Trees (2009) 23:1109–1121
DOI 10.1007/s00468-009-0354-8

ORIGINAL PAPER

Age-related nutrient content and carbon isotope composition in the leaves and branches of *Quercus aquifolioides* along an altitudinal gradient

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Received: 29 December 2008 / Revised: 8 May 2009 / Accepted: 17 May 2009 / Published online: 3 June 2009

Abstract *Quercus aquifolioides* Rehder & E.H. Wilson, an evergreen alpine and subalpine species, occupies a wide range of habitats in the Wolong Nature Reserve, southwestern China. We measured age-related carbon (C) and nutrient (N, P, K, Mg and Ca) contents, C/N, carbon isotope composition (δ^{13}C) and specific leaf area (SLA) in the leaves and branches of *Q. aquifolioides* trees along an altitudinal gradient ranging from 2,000 to 3,600 m. The results showed that both age and altitude significantly affected the morphological and physiological properties of *Q. aquifolioides*. Young tissues possessed higher contents of N, P, K and Mg, lower Ca contents, both on a dry mass basis (subscript "M") and on a unit area basis (subscript "A"), and lower C/N and δ^{13}C values than did the old ones. The levels of N_M and δ^{13}C increased with increasing altitude above 2,800 m, but decreased with increasing altitude below 2,800 m. In contrast, C/N and SLA showed opposite patterns, and other nutrient contents, including P_M, K_M, Ca_M and Mg_M, exhibited irregular changes with elevation. On the other hand, δ^{13}C was positively correlated with N_M in both leaves and branches, and negatively correlated with SLA in leaves along the altitudinal gradient. Our results also showed that both the Mg_M level of leaves and the Ca_M level of branches, besides the functional correlations between the N_M level and the structure of leaves, are responsible for or accompanied by variation in δ^{13}C. In addition, δ^{13}C was negatively correlated with C/N in both leaves and branches along an altitudinal gradient. It follows that high-altitude plants achieve higher water use efficiency (WUE) at the expense of decreasing nitrogen use efficiency (NUE, derived from C/N), whereas plants at 2,800 m can maintain relatively higher NUE but lower WUE. These characteristics probably reflect the physiological potential of *Q. aquifolioides* for vigorous growth and metabolism at the optimum altitude (around 2,800 m). With increasing distance from the optimum altitude, NUE decreases. The observed intraspecific variation in the trade-off between WUE and NUE may partially explain the altitudinal distribution of *Q. aquifolioides* in relation to moisture and nutrient availability.

Keywords Age · Altitude · Carbon isotope composition · Nutrients content · Specific leaf area · *Quercus aquifolioides*

Communicated by A. Gessler.

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Introduction

Carbon isotope composition (δ^{13}C) of plant tissues provides an integrated measurement for internal physiological and external environmental properties that influence photosynthetic gas exchange over the time when the carbon is fixed (Anderson et al. 1996; Brodribb and Hill 1998). Owing to its sensitivity to environmental constraints, δ^{13}C is now widely used to assess the effects of changing climatic conditions on plant ecophysiology. A great and consistent increase in δ^{13}C with altitude has been previously observed in many species (Marshall and Zhang 1994; Hultine and Marshall 2000; Li et al. 2004). Much effort has been made in the search for sources of variation in δ^{13}C in plant tissues along environmental gradients. Correlations of δ^{13}C with various abiotic factors over altitude gradients have been reported, including soil moisture (Sun et al. 1996), air temperature (Panek and Waring 1995), atmospheric CO_2 concentration (Ehleringer and Cerling 1995) and evaporative demand (Marshall and Zhang 1994). Compared with the extensive characterizations of the effects of abiotic factors on δ^{13}C, much less is known on how nutrient availability and δ^{13}C interact. Studies on the relationship between leaf nutrient concentration and δ^{13}C are still limited to N and exclude other nutrients (Saurer et al. 1995; Tsialtas and Maslaris 2006). In addition, to date, there have been only few detailed comparisons of nutrient characteristics of plant tissues of different ages in high-altitude regions. However, such knowledge of altitude effects is critical to our understanding of the potential of trees of different ages to respond to climate change, which can affect nutrient contents of plants by affecting soil moisture, biological activity of soil and plant growth.

As δ^{13}C reflects the relationship between the photosynthetic demand and diffusive (stomatal) supply of CO_2 (Sparks and Ehleringer 1997; Xu et al. 2000; Weih 2001; Hamerlynck et al. 2004), the use of stable isotopes provides fundamental insights into the interactions between the nutrients and ecophysiological processes of plants. Nitrogen (N) is an essential nutrient, which plants require in great quantity and the lack of which often limits growth. It has been positively associated with photosynthesis (Grassi and Minotta 2000) and chlorophyll pigment content (Major et al. 2007). Effects of phosphorus (P) on photosynthetic metabolism have been studied at many different levels, such as the effects of orthophosphate on Calvin cycle enzymes in vitro (Leegood et al. 1986). Potassium (K) is involved in photosynthesis in several ways, e.g., in the functioning of stomata, in ATP synthesis as a counter ion to the light-induced proton flux across the thylakoid membranes of chloroplasts, in phloem loading, and in phloem transport by creating osmotic pressure to drive mass flow in sieve cells (Marschner 1995). Calcium (Ca) is involved in the regulation of turgor pressure (Takagi and Nagai 1992) and is thus important in water loss regulation. Moreover, studies have demonstrated the importance of Ca^{2+} in the regulation of stomatal closure (Atkinson et al. 1990). These properties mean that calcium plays an important role in water use efficiency (WUE). Furthermore, magnesium (Mg), which occupies the center of the porphyrin nucleus of the chlorophyll molecule, is important in the photosynthetic pathway. It is an activity modulator of the RuBP carboxylase enzyme, which catalyzes the fixation of carbon. In brief, nutrient availability is of a special interest in respect to carbon isotope composition, as different nutrients influence δ^{13}C through their effects on photosynthetic capacity and stomatal conductance.

Quercus aquifolioides Rehder & E.H. Wilson, an endemic woody plant species in China, is widely distributed in the Yunnan and Sichuan provinces, southwestern China (Yang 1990; Xu and Guan 1992; Zhou and Guan 1992). Although it is mainly restricted to sunny, south-facing slopes, its large range of habitats across different elevations implies remarkable adaptation to different environments. *Q. aquifolioides* plays a very important role in preventing soil erosion and soil water loss, in regulating climate and in retaining ecological stability (Xu and Guan 1992; Zhou and Guan 1992). In the Wolong Nature Reserve, the species form clonal shrub stands and pure evergreen broad-leaved forests. It has been reported that the growth, spatial pattern and population structure of *Q. aquifolioides* are closely related to altitudinal gradients (Liu et al. 2006). Some morphological and physiological properties of leaves appear to change non-linearly along increasing altitudes (Li et al. 2006). However, the pattern of age-related changes in the nutrient content and δ^{13}C along an altitudinal gradient is unknown. Therefore, in the present study, we measured age-related nutrient contents and δ^{13}C in the leaves and branches of *Q. aquifolioides* growing along an altitudinal gradient. Our specific objectives were to: (1) elaborate variation in nutrient contents, carbon isotope composition and specific leaf area (SLA) in leaves and branches, as associated with age and altitude, and (2) analyze the age-related relationship between δ^{13}C and the nutrient contents and SLA.

Materials and methods

Site description

The study was conducted in the Balang Mountain in the Wolong Nature Reserve (200,000 ha; 102°52′–103°24′E; 30°45′–31°25′N), which is located on the southeastern slope of the Qionglashan Mountain at the upper reaches of the Minjiang River in the southeastern Qinhai-Tibet Plateau and which has a priority to protect the giant pandas and forest ecosystems (Fig. 1). The mean annual temperature at the Wonglong Field Station (2,800 m) is 8.4°C. The mean monthly temperature is highest in July (17.0°C) and lowest in January (−1.7°C). Annual precipitation averages 862 mm with 68% of precipitation occurring between May and September. In winter, precipitation is usually in the form of snow. There are about 271 frostless days during the year. The types of soil include mountain yellow loam soil, mountain gray cinnamon soil, mountain

Fig. 1 Map of the study area in southwestern China (*triangles* indicate the sample sites)

cinnamon soil, mountain brown soil, mountain brown podzolic soil and alpine meadow soil from bottom to peak, respectively, according to Chinese soil classification (Chinese Soil Taxonomy Research Group 1995).

Sampling

During summer 2003, samples were collected on the south-facing slopes of the Balang Mountain at a 400-m altitudinal interval along an altitudinal gradient (2,000–3,600 m). The gradient represents the altitudinal range of pure *Q. aquifolioides* stands in this area. Three temporary plots were established randomly 50–100 m away from each other at each altitude. We chose sample plots with similar slope, elevation, topographic position and climate in order to minimize, as much as possible, the impact of other environmental factors except altitude. The sample plots were chosen at each altitude to represent a typical stand structure: a pure *Q. aquifolioides* plantation with stand density of 14,600 trees ha^{-1}, canopy density of 0.8 and average height of 2.9 m. The main accompanying species of the shrub layer were *Cotoneaster dielsianus*, *C. horizontalis*, *Rosa moyesii* and *Deyeuxia scabrescens*. *Viola biflora*, *Polygonum viviparum* and *Potentilla saundersiana* were the main species of the herb layer with a coverage of 40%, while *Pleurozium schreberi* and *Rhytidiadelphus triquetrus* were the main species of the bryophyte layer with a coverage of 40%. The soil types at the five sites are brown soil with pH values of 6.2–6.9 and similar fertility. The geographic position of each plot was recorded using a Magellan GPS Field PRO VTM (Garmin, Kansas, USA). Within the plots, each plant was located with the precision of 5 cm within *X*–*Y* coordinates, using a measuring tape. Height, the number of stems at ground level, and the basal diameter were recorded for only live ramets larger than 0.3 m in height. The basal diameter was measured to the nearest 0.25 cm as close to the ground level as possible. Ramets of average size were selected as standard trees in each plot. The average heights of the ramets at each altitude were 1.83, 2.21, 2.62, 1.77 and 1.19 m, respectively. In total, ten trees at the growth stage were randomly selected at each altitude for sampling. Five leaves were randomly collected from the south side of the crowns and measured. After felling, leaves and branches were sorted according to their age (1 and 2 years old for leaves and 1–4 years old for branches). Fully mature leaves of the present growing season were regarded as 1-year-old leaves. The ages of leaves and branches were determined by using marks from overwintering buds on the stem and by counting back the internodes from the tip of the stems.

Specific leaf area

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

Nutrient analysis

All age-related leaf and branch samples were ground and passed through a 20 mesh screen after being first dried at 80°C for 36 h. The total concentrations of nitrogen (N) and organic carbon (C) were determined by the semi-micro Kjeldahl method (Mitchell 1998) and the rapid dichromate oxidation technique (Nelson and Sommers 1982), respectively. The total C to N ratio (C/N) (determined for the whole plant) was calculated as an estimate for the long-term nitrogen use efficiency (NUE) (Livingston et al. 1999). Total P was determined by colorimetrical blue of molybdate, and total K was determined by flame photometry. Ca and Mg were assayed by an atomic absorption spectrophotometer (Agrochemistry Commission and Soil Science Society of China 1983). The nutrient content of leaves per unit dry mass (subscript "M") was expressed as gram nutrient per kilogram leaf dry mass, and the nutrient content per unit of leaf area (subscript "A") was leaf nutrient content per unit dry mass divided by SLA.

Carbon isotope composition

The abundance of stable carbon isotopes in the leaf and branch samples was determined as described by Hubick et al. (1986). Oven-dried samples were finely ground, and the relative abundances of ^{13}C and ^{12}C were determined with an isotope ratio mass spectrometer (Finnigan MAT Delta-E). δ^{13}C values were expressed relative to the international measurement standard VPDB. The overall precision of the delta values was 0.1‰, as determined by repetitive measurements of standard material.

Statistical analyses

Altitude and age were used as treatments in the analyses of variance (ANOVA). Pearson's correlation coefficients were calculated to determine the relationships between variables. Partial correlation was used to exclude the possibility that the correlation between nutrient content and δ^{13}C was due to the impact of altitude or age, or both. Statistical analyses were conducted with SPSS 11.0 for Windows statistical software package.

Results

Changes of nutrient contents and δ^{13}C with age of leaves and branches

Age significantly affected SLA and all physiological properties of leaves and branches of *Q. aquifolioides*, and the interactions between age and altitude significantly affected the contents of N, K, P and Ca both on a dry mass basis (subscript "M") and on a unit area basis (subscript "A") (Figs. 2, 3, 4). Compared with old leaves, young leaves had higher SLA) (Fig. 2). In both leaves and branches of the same altitude, C/N and the carbon isotope composition (δ^{13}C) were lower in the young tissues when compared to the old ones (Fig. 2). The N, P, K and Mg contents on a dry mass basis and on a unit area basis were generally higher in the young tissues than in the old ones, whereas an opposite pattern was observed for the $\mathrm{Ca_M}$ and on $\mathrm{Ca_A}$ (Figs. 2, 3, 4). The $\mathrm{C_M}$ content showed a tendency to be higher in the old leaves than in the young ones, whereas it varied irregularly with age in branch samples (Fig. 2).

Variation of nutrient contents and δ^{13}C in leaves and branches at different altitudes

Except for the C content of branches, altitude significantly affected all variables in leaves and branches (Figs. 2, 3, 4). In both leaves and branches of the same age, the δ^{13}C and $\mathrm{N_M}$ increased with altitudes above 2,800 m, but decreased with increasing altitudes below 2,800 m. In contrast, C/N showed opposite patterns (Fig. 2). However, other nutrient contents, including P, K, Ca and Mg on a dry mass basis, exhibited irregular changes in response to elevation (Fig. 3). The nutrient content of leaves on a unit area basis was more regular. The $\mathrm{C_A}$, $\mathrm{N_A}$, $\mathrm{Ca_A}$ and $\mathrm{Mg_A}$ contents of leaves first decreased, but then increased with increasing altitude, with the lowest levels occurring at 2,800 m. The $\mathrm{P_A}$ and $\mathrm{K_A}$ contents of leaves changed little with increasing altitudes below 2,800 m, but promptly increased at above 2,800 m (Fig. 4).

Relationships between nutrient contents and δ^{13}C in leaves and branches

When partial correlations were used to statistically remove the influence of both age and altitude in leaves, δ^{13}C was positively correlated with $\mathrm{N_M}$ ($P < 0.001$) and $\mathrm{P_M}$ ($P < 0.05$) and negatively correlated with C/N ($P < 0.001$), SLA ($P < 0.001$), $\mathrm{K_M}$ ($P < 0.05$) and $\mathrm{Mg_M}$ ($P < 0.01$); C/N was positively correlated with SLA ($P < 0.001$) and $\mathrm{K_M}$ ($P < 0.001$), SLA was positively correlated with $\mathrm{K_M}$ ($P < 0.001$) and $\mathrm{Mg_M}$ ($P < 0.05$) and negatively correlated with $\mathrm{N_M}$ ($P < 0.001$), and all nutrient contents per unit area were positively correlated with each other (Table 1-a). Both age and altitude affected the relationships between nutrient contents and δ^{13}C. However, correlation between δ^{13}C and $\mathrm{Mg_M}$ showed little change besides that between δ^{13}C and SLA. In addition, correlations among nutrient contents per unit area (subscript "A") tended to be steadier than those per unit dry mass (subscript "M") (Table 1b).

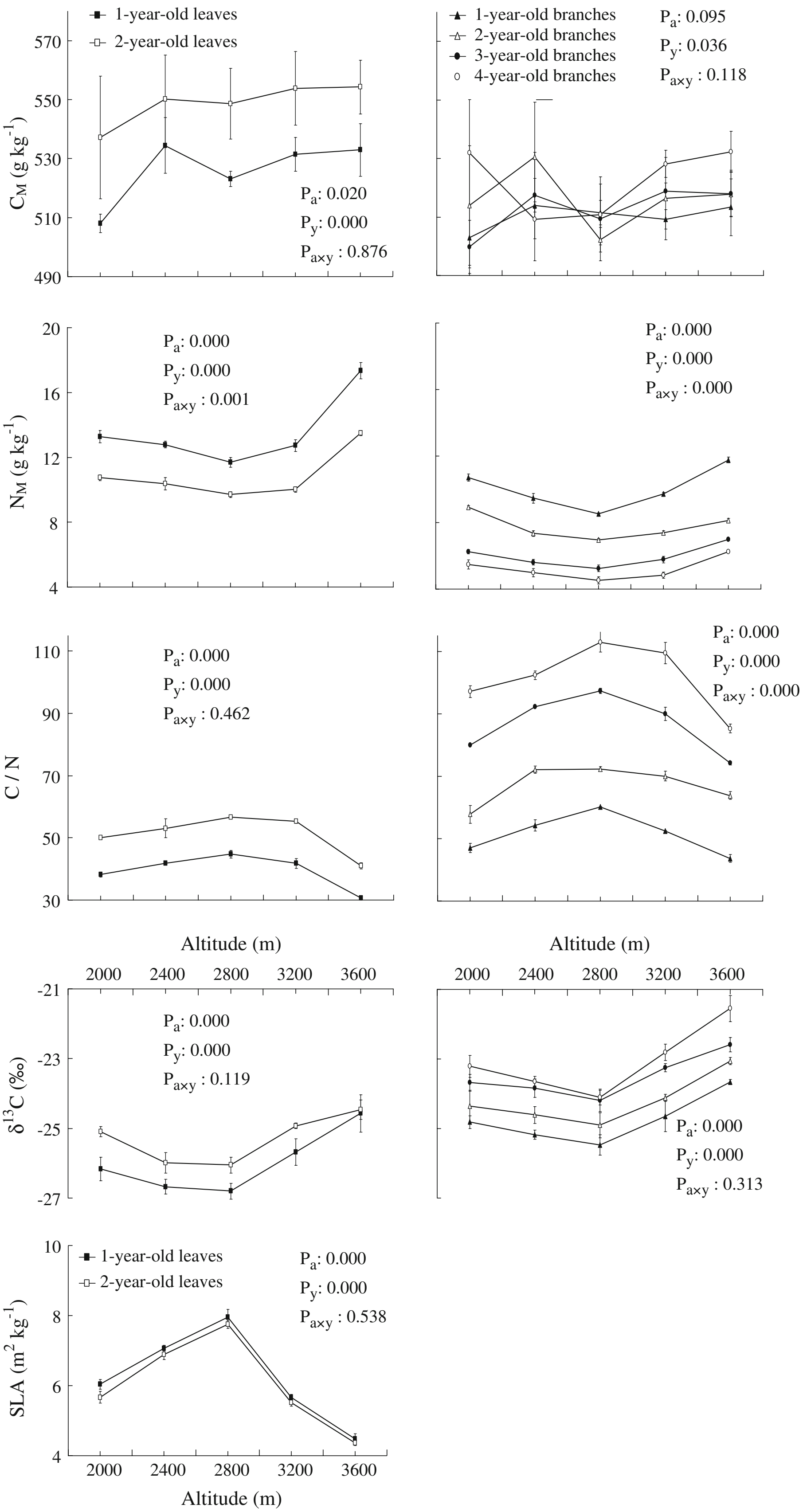

Fig. 2 Age-related carbon (C) and nitrogen (N) contents per dry mass, C/N, carbon isotope composition (δ^{13}C) and specific leaf area (SLA) of *Q. aquifolioides* along an altitudinal gradient. Values are means ± SE, $n = 3$. *P* values (ANOVA) are denoted as follows: P_a, altitude effect; P_y, age effect; $P_{a\times y}$, altitude × age interaction effect

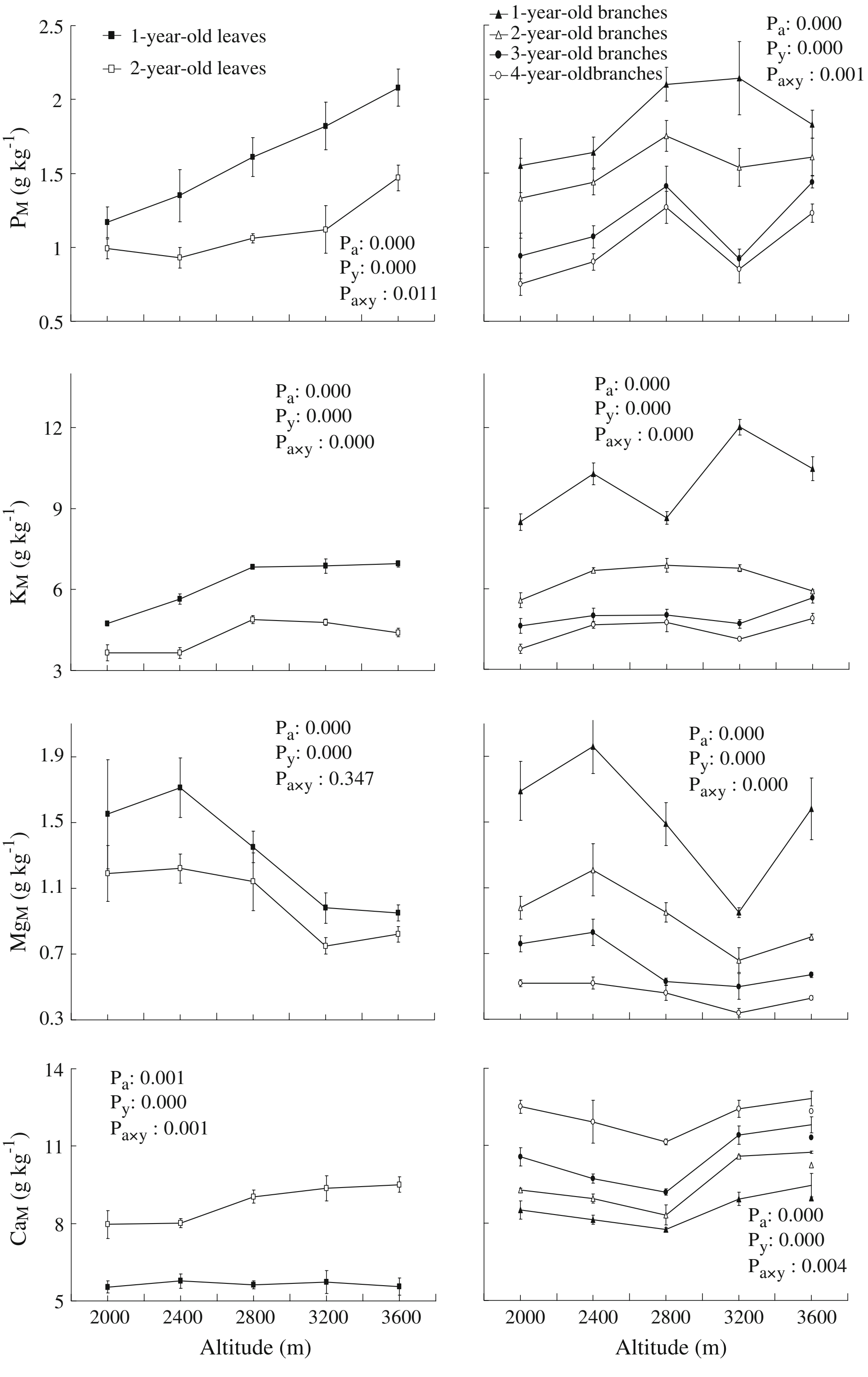

Fig. 3 Age-related phosphorus (P), potassium (K), magnesium (Mg) and calcium (Ca) contents per dry mass in the leaves and branches of *Q. aquifolioides* along an altitudinal gradient. Values are means ± SE, $n = 3$. *P* values (ANOVA) are denoted as follows: P_a, altitude effect; P_y, age effect; $P_{a\times y}$, altitude × age interaction effect

When partial correlations were used to statistically remove the influence of both age and altitude in branches, δ^{13}C was positively correlated with N_M ($P < 0.001$) and Ca_M ($P < 0.001$) and negatively correlated with P_M ($P < 0.01$) and C/N ($P < 0.001$), N_M was positively correlated with K_M ($P < 0.05$), Ca_M ($P < 0.001$) and Mg_M ($P < 0.01$), and C/N was negatively correlated with Ca_M ($P < 0.001$) (Table 2-a). Both age and altitude affected the relationships between nutrient contents and δ^{13}C in branches. However, the correlation between δ^{13}C and Ca_M was less affected in branches (Table 2-b).

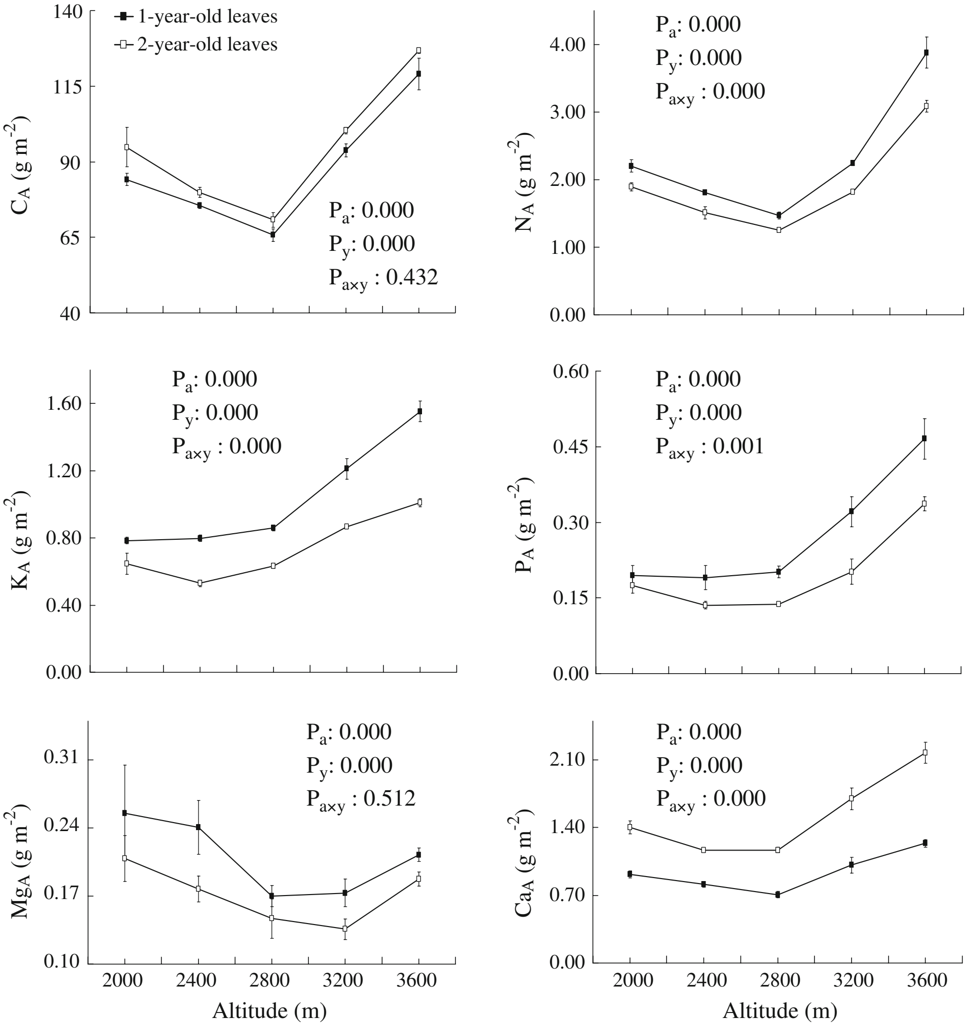

Fig. 4 Age-related carbon (C), nitrogen (N), potassium (K), phosphorus (P), magnesium (Mg) and calcium (Ca) contents per unit area in the leaves of *Q. aquifolioides* along an altitudinal gradient. Values are means ± SE, $n = 3$. P values (ANOVA) are denoted as follows: P_a, altitude effect; P_y, age effect; $P_{a\times y}$, altitude × age interaction effect

Discussion

Variation in the nutrient content, carbon isotope composition and SLA associated with age

As the whole plant is composed of tissues of different ages, in which the nutrient contents are not evenly distributed, the lower accumulation of N, P, K and Mg, both on a dry mass basis and on a unit area basis, with increasing leaf age can be explained by the nutrient availability and the mobility within the trees (Miller et al. 1979; Saur et al. 2000; Zas and Serrada 2003). It has also been reported that the contents of these nutrients are generally highest in the actively growing parts of the trees (e.g., leaves) and lowest in the structural and not actively growing parts (e.g., stem wood) (Wang et al. 1996; Wang and Klinka 1997; Laclau et al. 2001). C/N has been used to estimate the long-term NUE (Livingston et al. 1999), as this ratio indicates the amount of carbon fixed per unit nitrogen (Patterson et al. 1997; Chen et al. 2005). In our study, C/N increased with the age of tissues, which can be accounted for by both enhanced C and reduced N, as shown in Fig. 2. Although it has been suggested that NUE declines with the age of tissue (Escudero and Mediavilla 2003), we observed a slight increase in NUE with aging. This ensemble of age effects is consistent with the hypothesis that tissue aging not only represents deterioration, but also resource redistribution (Field and Mooney 1983). In addition, the higher C/N of branches, when compared to that of leaves, is expected to be due to the larger amount of wood and associated C-rich storage molecules and lignin in branches. Our results confirmed the existence of a significant age effect on δ^{13}C. Our data showed that old leaves are enriched in δ^{13}C, which suggests a higher assimilation rate or lower stomatal conductance, or both, in old leaves. Although there is no conclusive explanation, we believe that the most likely explanation for the old leaves being enriched in δ^{13}C is the combined effect of nutritional status (Ziegler 1995) and morphological difference (England and Attiwill 2006), as indicated by different SLA, but the

Table 1 Correlation coefficients among carbon isotope composition (δ^{13}C), carbon (C) and nitrogen (N) contents, C/N and other nutrient contents in leaves of different ages in *Q. aquifolioides* trees along an altitudinal gradient

	δ^{13}C	C/N	SLA	N_M	P_M	K_M	Ca_M	Mg_M	C_M	N_A	P_A	K_A	Ca_A	MG_A	C_A
(a)															
δ^{13}C		−0.680***	−0.902***	0.710***	0.383*	−0.399*	−0.277	−0.517**	−0.164	0.848***	0.850***	0.902***	0.665***	0.405*	0.894***
C/N	−0.155		0.770***	–	−0.316	0.709***	0.185	0.014	–	−0.931***	−0.794***	−0.598**	−0.726***	−0.753***	−0.829***
SLA	−0.878***	0.480**		−0.736***	−0.269	0.647***	0.162	0.387*	0.165	–	–	–	–	–	–
N_M	0.324	–	−0.578***		0.386*	−0.619***	−0.306	−0.050	-0.110	–	0.850***	0.697***	0.579**	0.705***	0.813***
P_M	0.203	−0.737***	−0.361*	0.783***		0.170	−0.641***	−0.312	0.190	0.392*	–	0.533**	−0.002	0.036	0.347
K_M	−0.161	−0.557***	0.032	0.548**	0.856***		−0.191	−0.053	0.127	−0.640***	−0.396*	–	−0.753***	−0.631***	−0.663***
Ca_M	0.432*	0.679***	−0.137	−0.561***	−0.563***	−0.676***		0.248	−0.179	−0.282	−0.497**	−0.460*	–	0.022	−0.193
Mg_M	−0.778***	−0.138	0.576***	−0.038	−0.227	0.008	−0.517**		−0.042	−0.193	−0.341	−0.452*	−0.147	–	−0.326
C_M	0.398*	–	−0.148	−0.315	−0.164	−0.335	0.678***	−0.570***		−0.159	−0.028	−0.099	−0.249	−0.224	–
N_A	0.689***	−0.793***	–	–	0.677***	0.319	−0.198	−0.377*	−0.045		0.911***	0.807***	0.706***	0.698***	0.928***
P_A	0.598***	−0.754***	–	0.863***	–	0.578***	−0.295	−0.450*	−0.004	0.925***		0.886***	0.538**	0.521**	0.873***
K_A	0.478**	−0.747***	–	0.838***	0.908***	–	−0.411*	−0.385*	−0.121	0.842***	0.955***		0.440*	0.398*	0.797***
Ca_A	0.808***	0.146	–	−0.021	−0.136	−0.461*	–	−0.696***	0.563***	0.389*	0.256	0.097		0.616***	0.840***
Mg_A	−0.195	−0.601***	–	0.480**	0.058	−0.001	−0.526**	–	−0.585***	0.292	0.102	0.083	−0.283		0.628***
C_A	0.912***	−0.424*	–	0.562***	0.397*	−0.021	0.248	−0.659***	–	0.859***	0.765***	0.631***	0.768***	0.019	
(b)															
δ^{13}C		−0.741***	−0.926***	0.804***	0.676***	0.286	0.161	−0.729***	0.182	0.905***	0.890***	0.886***	0.800***	0.000	0.934***
C/N	−0.003		0.813***	–	−0.498**	0.072	−0.092	0.326	–	−0.920***	−0.761***	−0.629**	−0.763***	−0.416*	−0.842***
SLA	−0.832***	0.412*		−0.809***	−0.553**	−0.060	−0.171	0.603***	−0.123	–	–	–	–	–	–
N_M	0.115	–	−0.464*		0.636***	0.118	0.099	−0.453*	0.187	–	0.865***	0.768***	0.728***	0.264	0.873***
P_M	−0.247	−0.765***	−0.052	0.734***		0.733***	0.214	−0.769***	0.504**	0.689***	–	0.878***	0.582***	−0.386*	0.689***
K_M	−0.589***	−0.504**	0.365	0.430*	0.814***		−0.353	−0.637***	0.454*	0.183	0.489**	–	0.201	−0.711***	0.201
Ca_M	0.401*	0.773***	−0.051	−0.722***	−0.916***	−0.892***		−0.313	0.148	0.168	0.208	0.296	–	−0.249	0.243
Mg_M	−0.657***	−0.475**	0.363	0.402*	0.412*	0.549**	−0.548**		−0.403*	−0.577***	−0.752***	−0.815***	−0.597***	–	−0.658***
C_M	0.257	–	0.040	−0.555**	−0.559**	−0.622***	0.666***	−0.478**		0.193	0.376*	0.376*	0.172	−0.402*	–
N_A	0.537**	−0.816***	–	–	0.475**	0.042	−0.393*	0.066	−0.345		0.924***	0.855***	0.827***	0.194	0.956***
P_A	0.336	−0.853***	–	0.898***	–	0.372*	−0.657***	0.135	−0.428*	0.897***		0.959***	0.781***	−0.100	0.916***
K_A	0.127	−0.837***	–	0.853***	0.836***	–	−0.822***	0.258	−0.605***	0.741***	0.902***		0.750***	−0.226	0.865***
Ca_A	0.739***	0.334	–	−0.293	−0.684***	−0.915***	–	−0.568***	0.477**	0.139	−0.182	−0.444*		0.083	0.909***
Mg_A	0.043	−0.815***	–	0.798***	0.469*	0.248	−0.502**	–	−0.515**	0.734***	0.663***	0.622***	−0.103		0.122
C_A	0.872***	−0.336	–	0.415*	−0.026	−0.485**	0.171	−0.392*	–	0.775***	0.569***	0.316	0.679***	0.397*	

(a) Partial correlation coefficients on removing the influence of both age and altitude (*upper triangle*); correlation coefficients without removing the influence of age and altitude (*lower triangle*)

(b) Partial correlation coefficients on removing the influence of age (*upper triangle*); partial correlation coefficients on removing the influence of altitude (*lower triangle*)

"M" and "A" subscripts indicate nutrient contents per unit dry mass and per unit area, respectively

SLA specific leaf area; *$\delta^{13}C$* carbon isotope composition; *N*, *P*, *K*, *Ca*, *Mg* and *C* indicate nitrogen, phosphorus, potassium, calcium, magnesium and carbon concentration, respectively; *C/N* ratio of carbon concentration to nitrogen concentration

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

"–" Correlation analyses rejected because of functional connection

Table 2 Correlation coefficients among carbon isotope composition ($\delta^{13}C$), carbon (C) and nitrogen (N) contents, C/N and other nutrient contents in branches of different ages in *Q. aquifolioides* trees along an altitudinal gradient

	$\delta^{13}C$	N_M	P_M	K_M	Ca_M	Mg_M	C_M	C/N
(a)								
$\delta^{13}C$		0.642***	−0.404**	−0.135	0.632***	0.114	0.133	−0.767**
N_M	−0.408**		−0.213	0.275*	0.605***	0.345**	0.178	–
P_M	−0.515**	0.700***		0.286*	−0.410**	−0.095	−0.159	0.195
K_M	−0.549**	0.852***	0.821***		0.057	0.270*	−0.021	0.019
Ca_M	0.863***	−0.612**	−0.688**	−0.679**		0.008	0.129	−0.620**
Mg_M	−0.652**	0.817***	0.591***	0.765***	−0.779**		0.166	−0.111
C_M	0.345**	−0.168	−0.248	−0.228	0.343**	−0.210		–
C/N	0.428**	–	−0.725**	−0.814**	0.652***	−0.793**	–	
(b)								
$\delta^{13}C$		0.590***	0.057	0.102	0.746***	−0.2132	0.220	−0.673**
N_M	−0.500**		−0.100	0.307*	0.592***	0.230	0.202	–
P_M	−0.782**	0.715***		0.397**	−0.034	−0.310*	−0.037	0.098
K_M	−0.712**	0.854***	0.822***		0.211	0.080	0.040	−0.024
Ca_M	0.863***	−0.659**	−0.847**	−0.762**		−0.232	0.206	−0.594**
Mg_M	−0.628**	0.862***	0.719***	0.837***	−0.764**		0.058	−0.037
C_M	0.300*	−0.183	−0.323*	−0.263*	0.310*	−0.174		–
C/N	0.512***	–	−0.746**	−0.817**	0.695***	−0.832**	–	

(a) Partial correlation coefficients on removing the influence of both age and altitude (*upper triangle*); correlation coefficients without removing the influence of age and altitude (*lower triangle*)

(b) Partial correlation coefficients on removing the influence of age (*upper triangle*); partial correlation coefficients on removing the influence of altitude (*lower triangle*)

"M" subscript indicates nutrient contents per unit dry mass

$\delta^{13}C$ Carbon isotope composition; *N*, *P*, *K*, *Ca*, *Mg* and *C* represent nitrogen, phosphorus, potassium, calcium, magnesium and carbon concentration, respectively; *C/N* ratio of carbon concentration to nitrogen concentration

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

"–" Correlation analyses rejected because of functional connection

contribution of old versus new carbon may also be relevant. Microenvironmental effects are not an explanation for relatively higher $\delta^{13}C$ in old leaves (D'Alessandro et al. 2004), because microenvironment was almost constant for old and younger leaves. We also observed that there is a consistent trend of more negative $\delta^{13}C$ values in leaves when compared to branches. Similar patterns have been observed in a range of other studies (Terwilliger et al. 2001; Scartazza et al. 2004; Brandes et al. 2006; Peuke et al. 2006). On one hand, this may be a result of a higher lipid content in the membrane-rich chloroplasts, as it has been reported that lipids are depleted in ^{13}C compared with organic matter, acids, sugars and respired CO_2 (Hobbie and Werner 2004). On the other hand, during the transport of organic C from leaves to roots, metabolic branching may result in C isotope fractionation and ^{13}C enrichment of sugars in sink when compared with source tissues (Damesin and Lelarge 2003; Franco et al. 2005).

Variation in the nutrient content, carbon isotope composition and specific leaf area associated with altitude

Our data showed that the responses of nutrient contents to altitudinal gradients on a dry mass basis are non-linear (irregular) with increasing altitude. The nutrient content of leaves on a unit area basis was more regular. Thus, the results on a dry mass basis are not as good a predictor of the altitudinal gradient as the results on a leaf area basis. Furthermore, because the leaf surface area is the interface between the leaves and the environment for gas exchange and light interception, it may be more appropriate in some cases to express properties on a unit area basis (Tegischer et al. 2002). An explanation for the occurrence of higher N_M and N_A at high altitudes in comparison to those in the lower altitude plants could be that a plant's N content is less diluted under cold conditions than under warm conditions (Körner and Larcher 1988), particularly if low temperatures and short growing seasons restrict shoot growth more than N uptake by roots, resulting in a higher concentration of N in leaves. Another explanation could be the way high-altitude plants adapt to lower resource availability (Chapin et al. 1990) and/or to higher abiotic stress level (cold hardiness). The altitudinal gradient of the trees examined in the present study was about 1,600 m. Over such elevation transects, plants encounter a variety of microclimates differing in temperature, soil moisture and vapor pressure gradient, each of which may influence nutrient contents. A species can persist in a heterogeneous environment either by means of phenotypic plasticity or genetic variation present among individuals (Scheiner and Goodnight 1984). Considering that there is only little genetic variation among *Q. aquifolioides* populations along an altitudinal gradient (Zhang et al. 2006), it is reasonable to attribute the observed altitudinal trends mainly to individual phenotypic plasticity.

It has been previously observed that decreasing δ^{13}C (Woodward 1986; Körner et al. 1988, 1991) or increasing δ^{13}C with increasing elevation are good indices when estimating long-term WUE (Marshall and Zhang 1994; Hultine and Marshall 2000; Li et al. 2004). However, our study involving an altitudinal gradient of 1,600 m showed that δ^{13}C increased with increasing altitude above 2,800 m, whereas it decreased with increasing altitude below 2,800 m. NUE (indicated by C/N) reached its maximum at about 2,800 m in both leaves and branches. When water is limited, the trade-off between WUE and NUE reflects the ability of the tree to maximize the efficiency of the use of resources. Our results showed that high-altitude plants achieved higher WUE at the expense of decreasing NUE, whereas plants at 2,800 m could maintain relatively higher NUE but lower WUE. Such intra-specific variation in the trade-off between NUE and WUE along an altitudinal gradient may partially explain the altitudinal distribution of the plants in relation to moisture and nutrient availability (Patterson et al. 1997).

Age-related relationship among δ^{13}C, nutrient contents and specific leaf area

We detected that δ^{13}C strongly correlated with N_M and N_A along an altitudinal gradient, which corroborated with the general pattern previously reported for other species (Flanagan and Johnsen 1995; Xu et al. 2003). This result also highlights the potential of using N contents of leaves as a useful trait for indirectly selecting *Q. aquifolioides* with improved WUE, when potential factors can be well controlled in common garden assays. Both age and altitude affected the relationships between nutrient contents and δ^{13}C. It is interesting to note that Mg_M in leaves and Ca_M in branches steadily and strongly correlated with δ^{13}C regardless of whether it was with or without statistically removing the influences of age or altitude, or both. Nevertheless, the relationships between other nutrients (P_M, K_M) and δ^{13}C were inconsistent due to either the age or the altitude effect. The hypothesis of scaling of leaf nutrient concentrations with δ^{13}C, due to nutrient-related alterations in photosynthetic efficiency and stomatal conductance does not completely explain why other nutrients, such as P_M and K_M, did not show consistent relationships with δ^{13}C. P_M and K_M can be involved in photosynthesis at various levels that would have implications for δ^{13}C. When we compared the detected concentrations of elements across all altitudes with data presented for *Quercus aliena* var. *acuteserrata* (Liu et al. 2001) and other tree species (Shen and Liu 1998), it was revealed that there are high (near maximal) concentrations of N_M, and average concentrations of P_M, K_M and Mg_M in branches, average concentrations of Ca_M in leaves and low concentrations (around minimal) of Mg_M in leaves and Ca_M in the branches. Such results may indicate that low concentrations of nutrients present in only functional cell compartments have the strongest relationships with δ^{13}C, as reported in other studies (Wright et al. 2001), while the elements with high or average concentrations may fluctuate more strongly due to the presence of non-metabolic storage. In fact, the nutrients in excess may be stored in the vacuoles (Rebeille et al. 1983). On the other hand, we found that the morphological responses, expressed as SLA, hold steady correlations with δ^{13}C, N_M and Mg_M along altitudinal gradients. We suggest that the adaptations to altitude in SLA, N_M and Mg_M have become constitutive and that these morphological and physiological adaptations are those that influence physiological functions, such as photosynthesis and stomatal conductance and, hence, WUE (δ^{13}C). Therefore, the functional correlations between the nutritional status and the structure of leaves are responsible for altitudinal variation in δ^{13}C. We propose that the potential factors causing an increase in δ^{13}C at altitudes above 2,800 m are as follows: (1) with water shortage resulting from damage to the cuticle by wind, from cold hardness at high altitude or from limited nutrients, stomatal conductance will decrease, (2) with higher nitrogen contents, there is a greater demand for carbon per unit leaf area, and (3) with thicker leaves (lower SLA), internal diffusion limitations will increase. For sclerophyllous evergreen species, as *Q. aquifolioides*, these limitations in the mesophyll seem to be especially large (Lloyd et al. 1992).

Although δ^{13}C, SLA and C_M in both leaves and branches, as well as C_A, Mg_M and Mg_A in leaves, responded to the age and altitude in an independent way, which predicts orthogonal effects between age and altitude. The data presented here showed that the interaction of age and altitude affected most measured nutrients, the overall consequence being that young tissues were more affected by altitude than old tissues. This age effect was supported by the fact that N, P, K and Ca contents, both on a dry mass basis and on a unit area basis, responded to altitude with much greater changes in the young tissues than in the older ones. The different patterns of N, P, K and Ca contents with altitude between young and old tissues suggest that, as well as being affected by ecological factors, the nutrient contents are related to the developmental stage. Variation in the fitness of different aged tissues may have important implications for understanding the potential of different aged trees to respond to climate change in subalpine mountainous regions.

Conclusion

The results from this study suggest that age-related changes observed in nutrient contents and δ^{13}C in the leaves and branches of *Q. aquifolioides* trees along altitudinal gradients in the Wolong Nature Reserve are a result of environmentally induced responses in ecophysiological processes. The detected responses to altitudinal gradients showed that the altitude of about 2,800 m was the optimum zone for *Q. aquifolioides*, as indicated by thinner leaves, lower N concentration, higher NUE and more negative δ^{13}C values. This information will contribute to the understanding of the growth potential of *Q. aquifolioides* and will assist in optimizing forest plantation management and reforestation practices. As a plant ages, changes in nutrient availability contribute to variation in WUE, NUE and their trade-off. It is still not easy to interpret how nutrient availability interacts to maximize instantaneous carbon gain, and long-term carbon, water and nutrient use efficiencies. Both the influence of altitude and age should be considered in research on the physiological ecology of plants. Moreover, our study showed that although a plant is simultaneously exposed to multiple nutrient factors under natural conditions, the strength of carbon isotope composition versus nutrient relationships may vary depending on the availability of specific nutrients that are in relatively short supply, such as Mg_M in leaves and Ca_M in branches. Future research aiming to exhaustively explain the observed altitudinal trends should involve direct measurements of local environmental and physical variables throughout the growing season to determine the main variables responsible for variation in the physiological activity of plants.

Acknowledgments The research was supported by the Program of “Knowledge Innovation Engineering” of the Chinese Academy of Sciences (No. KSCX2-YW-N-064) and the Outstanding Young Scientist Program of the National Science Foundation of China (No. 30525036).

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