# Leaf Morphological and Physiological Responses of *Quercus aquifolioides* along an Altitudinal Gradient

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*Quercus aquifolioides* Rehder & E.H. Wilson, an evergreen alpine and subalpine shrub species, occupies a wide range of habitats on the eastern slopes of the Himalaya in China. In this study, we measured leaf morphology, nitrogen content and carbon isotope composition (as an indicator of water use efficiency) of *Q. aquifolioides* along an altitudinal gradient. We found that these leaf morphological and physiological responses to altitudinal gradients were non-linear with increasing altitude. Specific leaf area, stomatal length and index increased with increasing altitude below 2800 m, but decreased with increasing altitude above 2800 m. In contrast, leaf nitrogen content per unit area and carbon isotope composition showed opposite change patterns. Specific leaf area seemed to be the most important parameter that determined the carbon isotope composition along the altitudinal gradient. Our results suggest that near 2800 m in altitude could be the optimum zone for growth and development of *Q. aquifolioides*, and highlight the importance of the influence of altitude in research on plant physiological ecology.

# 1   Introduction

*Quercus aquifolioides* Rehder & E.H. Wilson, an endemic woody plant species in China, is widely distributed in the Yunnan and Sichuan provinces, Southwest China. Its large range of habitat across different elevations implies strong adaptation to different environments, although it is mainly restricted to sunny, south facing slopes. It plays a very important role in preventing soil erosion, soil water loss and regulating climate, as well as in retaining ecological stability (Xu and Guan 1992, Zhou and Guan 1992). In the Wolong Nature Reserve, a main habitat of the giant panda, the species forms clonal shrub-stands and an evergreen broad-leaved pure forest. The earlier studies reported that the growth, spatial pattern and population structure of *Q. aquifolioides* were related closely to altitude; these properties changed non-linearly along increasing altitude (Li et al. 2005). However, its physiological ecology has been studied relatively little, in particular changes of leaf morphology, nitrogen content and carbon isotope composition along an altitudinal gradient need to be further elucidated.

The relation between carbon isotope composition ($\delta^{13}C$) and photosynthetic water-use efficiency (WUE) has led to wide-spread use of isotopic analyses in plant physiological ecology (Farquhar et al. 1989). There has been a considerable effort to elucidate the degree and nature of the genetic control over WUE on plants and much attention has been devoted to the study of $\delta^{13}C$ of plant tissues (Cregg 1994, Osorio and Pereira 1994, Leroux et al. 1996, Li 2000, Li et al. 2000). Water use efficiency is measured using $\delta^{13}C$ as a tool, because a strong positive correction is found between $\delta^{13}C$ and WUE. Plant tissue $\delta^{13}C$ provides an integrated measurement of internal plant physiological and external environmental properties influencing photosynthetic gas exchange over the time when the carbon was fixed (Anderson et al. 1996, Brodribb and Hill 1998). Considerable effort has gone into the description of sources of variation in $\delta^{13}C$, which varies among co-occurring species (Stewart et al. 1995, Schulze et al. 1998, Li et al. 2004), and genotypes within species (Zhang et al. 1993, Osorio et al. 1998, Li et al. 2000) along environmental gradients.

On the other hand, acclimation responses to environmental stresses are observed in leaf stomata and nitrogen (N) content, which are important factors for gas exchange (Farquhar et al. 2002). Stomata parameters reflect two important physiological processes, absorption of $CO_2$ in photosynthesis and transpiration of water. Environmental changes, such as atmospheric $CO_2$ concentration, temperature, light and humidity can influence stomatal parameters (Van de Water et al. 1994, Hultine and Marshall 2000, Li et al. 2002, Qiang et al. 2003). Moreover, levels of N in plant tissues have been positively correlated with altitude, and increased $CO_2$ demand at the site of carboxylation (Körner 1989, Sparks and Ehleringer 1997).

We hypothesized that there is an optimum altitudinal zone for *Q. aquifolioides* in the Wolong Nature Reserve where growth and metabolism of *Q. aquifolioides* are most vigorous and with increasing distance from this optimum the growth and metabolic rates decrease. Therefore, our aims were: 1) to show clearly how *Q. aquifolioides* acclimates to different environmental conditions; 2) to describe variations of leaf morphology, N content and $\delta^{13}C$ at different altitudes; 3) to determine the relationships among $\delta^{13}C$ and other leaf morphological and physiological responses.

# 2   Materials and Methods

## 2.1 Study Site and Sampling

The study was carried out in the Wolong Nature Reserve (200 000 ha; 102°52´–103°24´E; 30°45´–31°25´N), a national nature reserve giving priority to protecting the giant pandas and forest ecosystems. It is located on southeast slope of Qionglashan Mountain of upper reaches of Mingjiang River in the southeast Qinhai-Tibet Plateau. Our investigation was conducted in the Balang Mountain located in the nature reserve. Mean annual temperature at the Wonglong Field Station (2800 m) is close to 8.4 °C and the mean monthly temperature is the highest in July (17.0 °C) and the lowest in January (–1.7 °C). Annual precipitation averages 862 mm with about 68% of this between May and September. Precipitation is usually in the form of snow in

winter. There are about 271 frostless days during the year. The types of soil are mountain yellow loam soil, mountain grey cinnamon soil, mountain cinnamon soil, mountain brown soil, mountain brown podzolic soil, and alpine meadow soil from bottom to peak, respectively.

In the Wolong Nature Reserve, *Q. aquifolioides* forms clonal shrub-stands and an evergreen broad-leaved pure forest along an altitudinal gradient. We measured leaf morphological and physiological responses of *Q. aquifolioides* from eight sites (covering 2000, 2200, 2600, 2800, 3000, 3200, 3400 and 3600 m altitudes on the southeastern slope of the mountain) across an altitudinal gradient of 1600 m. The average height of shrubs was 1.83, 2.06, 2.37, 2.62, 2.21, 1.77, 1.38 and 1.19 m, respectively. Previous-year leaves were collected from the south side of open crowns of the average ramets (1.1–2.5 m) in each site as leaf samples. 10 individuals were selected randomly 30–50 m away from each other in each site. Sampling was carried out within one week. Due to the distance between individuals, we consider that individuals were independent samples of the population and analysis of variance type methods can be used in the data analysis.

## 2.2  Leaf Stomatal Parameters

Leaf samples, as described above, were used for measuring stomatal density (SD), stomatal length (SL) and stomatal index (SI). The leaves were placed between two plain sheets of white paper for three days to flatten the leaf surface and absorb moisture. Thereafter, a piece of leaf about 0.5 $cm^2$ in area was excised from the middle of the leaf close to the central vein and between two sub-veins. The leaf specimens were gold-plated to fix the stomata and the abaxial surfaces were observed at 400× magnification with a JSM-840 scanning electronic microscope (Model 840A, JIOL, Japan) (Li et al. 2002). The total stomatal length (described as stomatal index, SI) was calculated as mean stomatal length multiplied by stomatal density.

## 2.3  Specific leaf area

Leaf samples as described above were determined for leaf area and dry weight. Leaf area was measured using leaf area meter (CI-203, CID, USA). Leaf samples were dried (70 °C, 48 h) to constant weight and weighed. Specific leaf area (the ratio of one sided leaf area to dry weight, SLA) was then calculated.

## 2.4  Leaf Nitrogen Content

A mortar and pestle was used to grind the leaf samples as described above into fine powder. Leaf nitrogen content per unit mass ($N_{mass}$) was determined with a Leco 1000 CHN analyzer (Leco Corporation, St Joseph, Mich., USA). Leaf nitrogen content per unit area ($N_{area}$) was then calculated by $N_{mass}$ dividing by specific leaf area.

## 2.5  Carbon Isotope Composition

The abundances of stable isotopes of carbon in leaf samples as described above were determined as described by Hubick et al. (1986). Oven dried leaves of each sample were finely ground and relative abundance of $^{13}C$ and $^{12}C$ was determined with an isotope rationing mass spectrometer (Finnegan MAT Delta_E). The overall precision in δ-values was better than 0.1 $^0/_{00}$ determined by repetitive samples.

## 2.6  Statistical Analysis

Analyses of variance (ANOVA) for all variables from measurements were used for testing the differences among the different altitudes. Pearson's correlation coefficients and significances were calculated to determine the relationships between variables using individual data. Statistical analyses were done with the SAS and SYSTAT statistical software packages.

# 3   Results

## 3.1  Variations of Specific Leaf Area and Stomatal Parameters at Different Altitudes

Variations of specific leaf area (SLA), and stomatal parameters, including stomatal density (SD), stomatal length (SL) and stomatal index (SI, SI = SD × SL) of *Q. aquifolioides* showed that these responses to altitudinal gradients were non-linear with increasing altitude (Fig. 1). There was a critical altitude of these properties at 2800 m altitude. Below 2800 m, SLA, SL and SI increased significantly with increasing altitude (P < 0.001). From 2000 m to 2800 m altitude, SLA, SL and SI increased by 26.4%, 15.5% and 21.8%, respectively. In contrast, when altitude was above 2800 m, SLA, SL and SI decreased significantly with increasing altitude (P < 0.001).

From 2800 m to 3600 m altitude, SLA, SL and SI decreased by 44.5%, 42.6% and 47.35%, respectively. Variation in SD among the different altitudes was relatively small.

## 3.2.  Variations of Leaf N Content and $\delta^{13}$C at Different Altitudes

Changes in leaf N content per unit mass ($N_{mass}$), leaf N content per unit area ($N_{area}$), and carbon isotope composition ($\delta^{13}$C) of *Q. aquifolioides* at different altitudes showed that there was a critical altitude for variations of $N_{mass}$, $N_{area}$ and $\delta^{13}$C at 2800 m altitude (Fig. 2). These responses to altitudinal gradients were non-linear with increasing altitude. Below 2800 m, $N_{area}$ and $\delta^{13}$C decreased significantly with increasing altitude (P < 0.01). From 2000 m to 2800 m altitude, $N_{area}$ and $\delta^{13}$C decreased by 33.3% and 4.7%, respectively. In
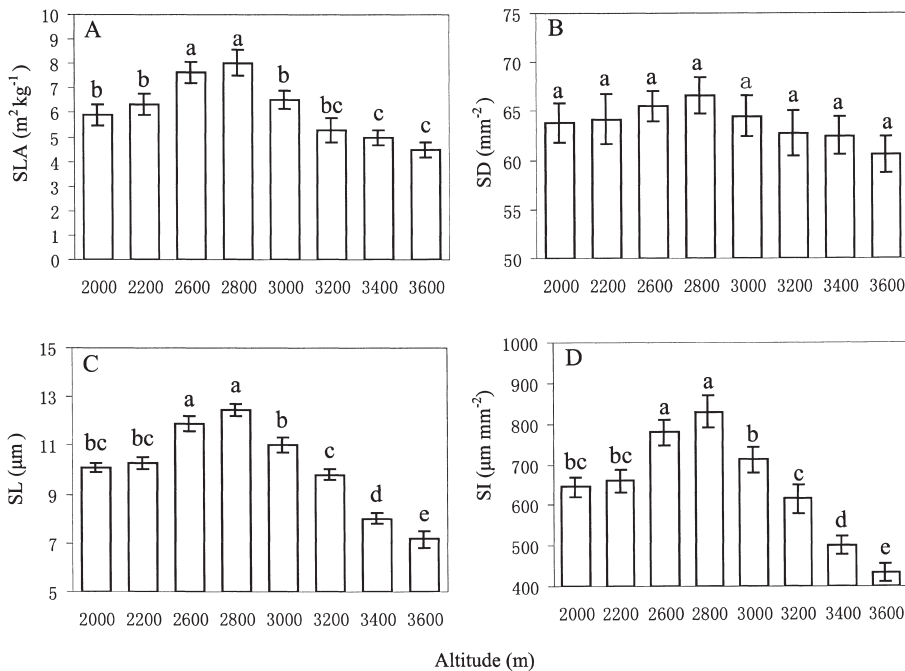
**Fig. 1.** Mean values of specific leaf area (SLA), stomatal density (SD), stomatal length (SL) and stomatal index (SI) at different altitudes. The different letters above the bars indicate statistically significant differences among the different altitudes at the P < 0.05 level in Tukey's HSD test. Values are means of 10 replicates ± SE.

**Fig. 2.** Mean values of leaf N content per unit mass ($N_{mass}$), leaf N content per unit area ($N_{area}$) and carbon isotope composition ($\delta^{13}C$) at different altitudes. The different letters above the bars indicate statistically significant differences among the different altitudes at the $P < 0.05$ level in Tukey's HSD test. Values are means of 10 replicates ± SE.
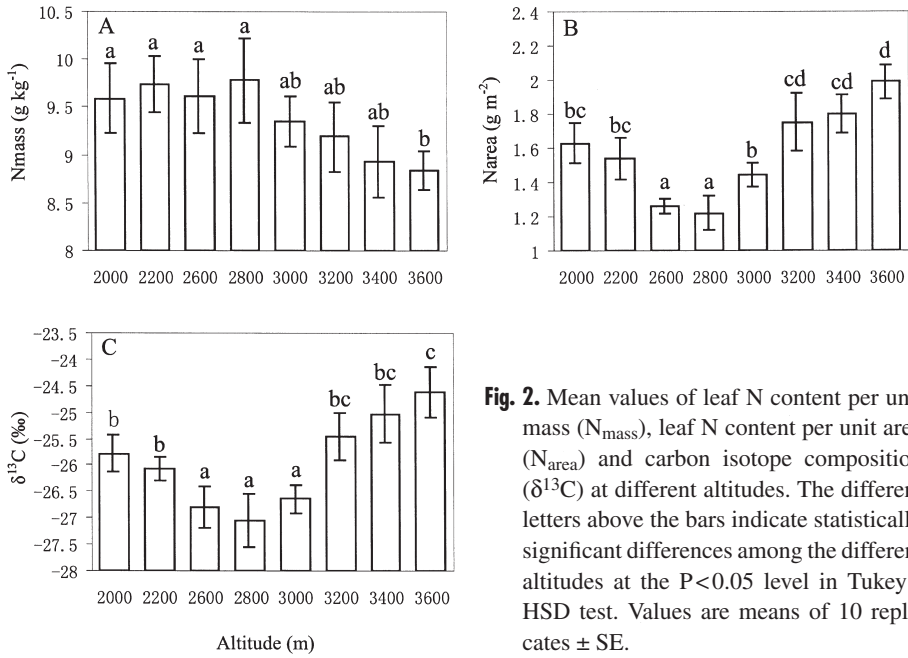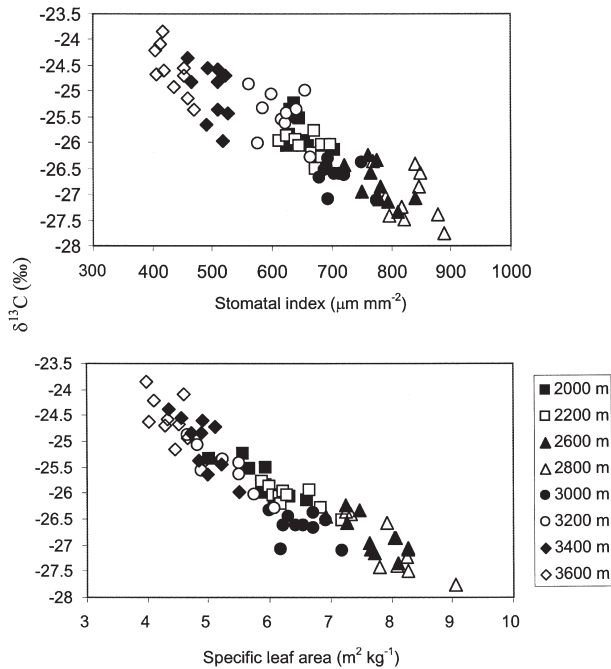


**Fig. 3.** The relationshpis among carbon isotope composition ($\delta^{13}C$), stomatal index and specific leaf area at different altitudes.

contrast, when altitude was above 2800 m, $N_{area}$ and $\delta^{13}C$ increased significantly with increasing altitude (P<0.01). From 2800 m to 3600 m altitude, $N_{area}$ and $\delta^{13}C$ increased by 61.8% and 9.0%, respectively. In addition, below 2800 m, $N_{mass}$ increased only slightly with increasing altitude. However, when altitude was above 2800 m, $N_{mass}$ decreased significantly with increasing altitude (P<0.01). From 2800 m to 3600 m altitude, $N_{mass}$ decreased by 9.6%.

### 3.3 Relationships among $\delta^{13}C$ and Other Leaf Morphological and Physiological Responses

The relationships among $\delta^{13}C$, SI and SLA along an altitudinal gradient are described in Fig. 3. It showed that $\delta^{13}C$ was negatively significantly correlated with SI (P<0.01) and SLA (P<0.001).

## 4 Discussion

Generally, leaf morphological and physiological properties of most woody plants are affected by different abiotic factors along environmental gradients, in particular over altitudinal gradients (Sparks and Ehleringer 1997, Cordell et al. 1998, Hultine and Marshall 2000, Qiang et al. 2003). These leaf morphological and physiological attributes change with changes in different environmental conditions, and appear to be a consequence of responses to abiotic factors such as soil moisture (Beerling et al. 1996, Sun et al. 1996), air temperature (Panek and Waring 1995) and atmospheric $CO_2$ concentrations (Ehleringer and Cerling 1995, Marshall and Monserud 1996). In this study, our results showed that specific leaf area, stomatal length and index of *Q. aquifolioides*, all decreased with increasing altitude above 2800 m, but these properties all increased with increasing altitude below 2800 m. In contrast, leaf nitrogen content per unit area and carbon isotope composition showed opposite change patterns. These leaf morphological and physiological responses to altitudinal gradients were non-linear with increasing altitude.

Our altitudinal gradient was 1600 m. Therefore, we have good reasons to assume that temperature decreases and humidity increases with increasing altitude. Indeed, the decrease in temperature along an altitudinal gradient is relatively large. Of the ecological variables we investigated, it could be interpreted in terms of increasing drought stress from lower to higher altitudes. The water stress is caused by damage to the cuticule of the trees due to wind and ice blasting in the winter time (Li et al. 2004). One possibility is that colder soils reduce the water uptake of the root system and induce the water stress (Magnani and Borghetti 1995). Another possibility is that colder air temperatures at the higher elevation maintain a higher state of frost resistance of the trees. Since abscisic acid is a central messenger for both frost resistance and drought, stomatal closure and allocation as apparent acclimations to drought might just be a side effect of a higher level of frost resistance (Quamruddin et al. 1993, Li et al. 2002).

The carbon isotope composition is indirect measure of water use efficiency and stomatal conductance (i.e. Hubick et al. 1986, Körner et al. 1988, 1991, Hultine and Marshall 2000), it has been the most common measure to describe differences in water use strategies (Marshall and Zhang 1994, Sun et al. 1996, Osorio et al. 1998, Li et al. 2000, Li and Wang 2003). Consistent with this, many previous studies reported that $\delta^{13}C$ increases with altitude, and is often correlated with leaf morphological and physiological properties that vary with altitude (Vitousek et al. 1990, Meinzer et al. 1992, Sparks and Ehleringer 1997, Cordell et al. 1998, Li et al. 2004). However, in our study the data showed that $\delta^{13}C$ increased with increasing altitude above 2800 m, whereas it decreased with increasing altitude below 2800 m.

Previous attempts to relate stomatal density to altitude have obtained mixed results. Some have found that stomatal density increases linearly with altitude (Körner and Cochrane 1985), that it increases and then decreases again (Körner et al. 1989), decreases with altitude (Schoettle and Rochelle 2000) or remains unchanged (Woodward 1986). If considering transpiration as a physical process, a physiologically more significant factor could be the total stomatal pore area per unit leaf area exposed to diffusive processes rather than the stomatal density. In our study, stomatal length and

the total stomatal length per unit leaf area (stomatal index) had a maximum at 2800 m altitude, and with increasing distance from this altitude the stomatal length and index decreased. Stomatal density had very little variation, although it had its highest values around 2800 m. One possibility is, that diffusion resistance to $CO_2$ depends primarily on specific leaf area (i.e. mesophyll resistance is dominating) and only to a lesser degree on stomatal characteristics.

Decreases in specific leaf area may be a disadvantage for $CO_2$ uptake. On the other hand, specific leaf area negatively correlated with $\delta^{13}C$, it could be that thicker leaves contain a higher quantity of photosynthetic enzymes, and hence have greater demand for carbon dioxide per unit area (Körner and Diemer 1987). Alternatively, the increase in $\delta^{13}C$ may be caused by an increase in the length of the internal diffusion pathway from the stomata to the chloroplasts, which would reduce carbon dioxide supply at the site of carboxylation (Evans et al. 1986, Vitousek et al. 1990). For sclerrophyllous evergreen species, as *Q. aquifolioides*, these limitations in the mesophyll seem to be especially large (Lloyd et al. 1992). In our data, the association between SLA and $\delta^{13}C$ was closer than relations between SI and $\delta^{13}C$. This could mean that diffusion limitation inside the leaf was dominant and probably more important than influences of stomatal size or density (as measured by SI). However, since SI and SLA were closely correlated it is not possible to draw firm conclusions from our data.

Photosynthetic capacity generally increases with leaf nitrogen content per unit area because photosynthetic enzymes such as Rubisco contain large quantities of N (Field 1983, Field and Mooney 1986, Friend and Woodward 1990). In our study, leaf nitrogen content per unit area decreased with increasing altitude below 2800 m, whereas it increased with increasing altitude above 2800 m. Increases in N could be associated with increased photoprotection of the leaf against high light stress (Körner 1989, Sparks and Ehleringer 1997). However, changes in the nitrogen concentration on a per mass basis were small, indicating that the plant nutritional status did not, necessarily, change very much on the gradient. There were, nevertheless, large changes in nitrogen in a per area basis, mainly due to the changes in the specific leaf area, which could affect the functioning of the photosynthetic system.

The present study shows that there were close correlations between morphological features and carbon isotope composition in our data. This indicates that a coordinated plant response modified these parameters simultaneously across different altitudes. Around 2800 m altitude there seems to be an optimum zone for growth and development of *Q. aquifolioides*, as indicated by thinner leaves, larger stomata and more negative $\delta^{13}C$ values.

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