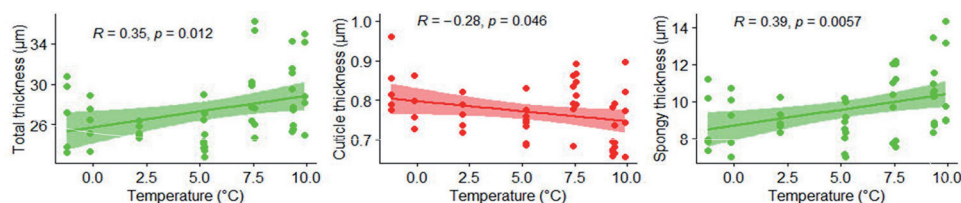


Figure 7. Regression plots for leaf traits of *R. anthopogon* and air temperature. The x-axis corresponds to temperature, whereas y-axis represents the values of anatomical traits estimated at different sampling time points. The red and green coloured regression lines in the graph represent negative and positive correlations between the two variables, respectively. Spongy, spongy parenchyma.

losses from leaf to atmosphere under low-temperature conditions, when solar radiations are also high [21, 22]. A significant positive correlation of total leaf thickness, mesophyll thickness and thickness of spongy parenchyma tissues with air temperature was also found. Also, these anatomical traits displayed a positive correlation with each other. So, a decrease in total leaf thickness with decreasing temperature could be attributed to simultaneous reduction in thickness of both mesophyll and spongy parenchyma tissues. The reduced thickness of different tissues might be an adjustment for protection from 'dehydration conditions induced due to low temperatures' (by decreasing the surface area of leaf tissues on exposure to low temperatures) [23, 24]. The spongy parenchyma have large inter-cellular spaces which have a role in mesophyll conductance (gas transportation and exchange) [25]. Due to slower metabolism in plants during low-temperature conditions, there is lesser requirement for mesophyll conductance, which probably explains the cause of reduction in the thickness of spongy parenchyma in this species during early winter conditions.

The peculiar anatomical structures such as epidermal appendages, act as evolutionary adaptive traits and help plants in protection against harsh environmental conditions [26]. In the present study, the presence of unique overlapping glandular scales on abaxial side of leaves (covering the entire surface) was observed (Figures 3–5). Scales of similar shape but larger in size could also be recognised on the adaxial surface of leaves which, however, were evident only during August. On this side of leaf, these scales were unnoticeable during rest of the time period, which could be due to deposition of epi-cuticular wax on the adaxial surface of leaves. The presence of such leaf scales has also been reported

in other *Rhododendron* species and have a specified function related to water relations, energy balance and gas exchange [27, 28]. The leaf surface topography, primarily represented by leaf hair/scales and the cuticular wax layer, is reported to shield it from the effects of low temperature and high solar radiations [29]. Similar physiological function of epidermal scales could be assigned for the studied species (i.e. protection from low temperature and high solar radiations). Further, the size of scales was observed to be lower during the onset of winter, which remained constant thereafter. This has not been reported in previous studies, which further evokes interest to investigate their functional role and dynamics. However, it can be proposed that the reduction in size could be due to increase in compactness, which may be the result of slight leaf curling as reported in other *Rhododendron* species [1, 6].

Overall, our results suggested that the leaf anatomy is relatively less sensitive to seasonal variations, as depicted by low variability observed in majority of the anatomical traits. The less variability could be attributed to the fact that the plant may not invest considerably in structural adjustments to counter seasonal variations. This can be explained by the fact that this evergreen shrub, occupying the highest elevations in the Himalaya, survives the harsh environmental conditions throughout the year, which is likely to be achieved via consistency in leaf anatomical traits. Thus, the results reinforce the idea that structural traits 'in general' are less variable and are 'evolutionary gained adaptive traits' [30, 31]. The low investment in structural adjustments may be due to their higher construction cost leading to diminishing returns [32, 33].

7. Conclusions

The findings presented here contribute to the understanding of intra-annual plant trait variability (the type of response and its magnitude) in an overwintering evergreen shrub. The evidences outlined above indicate that the leaf anatomy is less sensitive to seasonal variations. Low variability in leaf anatomical traits of *R. anthopogon* supports the concept of low-cost mechanism for attaining tolerance to harsh conditions and conservative use of resources. The results also indicated that certain evolutionary acquired adaptive traits such as epidermal glandular scales are supportive to successfully persist in harsh climates. Overall, the findings suggest that the plants in varying environments, such as high altitudes, reconfigure their anatomy to a little extent to sustain climatic variabilities.

Acknowledgements

Authors are thankful to the Director, IHBT, Palampur, for providing the necessary facilities. N.R. and N.E.H. were the recipients of senior research fellowship and junior research fellowship, respectively, from CSIR, India, during the study. Dr. Avnesh Kumari and Dr. Sita Kumari are acknowledged for their help during SEM analysis. Also, Rahul Kumar Rana, Lakhbeer Singh, Manish Kumar Sharma, Nandita Mehta and Om Prakash are acknowledged for their help with field survey.

Conflict of interest

The authors declare that they have no conflict of interest.

Author's contributions

N.R. carried out the plant sampling, anatomical trait measurements, SEM analysis, statistical analysis and drafting of the manuscript. D.T. performed the plant sampling, SEM analysis and statistical analysis. N.E.H. performed the plant sampling and anatomical trait measurements. A.C. conceived the study, designed the experiment, provided statistical guidance, edited and finalised the manuscript.

Author details

Nikita Rathore^{1,2}, Dinesh Thakur^{1,2}, Nang Elennie Hopak^{1,2} and Amit Chawla^{1,2*}

1 Environmental Technology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India

2 Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India

*Address all correspondence to: amitchawla21@gmail.com

IntechOpen

© 2022 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. 

References

- [1] Niinemets Ü. Does the touch of cold make evergreen leaves tougher? *Tree Physiology*. 2016;**36**:267-272. DOI: 10.1093/treephys/tpw007
- [2] Enquist BJ, Bentley LP, Shenkin A, Maitner B, Savage V, Michaletz S, et al. Assessing trait-based scaling theory in tropical forests spanning a broad temperature gradient. *Global Ecology and Biogeography*. 2017;**26**:1357-1373. DOI: 10.1111/geb.12645
- [3] Milla R, Giménez-Benavides L, Montserrat-Martí G. Replacement of species along altitude gradients: The role of branch architecture. *Annals of Botany*. 2008;**102**:953-966. DOI: 10.1093/aob/mcn187
- [4] Strimbeck GR, Schaberg PG, Fosdall CG, Schröder WP, Kjellsen TD. Extreme low temperature tolerance in woody plants. *Frontiers in Plant Science*. 2015;**6**:1-15. DOI: 10.3389/fpls.2015.00884
- [5] Stushnoff C, Cox SE. Temperature-related shifts in soluble carbohydrate content during dormancy and cold acclimation in *Populus tremuloides*. *Canadian Journal of Forest Research*. 2001;**31**:730-737. DOI: 10.1139/cjfr-31-4-730
- [6] Wang X, Arora R, Horner HT, Krebs SL. Structural adaptations in overwintering leaves of thermonastic and nonthermonastic *Rhododendron* species. *Journal of the American Society for Horticultural Science*. 2008;**133**:768-776. DOI: 10.21273/JASHS.133.6.768
- [7] Coldren GA. The multiple stress gradient hypothesis: Expansion of the revised stress gradient hypothesis using a mangrove and salt marsh study system [Dissertation]. Florida Atlantic University; 2013. p. 157
- [8] Henn JJ, Buzzard V, Enquist BJ, Halbritter AH, Klanderud K, Maitner BS, et al. Intraspecific trait variation and phenotypic plasticity mediate alpine plant species response to climate change. *Frontiers in Plant Science*. 2018;**9**:1548. DOI: 10.3389/fpls.2018.01548
- [9] Kofidis G, Bosabalidis AM, Moustakas M. Combined effects of altitude and season on leaf characteristics of *Clinopodium vulgare* L. (Labiatae). *Environmental and Experimental Botany*. 2007;**60**:69-76. DOI: 10.1016/j.envexpbot.2006.06.007
- [10] Lianopoulou V, Bosabalidis AM, Patakas A, Lazari D, Panteris E. Effects of chilling stress on leaf morphology, anatomy, ultrastructure, gas exchange, and essential oils in the seasonally dimorphic plant *Teucrium polium* (Lamiaceae). *Acta Physiologiae Plantarum*. 2014;**36**:2271-2281. DOI: 10.1007/s11738-014-1605-x
- [11] Liang E, Dawadi B, Pederson N, Eckstein D. Is the growth of birch at the upper timberline in the Himalayas limited by moisture or by temperature? *Ecology*. 2014;**95**:2453-2465. DOI: 10.1890/13-1904.1
- [12] Thakur D, Chawla A. Functional diversity along elevational gradients in the high altitude vegetation of the western Himalaya. *Biodiversity and Conservation*. 2019;**28**:1977-1996. DOI: 10.1007/s10531-019-01728-5
- [13] Felde VA, Kapfer J, Grytnes J-A. Upward shift in elevational plant species ranges in Sikkildalen, Central Norway. *Ecography (Cop.)*. 2012;**35**:922-932. DOI: 10.1111/j.1600-0587.2011.07057.x
- [14] Marian CO, Krebs SL, Arora R. Dehydrin variability among *Rhododendron* species: A 25-kDa dehydrin is conserved and associated with cold acclimation across diverse

- species. The New Phytologist. 2004;**161**:773-780. DOI: 10.1111/j.1469-8137.2003.01001.x
- [15] Tripp EA, Fatimah S. Comparative anatomy, morphology, and molecular phylogenetics of the African genus *satanocrater* (acanthaceae). American Journal of Botany. 2012;**99**:967-982. DOI: 10.3732/ajb.1100354
- [16] Rathore N, Thakur D, Kumar D, Chawla A, Kumar S. Time-series eco-metabolomics reveals extensive reshuffling in metabolome during transition from cold acclimation to de-acclimation in an alpine shrub. Physiologia Plantarum. 2021;**173**:1-17. DOI: 10.1111/ppl.13524
- [17] Lee TD, Reich PB, Bolstad PV. Acclimation of leaf respiration to temperature is rapid and related to specific leaf area, soluble sugars and leaf nitrogen across three temperate deciduous tree species. Functional Ecology. 2005;**1**:640-647. DOI: 10.1111/j.1365-2435.2005.01023.x
- [18] R Development Core Team. R: A language and environment for statistical computing. R Found. Stat. Comput. 2019;**1**:409. DOI: 10.1007/978-3-540-74686-7
- [19] Kassambara A. ggpubr:“ggplot2” based publication ready plots. R Package Version 0.1 6. 2017
- [20] Peguero-Pina JJ, Sisó S, Sancho-Knapik D, Díaz-Espejo A, Flexas J, Galmés J, et al. Leaf morphological and physiological adaptations of a deciduous oak (*Quercus faginea* Lam.) to the Mediterranean climate: A comparison with a closely related temperate species (*Quercus robur* L.). Tree Physiology. 2016;**36**:287-299. DOI: 10.1093/treephys/tpv107
- [21] Cai Y-F, Li S-F, Li S-F, Xie W-J, Song J. How do leaf anatomies and photosynthesis of three *Rhododendron* species relate to their natural environments? Botanical Studies. 2014;**55**:36. DOI: 10.1186/1999-3110-55-36
- [22] Oliveira MT, Souza GM, Pereira S, Oliveira DAS, Figueiredo-Lima KV, Arruda E, et al. Seasonal variability in physiological and anatomical traits contributes to invasion success of *Prosopis juliflora* in tropical dry forest. Tree Physiology. 2017;**37**:326-337. DOI: 10.1093/treephys/tpw123
- [23] Chabot BF, Chabot JF. Effects of light and temperature on leaf anatomy and photosynthesis in *Fragaria vesca*. Oecologia. 1977;**26**:363-377. DOI: 10.1007/BF00345535
- [24] Prozherina N, Freiwald V, Rousi M, Oksanen E. Interactive effect of springtime frost and elevated ozone on early growth, foliar injuries and leaf structure of birch (*Betula pendula*). The New Phytologist. 2003;**159**:623-636. DOI: 10.1046/j.1469-8137.2003.00828.x
- [25] He N, Liu C, Tian M, Li M, Yang H, Yu G, et al. Variation in leaf anatomical traits from tropical to cold-temperate forests and linkage to ecosystem functions. Functional Ecology. 2018;**32**:10-19. DOI: 10.1111/1365-2435.12934
- [26] Bosabalidis AM, Kofidis G. Comparative effects of drought stress on leaf anatomy of two olive cultivars. Plant Science. 2002;**163**:375-379. DOI: 10.1016/S0168-9452(02)00135-8
- [27] Nilsen ET, Webb DW, Bao Z. The function of foliar scales in water conservation: An evaluation using tropical-mountain, evergreen shrubs of the species *Rhododendron* in section *Schistanthe* (Ericaceae). Australian Journal of Botany. 2014;**62**:403-416. DOI: 10.1071/BT14072
- [28] Wang X, Mao Z, Choi K, Park K. Significance of the leaf epidermis fingerprint for taxonomy of Genus

Rhododendron. Journal of Forest Research. 2006;**17**:171-176.
DOI: 10.1007/s11676-006-0041-1

[29] Sheperd T, Griffiths DW. The effects of stress on plant cuticular waxes. The New Phytologist. 2006;**171**:469-499

[30] Aroca R. Plant Responses to Drought Stress: From Morphological to Molecular Features. Berlin, Heidelberg: Springer; 2013.
DOI: 10.1007/978-3-642-32653-0

[31] Matesanz S, Valladares F. Ecological and evolutionary responses of Mediterranean plants to global change. Environmental and Experimental Botany. 2014;**103**:53-67. DOI: 10.1016/j.envexpbot.2013.09.004

[32] Niklas KJ, Cobb ED, Niinemets Ü, Reich PB, Sellin A, Shipley B, et al. “Diminishing returns” in the scaling of functional leaf traits across and within species groups. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**: 8891-8896. DOI: 10.1073/pnas.0701135104

[33] Thakur D, Rathore N, Chawla A. Increase in light interception cost and metabolic mass component of leaves are coupled for efficient resource use in the high altitude vegetation. Oikos. 2019;**128**:254-263. DOI: 10.1111/oik.05538