How *Betula ermanii* Maintains a Positive Carbon Balance at the Individual Leaf Level at High Elevations

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Abstract

Generally, plant species with shorter leaf longevity maintain a positive carbon balance by decreasing leaf mass per area (LMA) and increasing photosynthesis. However, plants at high elevations need to increase LMA against environmental stresses. Therefore, plants need to increase both LMA and photosynthesis at high elevations. To examine how deciduous plants maintain a positive carbon balance at high elevations, photosynthesis and related leaf traits for deciduous broad-leaved tree *Betula ermanii* were measured at three elevations. LMA was greater at middle and high elevations than at low elevation. Leaf $\delta^{13}C$ was greater at higher elevations, and positively correlated with LMA, indicating greater long-term deficiency of CO_2 in leaves at higher elevations. However, the C_i/C_a ratio at photosynthetic measurement was not low at high elevations. Nitrogen content per leaf mass and stomatal conductance were greater at higher elevations. Photosynthetic rates and photosynthetic nitrogen use efficiency (PNUE) did not differ among the three elevations. Photosynthetic rate showed a strong positive correlation with stomatal conductance on a leaf area basis ($R^2 = 0.83$, $P < 0.001$). Therefore, this study suggests *B. ermanii* compensates the deficiency of CO_2 in leaves at high elevation by increasing stomatal conductance, and maintains photosynthesis and PNUE at high elevation as much as at low elevation.

Keywords

Leaf Mass per Area, Leaf Nitrogen, Photosynthesis, Stable Carbon Isotope Ratio, Stomatal Conductance

1. Introduction

Leaf longevity of deciduous plant species decreases at higher elevations because

the length of growing seasons for plants is shorter [1] [2]. It is often reported that leaf longevity positively and negatively correlates with leaf mass per area (LMA) and assimilative capacity (*i.e.*, photosynthetic rate at light saturation), respectively [3] [4] [5]. The photosynthetic rate is expected to be greater and LMA smaller for deciduous plant species at higher elevations because leaf longevity is shorter at higher elevations. However, environmental stresses, such as strong wind, low temperature and UV-B, also increase at high elevations [6]. Plants need to increase the mechanical stiffness of leaves (*i.e.*, LMA) to tolerate these stresses [6] [7]. Woodward [8] showed that LMA of grass plants increases at higher elevations, and stated that these plants adapt to strong wind and low temperature at high elevations by increasing mechanical stiffness. Thus, plants need to make tough leaves with high LMA at higher elevations, even though their leaf longevity is short. Namely, plants must acquire both high LMA and high photosynthetic rates at high elevations. Many studies have been done on elevational changes of photosynthetic characteristics. It is often reported that the photosynthetic rate of deciduous broad-leaved tree species increases at high elevations [9] [10]. However, whether deciduous plant species increase both LMA and photosynthesis at high elevations and how a positive carbon balance is maintained there have not been clarified.

Although plants tend to preferentially use $^{12}CO_2$ rather than isotopically heavier $^{13}CO_2$ during carbon assimilation, they have to assimilate more $^{13}CO_2$ than usual under CO_2-limited conditions [11]. Generally, the increase in LMA lengthens the internal diffusion pathway to chloroplasts, which decreases the CO_2 supply, which in turn increases a stable isotope ratio ($\delta^{13}C$) [12] [13]. Therefore, LMA often positively correlates with $\delta^{13}C$ [2] [14] [15] [16]. Deficiency of CO_2 in leaves possibly occurs at high elevations if plants increase LMA to intensify the mechanical stiffness, which in turn reduces photosynthesis. Photosynthetic enzymes, such as RuBP carboxylase and leaf chlorophyll with light harvest ability, are nitrogen compounds. Generally, photosynthetic rate positively correlates with nitrogen content per leaf mass [17] [18]. If LMA and nitrogen content per leaf mass are high, the nitrogen content per leaf area increases, which increases the photosynthetic rate per leaf area by increasing the CO_2 demand per leaf area [19] [20]. Therefore, it is possible that deciduous plant species maintain a positive carbon balance at the individual leaf level at high elevations if both LMA and nitrogen content per leaf mass are high at high elevations.

Deciduous broad-leaved tree species *Betula ermanii* Cham. is distributed widely in the subalpine zone in central Japan [21]. This study examined photosynthetic, leaf morphological and physiological traits of *B. ermanii* at three elevations to clarify the hypothesis that *B. ermanii* maintains a positive carbon balance at the individual leaf level at high elevations by increasing both LMA and nitrogen content per leaf mass.

2. Study Site

This study was done in the subalpine zone on the east slope of Mt. Norikura

(36°06'N, 137°33'E, 3026 m above sea level) in central Japan. Mean monthly temperatures of the coldest month (January) and the hottest month (August) at 2000 m a.s.l. were estimated as −9.2°C and 14.9°C, respectively, with 2.7°C annual mean temperature, from temperatures recorded at Nagawa Weather Station (1068 m a.s.l.) during 1979-2011 using the standard lapse rate of −0.6°C for each +100 m elevation. The annual total precipitation was 2206 mm at Nagawa Weaterh Station.

The study site was dominated by conifers *Abies mariesii* Mast., *A. veitchii* Lindl. and *Tsuga diversifolia* Mast. Although *Picea jezoensis* var. *hondoensis* Rehde also grows at this study site, the density was lower than for the three other species [22]. Subordinate trees were all deciduous broad-leaved trees: *Betula ermanii* Cham., *Sorbus commixta* Hedland and *Acer ukurunduense* Trantv. et Meyer. Miyajima *et al.* [22] and Miyajima and Takahashi [23] describe in detail the species composition and forest structure along an elevational gradient of Mt. Norikura.

3. Materials and Methods

3.1. Photosynthetic Measurements

This study measured the photosynthesis and related leaf traits of *B. ermanii*. *B. ermanii* is a shade-intolerant deciduous broad-leaved tree species distributing in subalpine forests and often forms secondary forests after large scale disturbances, such as forest fire and scarification [24] [25]. Leaf emergence of *B. ermanii* is the succeeding type [26]. Early leaves unfold on short shoots in the beginning of the growing season, and then late leaves unfold sequentially with elongation of long shoots until August. The leaf primordium of early leaves is formed in the previous year of growth [27]. Early and late leaves have different phenology and photosynthetic characteristics [28]. Generally, photosynthetic rate decreases with leaf age [29] [30]. The photosynthetic rate of early leaves decreases earlier than late leaves [28]. Therefore, this study examined the photosynthesis and the leaf traits of *B. ermanii* only for late leaves.

Five trees (2 - 5 m tall) were selected at open sites at each of 1600 m, 2000 m and 2500 m a.s.l. in August 2013. Study sites at 1600 m, 2000 m and 2500 m a.s.l. are indicated as low, middle and high elevations, respectively, in this study. A sun-exposed branch was sampled from each tree and was brought back to the laboratory of Shinshu University at Matsumoto Campus (622 m a.s.l.). Branches were kept at a room temperature 18°C overnight. Photosynthesis was measured the next morning until noon (6:00 - 12:00) because photosynthesis and transpiration are more active in morning than in the afternoon [31] [32] [33]. Photosynthesis of *B. ermanii* was measured by using a portable photosynthesis system (model LI-6400, Li-Cor Biosciences, Lincoln, NE, USA) equipped with a chamber, including a light source (6400-02B). Incoming CO_2 gas concentration from a CO_2 cartridge was set at 380 ppm. The leaf temperature was maintained at 20°C. The light-saturated maximum photosynthetic rate was measured at photosynthetic photon flux density (PPFD) 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ [28] [34]. Photosyn-

thesis was measured at least two leaves for each branch. Stomatal conductance and the ratio of CO_2 partial pressure of intercellular airspace (C_i) to that of ambient air (C_a) were also recorded at the photosynthetic measurement. Photosynthetic nitrogen use efficiency (PNUE) was calculated by the photosynthetic rate divided by leaf nitrogen on the leaf area basis.

3.2. Leaf Measurements

Leaf chlorophyll was extracted using dimethylformamide (4 ml). The absorbance of samples extracted from leaf samples at 663.8 nm ($A^{663.8}$) and 646.8 nm ($A^{646.8}$) was measured by using a spectrophotometer (UVmini-1240, Shimadzu, Kyoto), and was substituted into Porra's equations [35] to calculate chlorophyll a and b concentrations ($\mu g \cdot ml^{-1}$).

$$Chla = 12.00A^{663.8} - 3.11A^{646.8} \tag{1}$$

$$Chlb = 20.78A^{646.8} - 4.88A^{663.8} \tag{2}$$

Leaves were scanned using free software ImageJ 1.47 (http://rsbweb.nih.gov/ij/index.html), the leaf area was measured, and the leaves were oven-dried at 80°C for 48 hours and were weighed. The LMA of each leaf was calculated by leaf dry mass divided by leaf area. After the measuring leaf dry mass, the leaves were ground into a fine powder, was oven-dried for 24 hours, and the nitrogen content per leaf mass and stable carbon isotope ratio ($\delta^{13}C$) were measured by using an isotope ratio mass spectrometer (DELTA plus, Thermo Electron Ltd., Yokohama, Japan) equipped with an elemental analyzer (FlashEA1112, Thermo Electron, Ltd., Yokohama, Japan).

3.3. Statistical Analyses

Tukey multiple comparison tests were used to compare the following leaf traits among the three elevations: LMA, leaf nitrogen and chlorophyll contents, chlorophyll/nitrogen ratio, chlorophyll a/b ratio, $\delta^{13}C$, C_i/C_a, stomatal conductance, photosynthetic rate and PNUE. Proportional data were transformed by square-root-arcsine before the statistical analysis. Relationships between leaf $\delta^{13}C$ and LMA and between stomatal conductance and leaf $\delta^{13}C$ were analyzed by the regression analysis. All statistical analyses used free statistical software R 2.14.2 [36].

4. Results

4.1. Leaf Structure

LMA was greater at middle and high elevations than at low elevation (Tukey HSD test, $P < 0.05$, Figure 1(a)). Nitrogen contents per leaf mass and leaf area were greater at high elevation than at middle and low elevations (Tukey HSD test, $P < 0.05$, Figure 1(b), Figure 1(c)). No statistical significant difference was detected among the three elevations for chlorophyll content per leaf area, chlorophyll/nitrogen ratio and chlorophyll a/b ratio (Figures 1(d)-(f)). Therefore, LMA and the nitrogen content per leaf mass were high at high elevation, and ni-

trogen allocation ratios to chlorophyll *a* and *b* showed no elevational differences.

4.2. Gas Exchange

The $\delta^{13}C$ of leaves was less negative at higher elevations (Tukey HSD test, $P < 0.05$,
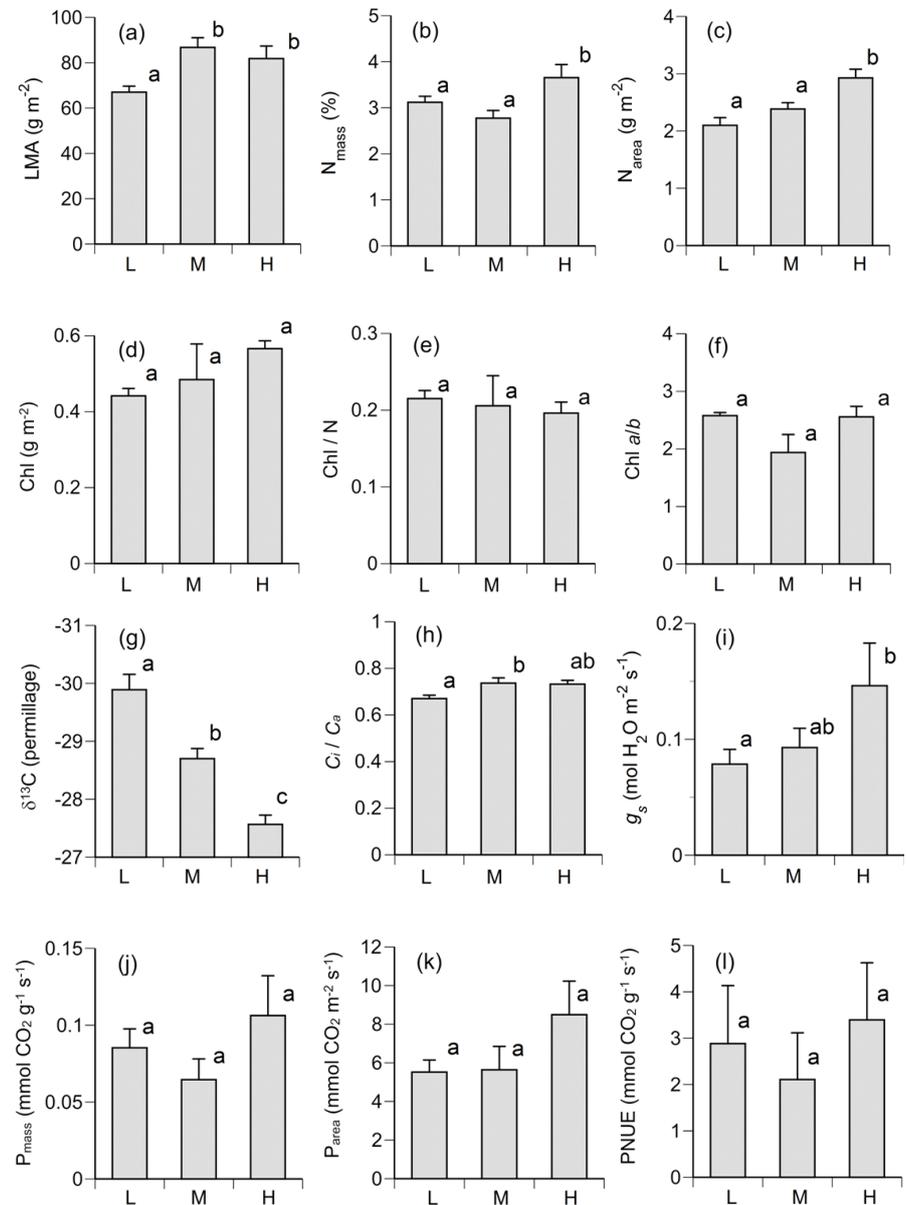
Figure 1. Leaf structural and physiological characteristics of *Betula ermanii* at low elevation (L, 1600 m a.s.l.), middle elevation (M, 2000 m a.s.l.) and high elevation (H, 2500 m a.s.l.). Mean values and standard errors are shown for (a) leaf mass per area (LMA), (b) nitrogen content per leaf mass (N_{mass}), (c) nitrogen content per leaf area (N_{area}), (d) chlorophyll content per leaf area (Chl), (e) ratio of chlorophyll to nitrogen (Chl/N), (f) Chl *a*/*b* ratio, (g) stable carbon isotope ratio ($\delta^{13}C$), (h) ratio of intercellular to the ambient CO_2 partial pressure (C_i/C_a), (i) stomatal conductance (g_s), (j) photosynthetic rate per leaf mass (P_{mass}), (k) photosynthetic rate per leaf area (P_{area}), and (l) photosynthetic nitrogen use efficiency (PNUE). Stomatal conductance and photosynthetic rates were measured at photosynthetic photon flux density 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Same letters indicate no significant difference at the level of 0.05 by the Tukey HSD test in each graph.

Figure 1(g)) and it positively correlated with LMA (R^2 = 0.224, $P < 0.01$, Figure 2). On the contrary, the C_i/C_a ratio at high elevation did not differ from this ratio at low and middle elevations (Figure 1(h)).

Stomatal conductance tended to be greater at higher elevations (Tukey HSD test, $P < 0.05$, Figure 1(i)). A positive correlation was found between $\delta^{13}C$ and stomatal conductance (R^2 = 0.149, $P < 0.05$, Figure 3). The photosynthetic rate per leaf area showed a high positive correlation with stomatal conductance (R^2 = 0.830, $P < 0.001$, Figure 4). Photosynthetic rates per leaf mass and per leaf area (Figure 1(j), Figure 1(k)), and PNUE (Figure 1(l)), showed no statistical significant difference among the three elevations. Therefore, the photosynthetic rate and PNUE were maintained at high elevation as much as at low and middle elevations.
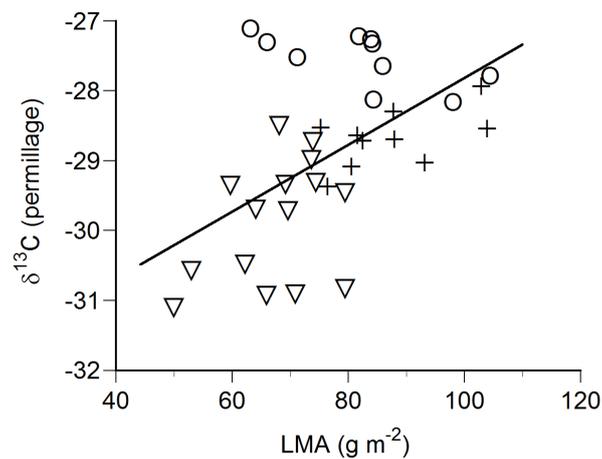
Figure 2. Relationship between leaf mass per area (LMA) and stable carbon isotope ratio ($\delta^{13}C$) for *Betula ermanii* leaves from low elevation (1600 m a.s.l., triangle), middle elevation (2000 m a.s.l., cross) and high elevation (2500 m a.s.l., circle). The regression line is Y = 0.043 X − 32.19 ($F_{1,33}$ = 10.8, $P < 0.01$, R^2 = 0.224) for pooled data of the three elevations.
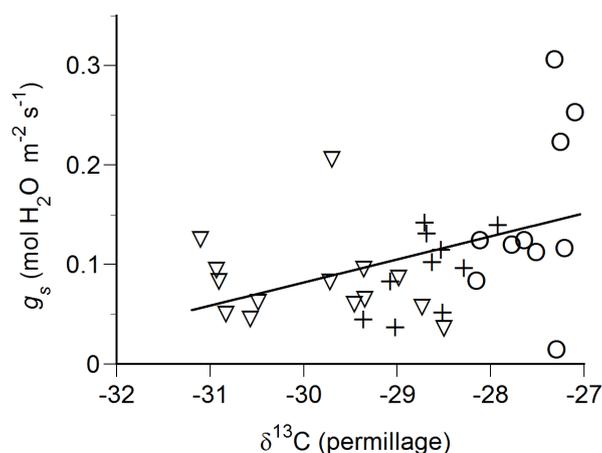
Figure 3. Relationship between stable carbon isotope ratio ($\delta^{13}C$) and stomatal conductance (g_s) for *Betula ermanii* leaves from low elevation (1600 m a.s.l., triangle), middle elevation (2000 m a.s.l., cross) and high elevation (2500 m a.s.l., circle). The regression line is Y = 0.0225 X + 0.752 ($F_{1,33}$ = 6.95, $P < 0.05$, R^2 = 0.149) for pooled data of the three elevations.
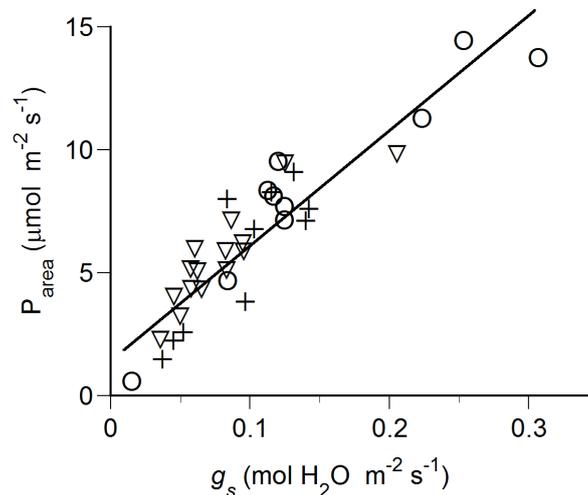
Figure 4. Relationship between stomatal conductance (g_s) and photosynthetic rate per leaf area (P_{area}) at photosynthetic photon flux density 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for *Betula ermanii* leaves from low elevation (1600 m a.s.l., triangle), middle elevation (2000 m a.s.l., cross) and high elevation (2500 m a.s.l., circle). The regression line is $Y = 46.23 \, X + 1.688$ ($F_{1,33} = 167.4$, $P < 0.001$, $R^2 = 0.830$) for pooled data of the three elevations.

5. Discussion

The LMA of *B. ermanii* was greater at middle and high elevations than at low elevation, and positively correlated with $\delta^{13}C$. CO_2 is suggested to be deficient in leaves with high LMA because of the long internal diffusion pathway to chloroplasts from stomata. Therefore, the photosynthetic rate was expected to be low at high elevation because of the CO_2 deficiency in leaves due to high LMA. However, no reduction was observed in the C_i/C_a ratio at photosynthetic measurement of leaves at high elevation. The nitrogen content per leaf mass also increased at high elevation. The high nitrogen content per leaf mass indicates an increase in photosynthetic enzymes, such as RuBP carboxylase [18]. The CO_2 demand increases for leaves with greater leaf nitrogen because of greater activity of photosynthesis. In fact, the stomatal conductance was high for leaves at high elevation; stomatal conductance and photosynthetic rate showed a high positive correlation on a leaf area basis. The stomatal conductance also positively correlated with $\delta^{13}C$. These results indicate that CO_2-deficient leaves with high LMA at high elevation increased the stomatal conductance. Therefore, this study suggests that *B. ermanii* maintains a positive carbon balance at the individual leaf level at high elevations by increasing the nitrogen content per leaf mass and stomatal conductance, even if *B. ermanii* intensifies the mechanical stiffness of leaves (*i.e.*, LMA), which supports the hypothesis.

The LMA increases in many plant species at high elevations [12] [20] [37] [38] [39] [40]. The increase in mechanical stiffness (*i.e.*, LMA) at high elevations is thought to be an adaptation to strong wind, low temperature and UV-B [8] [38] [41] [42]. Although many studies have examined elevational differences of photosynthetic rates, their results are not consistent, *i.e.*, an increase [43] [44], a decrease [45] [46] [47] [48] or no tendency [49] [50] in photosynthetic rates at

high elevations. Generally, leaf nitrogen content positively correlates with photosynthetic rate at light saturation [3] [4] [5]. However, some studies showed that photosynthetic rates per leaf area do not increase even if the nitrogen content per leaf area increases [46] [47]. Therefore, the change in leaf nitrogen contents is suggested to be not a causal factor for elevational differences in photosynthetic rates. The LMA was greater and $\delta^{13}C$ was less negative at higher elevations in these previous studies [46] [47]. Increase in LMA lengthens the internal diffusion pathway to chloroplasts, which decreases the CO_2 supply. Therefore, the long-term C_i/C_a ratio decreases and discrimination between $^{12}CO_2$ and $^{13}CO_2$ becomes less as the CO_2 supply decreases, which in turn increases the $\delta^{13}C$ of leaves [2] [12] [13] [16]. Thus, elevational change in $\delta^{13}C$ is suggested to be strongly regulated by the elevational change in LMA. Often reported is that photosynthetic rate positively correlates with stomatal conductance [19] [51] [52], and elevational changes in photosynthetic rates also tend to be in accordance with elevational changes in stomatal conductance [46] [49].

Plants not only absorb CO_2 by opening stomata, but also emit water through stomata (*i.e.*, transpiration) simultaneously. Plants close stomata to prevent transpiration in response to drought of the atmosphere and soil [53] [54] [55] [56]. Conversely, stomatal conductance is an indicator of the ability of soil water acquisition [57] [58]. *In situ* stomatal conductance of *B. ermanii* on Mt. Norikura is greater and pre-dawn water potential of leaves is also less negative at higher elevations [2]. Greater stomatal conductance of *B. ermanii* at higher elevations indicates more opening of stomata because the stomatal density of *B. ermanii* does not differ along an elevational gradient [2]. Greater stomatal conductance of *B. ermanii* at high elevations is thought to be due to less drought stress because precipitation is greater at higher elevations in the region of the study site [59]. On the contrary, radiation energy on fine days is greater at higher elevations [60]. Shade-intolerant *B. ermanii* may be able to utilize high radiation energy effectively for photosynthesis by having high stomatal conductance, which contributes to annual photosynthetic production.

Plant species with short leaf longevity are thought to maintain a positive carbon balance at the individual leaf level by producing leaves with low LMA and high photosynthetic rates [61]. However, although LMA of *B. ermanii* at high elevation was high in this study, *B. ermanii* could maintain the photosynthetic rate as much as at low elevation by increasing stomatal conductance. Namely, the results of this study cannot be explained by the conventional theory of leaf longevity. This study provides a new finding of elevational differences in leaf longevity, photosynthesis, morphological and physiological traits of leaves. However, further studies are necessary to show elevational patterns in photosynthesis and related leaf traits of other plant species for understanding of the adaptation of plants at the individual leaf level to high elevations.

Acknowledgements

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