J. Orchid Soc. India, 38: 67-75, 2024 ISSN 0971-5371

ECOPHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF *DACTYLORHIZA HATAGIREA* (D.DON) SOO ALONG AN ALTITUDINAL GRADIENT IN THE WESTERN HIMALAYAS

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Abstract

Environmental adaptations necessitate the modulation of photosynthetic processes and water vapor exchange in plant species, thereby influencing key biochemical and physiological processes that govern overall plant function and productivity. Alpine plant communities are particularly susceptible to these environmental perturbations, requiring intricate adaptive mechanisms to sustain their ecological viability. Given the heightened sensitivity of high-altitude flora to climate-induced shifts, it is crucial to elucidate species-specific responses to these changes. The present study investigates the eco-physiological and biochemical variations in *Dactylorhiza hatagirea* along an altitudinal gradient, providing insights into the adaptive strategies of high-elevation species under climate warming scenarios.

Introduction

ALPINE ENVIRONMENTS changing at an unprecedented rate across the globe have attributed to climate variability, land use, land cover change, and anthropogenic pressures. Therefore, the ability of populations to track climate change in a timely manner may prove pivotal for its sustained existence. Plants exhibit remarkable plasticity to endure shifting environmental conditions corresponding to the altitude. The adaptability of species in their reaction to environmental factors may encompass modifications in structure, form, and functions (Singh *et al*., 2021, 2022). This allows plants to withstand challenging climatic conditions at high altitudes and maintain an effective carbon acquisition system to compensate for the relatively brief growing season (Wehn *et al*., 2014). When studying plant adaptation and stress responses in the presence of abiotic variables, altitudinal gradient studies are invaluable and can be used to comprehend how climate change impacts plants for adaptation and survival. Examining these patterns of plant activity along elevation gradients allows us to indirectly gauge the potential effects of climate change. In totality, it can serve for the rapid selection of plants for a particular environmental condition or the selection of suitable environmental conditions for a particular plant species (Chandra *et al*., 2008; Joshi and Palni, 2005; Singh *et al*., 2022). Plant eco-physiological studies are important, particularly when dealing with plant conservation, reintroduction, and developing cultivation packages. Species in the mountains are confronted by steep fluctuation in environmental gradients associated with increasing altitude, *i.e.,* reduced atmospheric pressure and temperature, increased light intensity and UV radiation, decrease in the length of the growth period, increase in precipitation, reduction in soil microbial activity, prolonged duration of snow cover and cryoturbation *etc*. (Körner, 2007; Stastna *et al*., 2012; Stöcklin *et al*., 2009). Further, changes in land use, land cover, and climate (mainly temperature and precipitation) have indirectly impacted the biotic interaction, thus affecting plant-pollinator relationship (Olsen and Klanderud, 2014; Prakash and Pathak, 2020, 2022; Singh *et al*., 2023). Temperature stress, in particular, imbalances the tissue water status, reduces photosynthesis efficiency, changes resource allocation cycle, affecting different life cycle stages (Wehn *et al*., 2014). Overall, it impacts the distribution and abundance of the species in a given region. The ability of species to acclimatize and adapt to a set of environmental variations, depends (directly/indirectly) on their ability to modulate photosynthesis and water vapor exchange rates. This in turn, affects biochemical and physiological processes and subsequently the physiology and productivity of the species (Chandra *et al*., 2008). Studies involving gas exchange characteristics (infrared gas analyzer) have provided valuable information on the functioning of plants in a set of environmental conditions. However, much of the emphasis has mainly been conducted in crop plants; only a few studies have focused on medicinal plants, but the number in the Himalayan region is much lower. Some of the prominent species undertaken for eco-physiological studies include *Boehmeria rugulosa* Wedd., *Cannabis sativa* L. (Chandra *et al*., 2008), *Dendrobium moniliforme* (L.) Sw. (Wu *et*

Received: April 29, 2024; Accepted: August 20, 2024

al., 2016), *Hedychium spicatum* Sm. (Pandey *et al*., 2021), *Hypericum perforatum* L. (Sharma *et al*., 2020), *Megacarpaea polyandra* Benth. ex Madden, *Olea glandulifera* Desf. (Joshi *et al*., 2007, *Plantago major* L. (Mudrik *et al*., 2003), *Rheum emodi* Wall., *R. moorcroftianum* Royle, *Rumex nepalensis* Spreng. (Kumar *et al*., 2008; Rawat and Purohit, 1991), *Selinum vaginatum* C.B. Clarke (Pandey *et al*., 1984), *Sinopodophyllum hexandrum* Royle (Vats and Kumar, 2006), *Stevia rebaudiana* (Bertoni) Bertoni (Kumar *et al*., 2013), and *Valeriana jatamansi* Jones (Pandey and Kushwaha, 2005; Vats *et al*., 2002). Therefore, the present study was aimed to study the adaptability of *Dactylorhiza hatagirea* (D.Don) Soo (Fig. 1), an important terrestrial orchid along the elevation gradient by studying ecophysiological and biochemical traits which may be of significance for species cultivation and re-introduction programmes.

Material and Methods

Study Area and Instrument Used

Experiments on eco-physiological responses of *Dactylorhiza hatagirea* were conducted in Pinder valley

Fig.1. Flower and tuber characteristics of *Dactylorhiza hatagirea* in natural habitat.

(30°05'E - 30°11' N; 79°54' - 79°59'E) of Kumaun region along the elevation gradients from 3000 to 4000 m amsl. A portable photosynthesis system (Li-COR 6800; Lincoln, NE, USA) was used to monitor gas exchange behaviour. The instrument was calibrated for CO_2 and H_2O using a CO_2 scrubber and desiccators. Gas exchange behaviour was measured by calculating net photosynthetic rates (PN) and stomatal conductance (Gs) at different photosynthetic photon flux density (PPFD) ranging between 0-2000 μmol $m^{-2}s^{-1}$. Similarly, net photosynthetic rates versus intercellular CO_2 concentration (PN versus Ci) curves were obtained using a 6800-01CO_2 injector. In each calculation, the time interval of 90 sec was provided for the leaf to equilibrate to the new conditions. Data was obtained by automated logging in triplicates.

Light Response Curve

Light response curve was prepared between 8:00 am to 12.00 am for leaves of 5 different plants of *D. hatagirea*. The airflow rate, relative humidity (RH; %), leaf temperature (TL; °C), vapour pressure deficit (VPD; KPa), reference CO_2 concentration were kept constant. The light response curves were prepared at different PPFD starting from 0 to 2000 μmol $m^{-2}s^{-1}$ (Fig. 2a). For measuring photosynthetic rate (PN) against varying PPFD, a healthy, fully mature leaf from the top was selected and illuminated with the respective PPFD for 20 min before recording the PN response rate. The light saturation point (LSP) was determined by plotting the light response curve at different PPFD points (*i.e.*, 0, 100, 150, 300, 600, 900, 1200, 1500, 1800, and 2000 μmol $m^{-2}s^{-1}$) versus photosynthetic rate (PN).

CO_2 Response Curves

Photosynthetic rates (PN) versus CO_2 curve were determined with leaf chamber fluorometer (Li-COR 6800-040 LCF) and 6800 CO_2 mixers, which control the flow of CO_2 concentration inside the leaf chamber. Beforehand, leaves were illuminated with a light source determined from LSP (2000 μmol $m^{-2}s^{-1}$) for 20 min. CO_2 response curves were prepared at different CO_2 concentration points (CO_2 = 0, 50, 100, 200, 300, 400 μmol mol^{-1}) versus photosynthetic rate (PN; Fig. 2b).

Physiological Measurements

The gas exchange behaviour was performed on clear days (except for passing clouds). Physiological measurements were measured with an open flow gas exchange system (Li-COR 6800). Leaf (abaxial side) was clamped in dimensions of 2×2 cm in a clear bottom chamber (Li-COR 6800) with a fluorescence head. Early morning measurements often coincided with dew present on the leaves; in that case, leaves were blotted dry. The different parameters such as net photosynthetic rate (PN), transpiration rate (E), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were determined through green, healthy leaves situated at the top position in the plants. A total of 3 leaves from 5 individuals were selected for measurement in different altitude sites.

Content of Photosynthetic Pigments

Photosynthetic pigments content was measured following cutting the leaves into small pieces (1.0 × 1.0 cm); these were then put in a mortar-pestle and ground in 5 mL of 80% acetone. The homogenized mixture was then centrifuged (6000 rpm for 20 min at 4°C). The supernatant was removed, the volume made up to 10 mL using 80% acetone and used to measure photosynthetic pigments such as chlorophyll-a (Chl-a), chlorophyll-b (Chl-b), total chlorophyll (T-Chl) and carotenoid content which were calculated using the following equations as described earlier (Wu *et al.*, 2016):

Chl-a (mgg^{-1}) = 1.526 × A663 - 0.351 × A645

Chl-b (mgg^{-1}) = 2.516 × A645 - 0.629 × A663

T-Chl (mgg^{-1}) = 2.165 × A645 + 0.898 × A663

Carotenoid (mgg^{-1}) = 4.367 × A470 - 0.016 × Chl-a - 0.454 × Chl-b

Measurement of Antioxidant Enzyme

Antioxidant enzyme activity was determined by taking 1.0g fresh leaves homogenized in liquid nitrogen and adding 5 mL of enzyme extraction buffers prepared out of 0.05 M phosphate buffer (pH- 7.8) and 3.0 g polyvinyl-pyrrolidone (PVP). The total extract was made to 10 mL by adding phosphate buffer (pH- 7.8) in centrifuge tubes and centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was collected in separate vials, sealed and stored at -20°C. The extract was used for the analysis of antioxidant enzyme activities. Various assays, namely, catalase (CAT), peroxidase (POD) (Lee and Lee, 2000), superoxide dismutase (SOD) (Das *et al.*, 2000), hydrogen peroxide (H_2O_2) (Lorento and Velikova, 2001), and lipid peroxidation (MDA) (Xiaolu *et al.*, 2016) were carried out.

Results

Light and CO_2 Response Curves

Gas exchange parameters were measured using a portable photosynthesis system (LI-6800, LI-COR Biosciences, Lincoln, NE, USA) on open habitat conditions of the species. Topmost (second or third), undamaged, and most fully expanded leaves were used for measurements between 8:00 am to 12:00 am. The light response curve was prepared by varying photosynthetic photon flux densities (PPFD, 0-2000 μmol $m^{-2}s^{-1}$) with the help of an artificial light source (light-emitting silicon diode; LI-COR), fixed on the top of the leaf chamber, while the reference CO_2 concentration was set to 380 μmol mol^{-1}. Along the altitudinal gradient, plants at 3800 m reached photon saturated photosynthesis [PN 8.18 μmol (CO_2) $m^{-2}s^{-1}$] at PPFD around 1800 μmol $m^{-2}s^{-1}$, while plants at 3200 m, 3400 m, and 3600 m reached around 2000 μmol $m^{-2}s^{-1}$. Plants at 3400 m recorded maximum photosynthesis 9.45 μmol (CO_2) $m^{-2}s^{-1}$, while lowest [8.01 μmol (CO_2) $m^{-2}s^{-1}$]) was recorded at 3800 m (Fig. 3a). Similarly, CO_2 response curves were determined as a ratio of PN to Ci, while PPFD was kept constant at 2000 μmol $m^{-2}s^{-1}$. For generating PN/Ci curves, different concentration of CO_2 concentration (100-400 μmol mol^{-1}) were supplied in leaf chamber using 3800-01 CO_2 injector. PN determined at different Ci was higher in plants growing at 3400m [9.05 μmol (CO_2) $m^{-2}s^{-1}$] than plants at 3200 m [lowest, 8.81 μmol (CO_2) $m^{-2}s^{-1}$] at maximum (400 μmol mol^{-1}) CO_2 concentration (Fig. 2b). In conclusion, the study highlights, PPFD as one of the important abiotic factors for gas exchange behavior of the species along the altitudinal gradient (Fig. 2c).

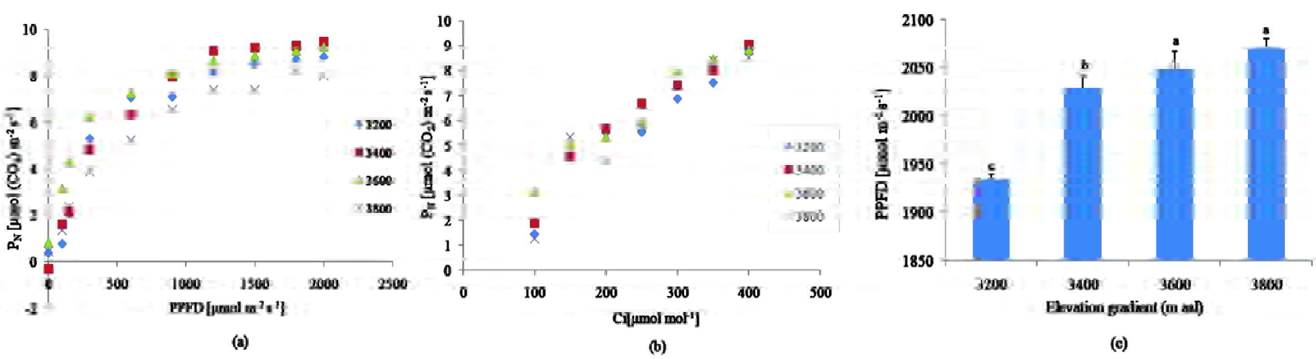

Fig. 2a-c. Response of net photosynthetic rate (PN): a, PPFD; b, Different CO_2 concentration; c, Average PPFD perceived at respective altitude. Results are represented as a means (±standard error). PPFD means with different superscript letters are significantly different (p <0.05) according to Duncan's multiple range test (DMRT).

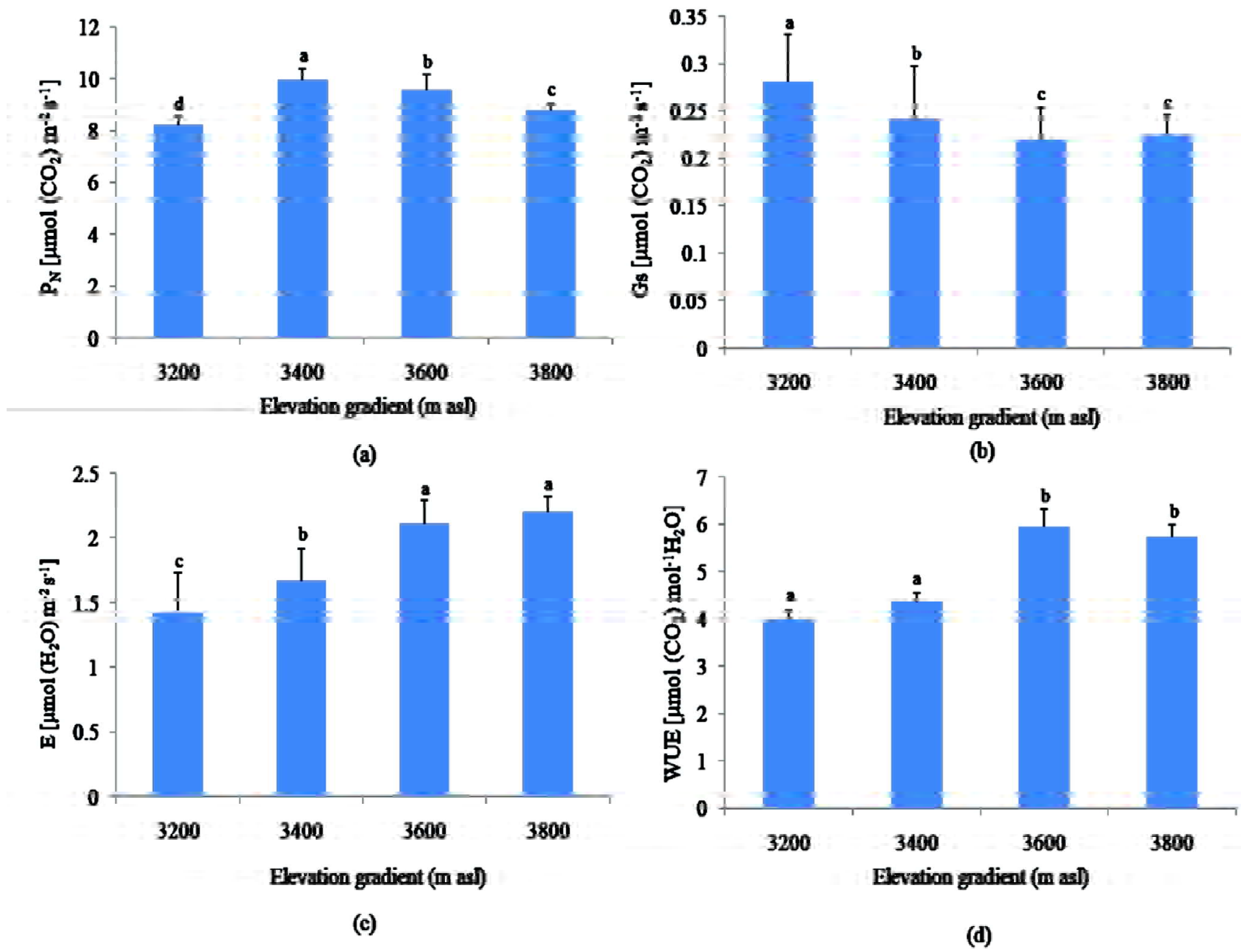

Fig. 3a-d. Variations in different parameters: a, Variations in net photosynthetic rate (PN); b, Stomatal conductance (Gs); c, Transpiration rate (E); d, Water use efficiency (WUE) along the elevation gradient. Results are represented as a means (±standard error). Means with different superscript letters are significantly different ($p<0.05$) according to Duncan's multiple range test (DMRT).

Gas-Exchange Behaviour

Various physiological parameters like photosynthesis rate (PN), transpiration rate (E), stomatal conductance (Gs), water use efficiency (WUE) and carboxylation efficiency (CE) rate *etc*. were determined. *Dactylorhiza hatagirea* showed enormous plasticity when it comes to survival under different altitudinal and environmental conditions. Briefly, net photosynthetic assimilation (PN) rate was recorded maximum [9.94 µmol (CO_2) $m^{-2}s^{-1}$] at 3400 m, while minimum [8.21 µmol (CO_2) $m^{-2}s^{-1}$] at 3200 m. In addition, increase in altitude range from 3400 m to 3800 m, a significant ($p<0.05$) decrease (*i.e.*, 21.11% to 13.37%) in PN rate was recorded (Fig. 3a). The stomatal conductance (Gs) decreased (27.81% to 9.63%) significantly ($p<0.05$) with the increase in latitude, while no definite relationship would be developed between Gs versus PN (Fig. 3b). In contrast, the definite relationship was only developed between transpiration rate and respective altitude PPFD. In both cases, an increase in altitude increased the transpiration rate (16.21% to 31.12%) and directs PPFD (4.88% to 7.02%) on the species (Fig. 3c and Fig. 2c). Furthermore, based on the data of photosynthesis rate (PN) and transpiration (E), water use efficiency (WUE) was calculated. It was found that the WUE increases (29.99% to 42.85%) with increasing PPFD and altitude; minimum (29.99%) water use efficiency was recorded at 3200 m, whereas at 3600 m showed maximum (42.85%) water use efficiency irrespective of light condition (Fig. 3d). Studies on carboxylation efficiency (CE) corroborates with PN. Maximum (21.11%) CE per cent was recorded for plants species growing at 3400m and least (13.37%) for species at 3200 m. As off PN, no clear trend could be developed between CE and altitude.

Photosynthetic Pigments Content

Photosynthetic pigments play central roles in the light reaction of the photosynthetic process of plants by

Table 1.Variation in photosynthetic pigments and biochemical activity along the elevation gradient.

Altitude (m)	Chl a (mgg^{-1} FW)	Chl b (mgg^{-1} FW)	T-Chl (mgg^{-1} FW)	Caro (mgg^{-1} FW)	CAT (U $min^{-1}g^{-1}$ FW)	POD (U $min^{-1}g^{-1}$ FW)	SOD (U $min^{-1}g^{-1}$ FW)	H_2O_2 (mgg^{-1} FW)	MDA (mgg^{-1} FW)
3200	0.78±0.01^{c}	0.58± 0.034^{ab}	1.95±0.061^{b}	0.29 ± 0.027^{c}	0.11± 0.014^{b}	2.25 ± 0.22^{c}	2.18 ± 0.08^{d}	1.37 ± 0.13^{b}	0.28 ± 0.03^{c}
3400	0.92±0.03^{a}	0.61± 0.02^{ab}	2.15±0.08^{a}	0.31± 0.04^{c}	0.21± 0.011^{a}	2.91 ± 0.28^{b}	2.36 ±0.038^{c}	1.58± 0.07^{ab}	0.36 ± 0.08^{b}
3600	0.86±0.02^{b}	0.63± 0.029^{a}	2.12±0.025^{a}	0.47 ± 0.013^{a}	0.23± 0.017^{a}	3.1 ± 0.58^{b}	3.21 ± 0.04^{b}	1.85 ± 0.08^{a}	0.40 ± 0.01^{a}
3800	0.81±0.011^{bc}	0.54±0.011^{b}	1.91±0.034^{b}	0.38± 0.081^{b}	0.26± 0.014^{a}	3.57 ± 0.1^{a}	3.54 ± 0.06^{a}	1.91± 0.018^{a}	0.41 ± 0.05^{a}

FW, fresh weight; Chl-a, chlorophyll a; Chl-b, chlorophyll b; T-Chl, total chlorophyll; Caro, carotenoids content; CAT, catalase activity; POD, peroxidase activity; SOD, superoxidase dismutase activity; H_2O , hydrogen peroxide accumulation; MDA, malondialdehyde formation. Means with different superscript letters are significantly different (p<0.05) according to Duncan's multiple range test (DMRT).

capturing solar energy to be used for CO_2 fixation. In addition, these are also widely used to assess the relative impact of environmental stresses on photosynthetic properties. Quantification of photosynthetic pigments studied along the elevation gradient revealed significant ($p<0.05$) high Chl-a, at 3400 m (0.92 mgg^{-1} FW), followed by plants at 3600 m (0.86 mgg^{-1} FW) and least (0.78 mgg^{-1} FW) at 3200m. In contrast, Chl-b and carotenoid were recorded maximum (0.63 mgg^{-1} FW, 0.47 mgg^{-1} FW) at 3600 m that significantly varied with respective altitudes (Table 1). Furthermore, T- Chl content revealed a similar finding as Chl-a, with maximum (2.15 mgg^{-1} FW) T-Chl at 3400 m, while minimum (1.91 mgg^{-1} FW) T-Chl was reported at 3800 m. T-Chl content was found to decline from 26.04% to 23.04% in plants growing at 3400 m to 3800 m (Table 1). The effect due to different intensities of environmental stress and altitudinal variation was clearly reflected in the photosynthetic pigment levels and photosynthetic responses.

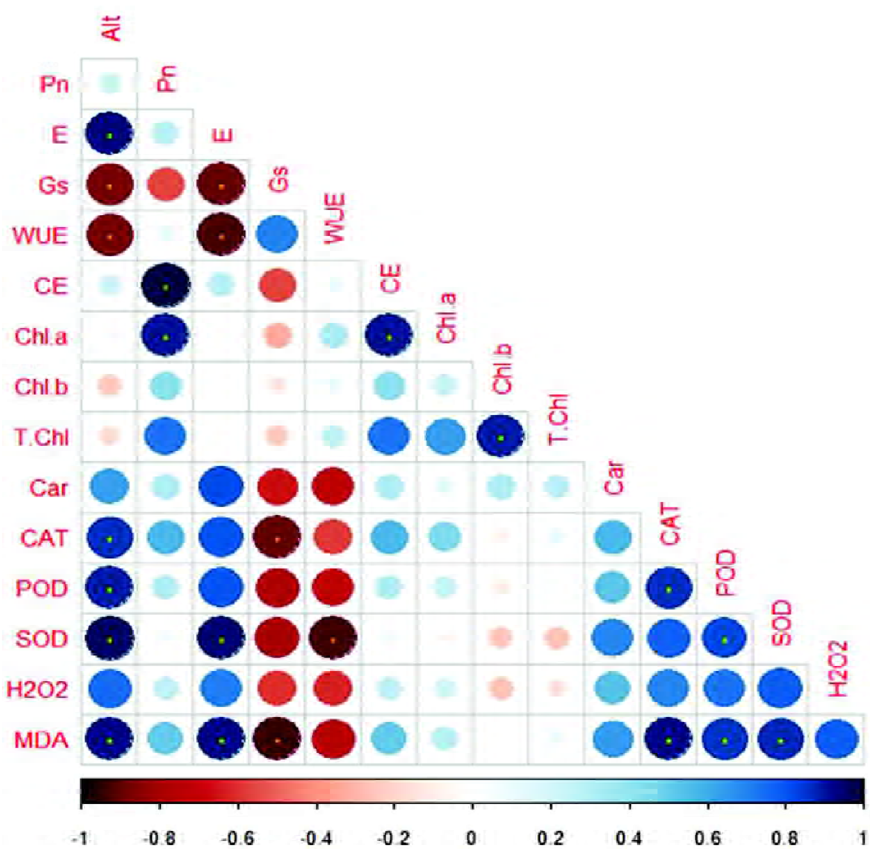

Fig. 4. Pearson correlation analysis between gas exchange parameters, photosynthetic pigments and biochemical activity and altitude. P_N - net photosynthesis rate; Gs - stomatal conductance; E - transpiration rate; WUE - water use efficiency; CE - carboxylation efficiency; Chl-a - chlorophyll a; Chl-b - chlorophyll b; T-Chl - total chlorophyll; Caro - carotenoids content; CAT- catalase activity; POD- peroxidase activity; SOD- superoxidase dismutase activity; H_2O_2- hydrogen peroxide accumulation; and MDA-malondialdehyde formation. Level of significance- *p<0.05; ** p<0.01

Antioxidant Enzyme Activity

Several sets of reactive oxygen species (ROS) are produced [*i.e.*, superoxide anion ($O_2^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), singlet oxygen ($^1O_2$), peroxynitrite (ONOO), and hypochlorous acid (HOCl)] as an essential metabolic process in response to environmental stress. As a result, ROS species are often overproduced, which result in causing detrimental effects. To counter this, important enzymes such as CAT, POD, and SOD are produced to protect the plant cells from the adverse effect of free radicals. In the present study, with an increase in altitude from 3200 m to 3800 m, a significant ($p<0.05$) up-regulation in antioxidant enzyme activity was investigated. Briefly, maximum antioxidant enzyme (CAT, POD, and SOD) activity (0.26, 3.57, 3.54 U min^{-1} g^{-1} FW) was recorded at 3800 m followed by at 3600 m (0.23, 3.1, 3.21 U $min^{-1}g^{-1}$ FW), while minimum activity was recorded at 3200 m (0.11, 2.25, 2.18 U $min^{-1}g^{-1}$ FW) (Table 1). In other words, CAT activity showed an overall increase from 12.60% (3200 m) to 32.11% (3800 m), POD activity from 25.50% to 40.38% and SOD activity from 19.33% to 31.35%.

H_2O_2 Determination and Lipid Peroxidation

Plant cells under stressful conditions lead to excess ROS accumulation, including H_2O_2 (hydrogen peroxide), leading to oxidative damage to cellular components. One such stance is a direct attack on membrane lipids or initiates a damaging autocatalytic process of lipid peroxidation and malondialdehyde (MDA) formation. MDA, the main byproduct accumulated due to membrane lipids peroxidation is usually utilized as an important biomarker highlighting cell membrane damage in plants. In the present study, H_2O_2 accumulation significantly ($p<0.05$) increased (1.37 mgg^{-1} FW to 1.91 mgg^{-1} FW) with altitude from 3200 m to 3600 m, while an insignificant increase between 3600 m to 3800 m was recorded (Table 1). Overall, with increase in altitude, H_2O_2 accumulation increased from 20.48% to 28.47%. Likewise, MDA content was quantified, which showed a similar trend as the H_2O_2 accumulation rate. Maximum MDA content (0.41 mgg^{-1} FW) was recorded at 3800 m, followed by at 3600 m (0.40 mgg^{-1} FW), and least at 3200 m (0.28 mgg^{-1} FW) (Table 1). Overall, with the increase in altitude, MDA content increased from 19.45% to 28.29%. The increase in H_2O_2, MDA or other antioxidant enzyme activity with an increase in altitude is an index of oxidative damage to cell membranes and plant innate resilience mechanism to thrive in such conditions.

Relationship amongst Gas Exchange Behavior, Photosynthetic Pigments, Enzymatic Assays and Non-Enzymatic Assays

Pearson correlation analysis was conducted to develop relationships amongst net photosynthesis rate (PN), stomatal conductance (Gs), transpiration rate (E), water use efficiency (WUE), carboxylation efficiency (CE), photosynthetic pigments (Chl-a, Chl-b, T-Chl, and carotenoid content), enzymatic assay (CAT, POD, and SOD), non-enzymatic assay (H_2O_2 and MDA) and altitudes are presented (Fig. 4). Based on correlation (r) analysis, altitude revealed positive and significant ($p<0.05$) correlation with POD ($r = 0.979$), SOD ($r = 0.970$) and H_2O_2 ($r = 0.975$), while bear negative and insignificant correlation with Gs ($r = -0.882$), WUE ($r = -0.902$), Chl-b ($r = -0.330$), and T-Chl ($r = -0.161$). Amongst the gas exchange behaviour/parameters, net photosynthesis rate (PN) was positively and significantly correlated with CE ($r = 0.99$, $p<0.01$) and Chl-a ($r = 0.975$, $p<0.05$). Transpiration rate was positively correlated with SOD ($r = 0.971$, $p<0.05$) and H_2O_2 ($r = 0.992$, $p<0.01$), while exhibited a negative and insignificant correlation with Gs ($r = -0.927$), and WUE ($r = -0.930$). Stomatal conductance (Gs) bears negative correlation with all the studied parameters except WUE, while this correlation is significant only with CAT ($r = -0.966$, $p<0.05$) and MDA ($r = -0.990$, $p<0.05$). Likewise, WUE revealed significant and negative correlation with SOD ($r = -0.979$, $p<0.05$). Prominently, CE shows significant and positive correlation with Chl-a (0.975, $p<0.05$), while with altitude ($r = 0.216$), E ($r = 0.291$) and WUE ($r = 0.079$), it showed positive but insignificant relation (Fig. 4). Furthermore, antioxidant enzyme such as CAT showed significant and positive correlation with POD ($r = 0.972$, $p<0.05$), and MDA ($r = 0.989$, $p<0.05$) activity, while POD showed significant relationship only with MDA ($r = 0.957$, $p<0.05$). SOD activity showed meaningful and positive relationship with H_2O_2 ($r = 0.967$, $p<0.05$), and insignificant relationship with MDA ($r = 0.872$). MDA a biomarker for lipid peroxidation showed significant and positive correlation with H_2O_2 ($r = 0.966$, $p<0.05$), while with Gs bear negative but significant correlation ($r = -0.990$, $p< 0.05$) (Fig. 4).

Discussion

Eco-physiological Responses Along the Altitudinal Gradient

Mountainous regions are acknowledged for being centers of endemism besides early indicators of climate change (Singh *et al*., 2023). For instance, temperature is reported to increase by 1.5°C in the Himalayas than an average increase of 0.74°C globally in last century (Singh *et al*., 2022). Climate change in the region is posing new threats to biodiversity elements, crop production, livestock husbandry, and availability of water and livelihood of subsistence-oriented communities (Tripathi and Singh, 2013). Therefore, understanding the driving force that plays a pivotal role in the form and distribution of alpine plant communities has become more important, considering the vulnerability of these ecosystems. Further, the dominant role of temperature, precipitation and micro/meso topography in influencing plant distribution and shaping the fundamental niche limits in the alpine zone has been well recognized (Carlson *et al*., 2013). The domino effect, *i.e.,* increase in temperature and reduced precipitation, leads to increased water stress in plants, thus altering the photosynthetic efficiency and changing phenotypic agility, limiting their further distribution (Wahid *et al*., 2007). These varied environmental conditions result in different eco-physiological adaptations strategies along the elevation gradient (Sandoval *et al*., 2019). A restricted water supply alters transpiration, stomatal conductance, chlorophyll content, inhibits photochemical activities, and reduces the activity of enzymes in the plant (Pons, 2012).

In the present study, eco-physiological responses, mainly gas exchange behaviour and the biochemical profile of *D. hatagirea* were studied along the elevation gradient. Studies involving altitudinal gradients offer an excellent understanding of species adaptive and survival potential to climate change in the Himalayas (Rahman *et al*., 2019). However, very little research has been conducted to draw definitive conclusions on this topic. The gas exchange behaviour investigated highlights, maximum [9.94 µmol (CO_2) $m^{-2}s^{-1}$] net photosynthetic assimilation (PN) rate at 3400 m; after that, further increase in altitude results in a significant ($p<0.05$) decrease in PN response. Likewise, stomatal conductance (Gs) showed a significant ($p<0.05$) decrease in stomatal conductivity, being from 27.81% at 3200 m to 9.63% at 3800 m. The observed variations in photosynthetic response at various elevations are consistent with the findings of Hovenden and Brodribb (2000), and Vats and Kumar (2006). An increase in altitude was reported to exhibit increased photosynthetic rates (PN), and increased stomatal conductance (GS) as presented earlier (Vats and Kumar, 2006). However, we did not find any definite relationship between increased photosynthetic rates (PN) and stomatal conductance (GS). Similar findings are reported elsewhere. For instance, Meinzer *et al*. (1992) recorded constant photosynthetic rates under varied stomatal conductance in the altitudinal range 200-300 m. It is further hypothesized that photosynthetic rate (PN) may not always respond to change to stomatal conductance (GS) (Pons, 2012).

Further, photosynthetic photon flux densities (PPFD) significantly increased with altitude (4.88% to 7.02%). This increase in PPFD was reflected in the rate of transpiration (E) and stomatal conductance (GS). The transpiration rate increased from 16.21% to 31.12% at 3200 m and 3800 m, respectively, while stomatal conductance (GS) showed a decreasing trend. Transpiration rate depends on stomatal conductance (GS) (Chandra *et al*., 2008). However, this is not absolute; report on an increase in transpiration rate (E) and decrease in stomatal conductance are reported (Elferjani and Soolanayakanahally, 2018). Preferred habitat conditions for *D. hatagirea* are moist environments, and therefore avoid drought stress conditions. As PPFD increases with altitude, the species faces heat stress, therefore to counter this, the rate of transpiration increases, ensuring leaf transpirational cooling. This increase in transpiration with altitude and PPFD was reflected in increased water use efficiency (WUE). The results indicate plant phenotypic plasticity and divergent adjustments of the species along the altitudinal gradient. Similar reports were presented on reduced stomatal conductance (GS) under heat stress and non-significant effect on drought stress (Barbour and Kaiser, 2016). Thus, it is concluded that *D. hatagirea* can utilize a fairly high level of PPFD at increased altitude for its gas and water exchange processes and perform much better if grown at ~2000 µmol $m^{-2}s^{-1}$ PPFD. Furthermore, higher PN at higher altitude (*i.e.*, 3400-3800 m) compared to 3200 m reflects its potential for improved growth, productivity, and better adaptability in these environments.

Biochemical Responses of the Target Species Along the Altitudinal Gradient

Several defensives (enzymatic and non-enzymatic) mechanisms get activated to counterbalance the oxidative stresses and maintain cell homeostasis. Enzymatic assays such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione-S-transferase (GST), and non-enzymatic assays such as carotenoids, phenolics, flavonoids, ascorbic acid, *etc*. are key defensive attributes (Das and Roychoudhury, 2014). Some of the prominent assays commonly investigated in the present study included superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), *etc*. Besides these, stress makers such as hydrogen peroxide (H_2O_2) and MDA were studied. In this study, with an increase in altitude from 3200 m to 3800 m, a significant ($p<0.05$) up-regulation in antioxidant enzyme activity was observed. Briefly, maximum antioxidant enzyme (CAT, POD, and SOD) activity (0.26, 3.57, 3.54 U $min^{-1}g^{-1}$ FW) was recorded at 3800 m followed by at 3600 m (0.23, 3.1, 3.21 U $min^{-1}g^{-1}$ FW), while minimum activity was recorded at 3200 m (0.11, 2.25, 2.18 U $min^{-1}g^{-1}$ FW). As the altitude increases, environmental variables such as high-intensity direct sunlight, low temperature, UV radiation *etc*. vary, and plants in response to these changes increase in enzymatic and non-enzymatic compounds to overcome such stressful condition (Rahman *et al*., 2019). Influence of these attributes explicitly showed significant variations in the present study as CAT activity showed an overall increase from 12.60% (3200 m) to 32.11% (3800 m), POD activity from 25.50% to 40.38% and SOD activity from 19.33% to 31.35%. Similarly, oxidative markers such as H_2O_2 accumulation significantly ($p<0.05$) increased (1.37 mgg^{-1} FW to 1.91

mgg^{-1} FW) with altitude. Overall, with the increase in altitude, H_2O_2 accumulation increase from 20.48% to 28.47%. Likewise, MDA content showed a similar trend as the H_2O_2 accumulation rate. Maximum (0.41 mg g^{-1} FW) MDA content was recorded at 3800 m, followed by 3600 m (0.40 mg g^{-1} FW), and least at 3200 m (0.28 mg g^{-1} FW). Overall, with the increase in altitude, MDA content increased from 19.45% to 28.29%. The mechanism of plant defensive response (*i.e.,* antioxidant activity) against oxidative stress is discussed in the above section. The increase in H_2O_2, MDA or other antioxidant enzyme activity concomitant with an increase in altitude is an index of oxidative damage to cell membranes and plant innate resilience mechanism to thrive in such conditions (Laxa *et al*., 2019).

Conclusion

Understanding gas-exchange behaviour and biochemical analysis highlights divergent responses of *D. hatagirea* towards various environmental stresses with inherent plasticity towards modulating gas exchange behaviour. The intrinsic flexibility shown by the species will allow the species to survive in varied environmental/habitat conditions. The present study suggested that mid-altitude (*i.e.,* 3400 m to 3600 m) with better growth performance of the species be considered for species re-introduction/species augmentation programmes.

Acknowledgments

The authors are thankful to the Director, G B Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand, India for encouragement and providing necessary facilities. Financial support from the National Mission for Sustaining the Himalayan Ecosystem, Task Force-3 (Phase-II) 'Forest Resources and Plant Biodiversity' (Department of Science and Technology, Govt. of India) is gratefully acknowledged.

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