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Multielement stoichiometry in *Quercus variabilis* under natural phosphorus variation in subtropical China

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Plant stoichiometry in relation to environmental factors has recently received increasing attention. However, regulations and variations of plant elements in different environments are not well understood. We investigated homeostasis and variation of macroelements (C, N, P, K, Ca, Mg, and S), essential microelements (Fe, Mn, and Zn) and non-essential elements (Al) in *Quercus variabilis* leaves at a range of natural P concentration from P-rich to P-deficient (typical subtropical conditions) soils. The results showed that element ratios were more stable (except for C : P and Mn : P) than individual element concentrations. Of the individual elements, protein-related elements (e.g. N, S, and Fe) were correlated with leaf P while non-protein elements (e.g. C, K, and Ca) were not. The degree of homeostasis indicated that macroelements (N, P, and Ca) concentrations were more variable than microelements (Mn, Zn, and Al) under a varying element concentration in soils. These results suggest that local P-rich geochemistry alters leaf element concentrations, but not element ratios, and that plants are capable of meeting their needs for elements in certain proportions to achieve optimal performance under varying elemental conditions.

Various nutrients in plants need to be balanced for normal growth and development[1]; some nutrients are generally required in large quantities (macroelements, e.g. N, P, S, K, Ca, and Mg), while others are needed in notably low quantities (microelements, e.g. B, Fe, Mn, Zn, and Mo). Ecological stoichiometry is the study of the balance of these elements in living systems[2–4]. Stoichiometric homeostasis, a core concept in ecological stoichiometry, describes the ability of organisms to ensure minimum nutrient uptake while avoiding excess accumulation, in order to live in environments that vary greatly in elemental availability[5]. Various studies have investigated homeostasis in zooplankton[6,7] and vascular plants[8] with artificial addition experiments; however, to date little work has been done to investigate homeostasis regulation in plants under natural element gradients[9]. Comparatively, the artificial nutrient addition experiments examines *acclimation*, responses to short-term environmental changes; while assessments of stoichiometric traits along natural element gradients reveal long-term *adaptation*[10].

The degree of stoichiometric homeostasis varies with trophic types (often being stricter in heterotrophs than in autotrophs)[4], species[11–13], organs[8], and elements. For instance, Karimi and Folt (2006) studied freshwater invertebrates and found that homeostasis differed among elements, with macroelement (C, N and P) more tightly regulated than non-essential elements (Pb, Hg and Cd). The studies that examine the changes of N and P concentrations in response to nutrient supply across different taxa, including algae[14], zooplankton[15,16], and higher plant[17,18] show that the homeostasis of macroelements is weak. N and P ratios, however, are found more stable than their concentrations[8,11]. It is not known how other elements, such as K, Ca, Mg, S, Fe, and Mn that have been much less studied, respond to variation of element availability in the environment.

P loading is a worldwide issue in terrestrial ecosystems as excess P is released into soils[19], which can greatly affect P concentration[8,20] and C : P and N : P ratios in plants[21]. The growth rate hypothesis (GRH) assumes that high P in organisms results from the high amount of RNA[3] required for synthesis of N-rich proteins and structure of C-containing organs[4,21], leading to high growth rate. Other than P and N, proteins also contain microelements for structural integrity[1,22]. For example, Zn and Mg act as cofactors for many enzymes and a large amount of proteins contains zinc-binding structural domains[23]. Similarly, iron-containing proteins are required

for many energy-transducing biological reactions in the processes such as photosynthesis, hormone synthesis, and respiration[24]. Although essential to plants, K generally exists in the form of cation within plant cells and Ca serves as a structural component of cell walls and cell membranes, both of which are not protein-related[22]. Up to now, a close P-N relationship has been confirmed[25,26], but the relationships of P with other elements in plants are generally unknown.

Kunming and the surrounding area is one of the largest P-rich regions in Yunnan Province in subtropical China[27]. Within this area, P-rich soil developed from the weathering of natural deposits is distributed in a mosaic pattern among prevalent phosphate-deficient subtropical soil[28] that is also deficient in Ca and Mg, but rich in Fe and Al due to strong weathering and leaching by high temperature and precipitation through long-term biogeochemical processes[29]. P-rich soil contains relatively high P and phosphate-associated elements (e.g. Ca, Mg, Fe and Al)[30]. This difference in soil element concentrations is expected to influences plant stoichiometric traits as well as forest ecosystem processes and functions[31]. For instance, soil P in subtropical China is remarkably lower than the global average (0.80 mg g^{-1}), resulting in high leaf C:P ratio[18,32]. In Yunnan

Province, deciduous *Quercus variabilis* Bl. is widely distributed as single species stands or in mixtures with other broadleaf trees. This provides a good opportunity to examine the impacts of soil elemental concentrations on plant stoichiometry for a single species along a natural soil P gradient from P-rich to P-deficient soils in subtropical biomes.

To better understand how soil P concentration affects plant stoichiometric traits, we studied *Q. variabilis* leaf elemental concentrations in central Yunnan near Kunming where P-rich soil develops from natural, long-term mineralization of phosphorus rock. We hypothesized that i) stoichiometric homeostasis of element ratios is greater than that of elemental concentrations to ensure the needs of plants for elements in certain proportions[38], ii) stoichiometric homeostasis of macroelements is stronger than that of microelements according to previous studies[7], and iii) varying P concentration has greater impacts on protein-related elements than on non-protein elements in *Q. variabilis* leaves. Our ultimate objective was to determine whether P enrichment affects plant stoichiometry and growth rate and to evaluate how increasing soil P in the future may affect primary productivity and ecological functions.
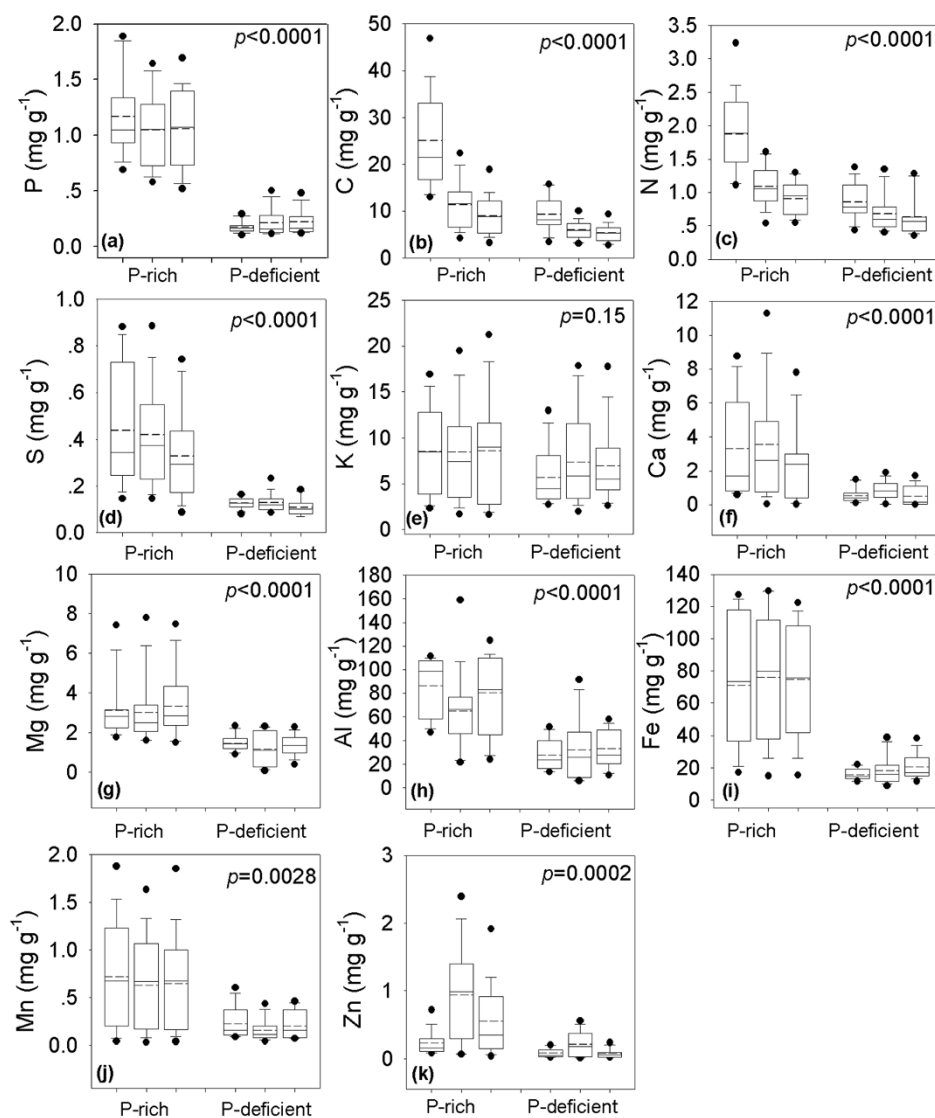

Figure 1 | **Concentrations of soil P, K, Ca, Mg, Al, Fe, Mn, and Zn by different soil depths (0–10 cm, 10–20 cm, and 20–30 cm from left to right) at P-rich (n = 16) and P-deficient (n = 18) sites in** *Q. variabilis* **stands across the study area, in Yunnan province, southwestern China.** Middle solid lines represent for 50th percentile (median), middle dash line for mean, box limits for 25th and 75th percentiles, and bars for minimum and maximum values.

Table 1 | Element concentrations and element:P ratios (molar ratios) of *Q. variabilis* leaves by P-rich and P-deficient sites. The values in bold indicate significant differences (ANOVA) between the two site types (***$P < 0.001$; **$P < 0.01$;*$P < 0.05$)

Elements (mg g^{-1})	P-rich sites		P-deficient sites		Element: P ratios	P-rich sites		P-deficient sites	
	Mean	CV	Mean	CV		Mean	CV	Mean	CV
C	508.9	1.28	508.6	2.04	C:P	**1105.6** *	16.14	**1255.8** *	15.83
N	**19.44** *	15.86	**17.33** *	14.83	N:P	36.47	27.44	36.52	19.06
Ca	**9.48** **	15.62	**7.70** **	21.29	Ca:P	6.20	24.30	5.75	30.56
K	5.69	17.19	5.04	24.81	K:P	3.76	18.17	3.83	30.25
Mn	**1.68** *	27.95	**2.48** *	48.47	Mn:P	**0.79** **	33.71	**1.31** **	46.22
Mg	1.65	20.28	1.46	19.94	Mg:P	1.82	34.98	1.80	23.49
S	1.43	11.98	1.35	7.29	S:P	1.15	17.10	1.24	17.01
P	**1.22** *	16.49	**1.07** *	17.22	P:P	--	--	--	--
Fe	**0.15** ***	16.88	**0.11** ***	25.36	Fe:P	0.10	22.18	0.11	22.39
Al	0.10	15.60	0.10	21.50	Al:P	0.12	38.31	0.08	33.85
Zn	**0.03** *	34.14	**0.02** *	29.10	Zn:P	0.01	33.66	0.01	25.48

Results

Leaf stoichiometric traits. The results of cluster analysis indicated two groups of the 11 sites: P-rich and P-deficient sites (Fig. 1a, Supplementary Table S1). Except for K, soil elements concentrations differed significantly between the two site types (One-way ANOVA, $p < 0.0001$) (Fig. 1, Supplementary Table S2).

Leaf element concentrations of N, P, Ca, Fe, and Zn at P-rich sites were higher than those at P-deficient sites (ANOVA, $p < 0.05$, Table 1). The only exception was Mn that displayed an opposite pattern ($p < 0.05$, Table 1). Leaf element : P ratios, however, did not differ significantly between the two site types; the exception was C : P and Mn : P that were lower at P-rich sites than at P-deficient sites (Table 1). P-rich sites were statistically separated from P-deficient sites along the first component (explaining 22% of the variation, $F = 15.7$, $p = 0.0004$) of PCA using leaf element concentrations (C, N, P, S, K, Ca, Mg, Al, Fe, Mn, and Zn) (Fig. 3).

Homeostasis of leaf elements and element ratios. Among the 11 elements, only leaf N, P, Ca, and Fe concentrations were positively correlated with the soil counterparts ($p = 0.01$ to 0.04), while no significant correlation between leaf and soil was observed for other elements (Fig. 2, Supplementary Table S3). The homeostasis (expressed as H) of leaf N, P, Ca, and Fe (from 6.58 (Fe) to 16.67 (P)) was lower than that of other elements (S, K, Mg, Al, Mn, Na, and Zn, from 21.74 to 33.33) (Fig. 4, Supplementary Table S4). Comparatively, the homeostasis of element : P ratios was much higher than that of element concentrations (from 6.21 (Mn : P) to 163.93 (Fe : P)). The highest homeostasis of leaf element ratios was in K : P (71.43) and Fe : P (163.93) and the lowest in microelement : P
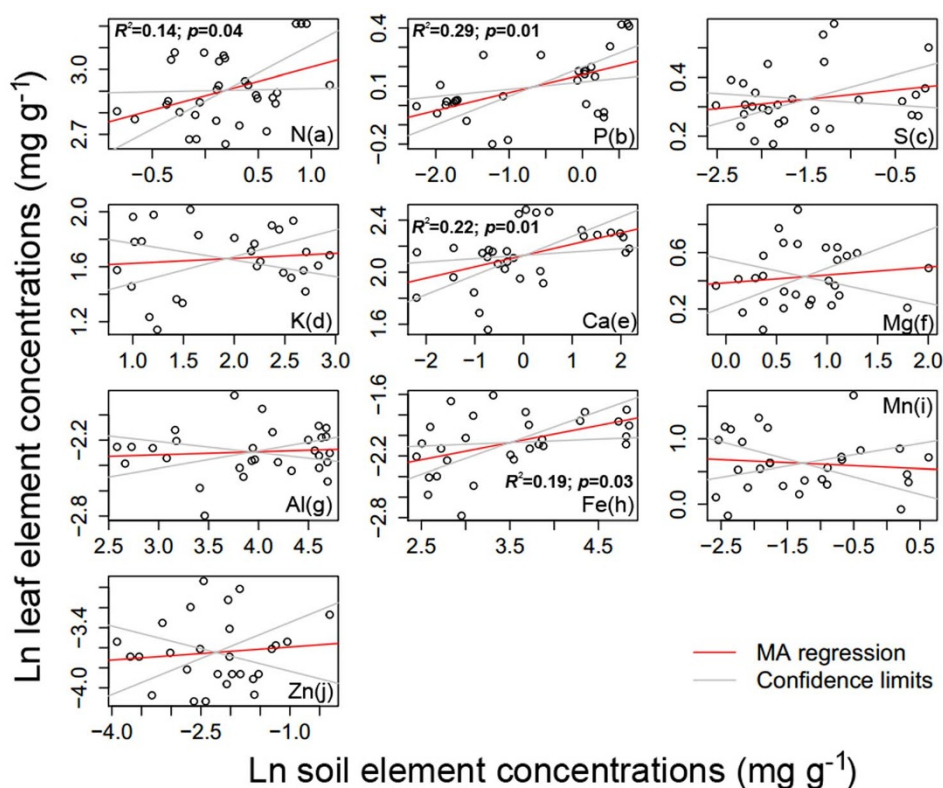

Figure 2 | Relationships of element concentrations between soil and *Q. variabilis* leaves. Major axis (MA) regression (red) and 95% confidence lines (grey) are shown.
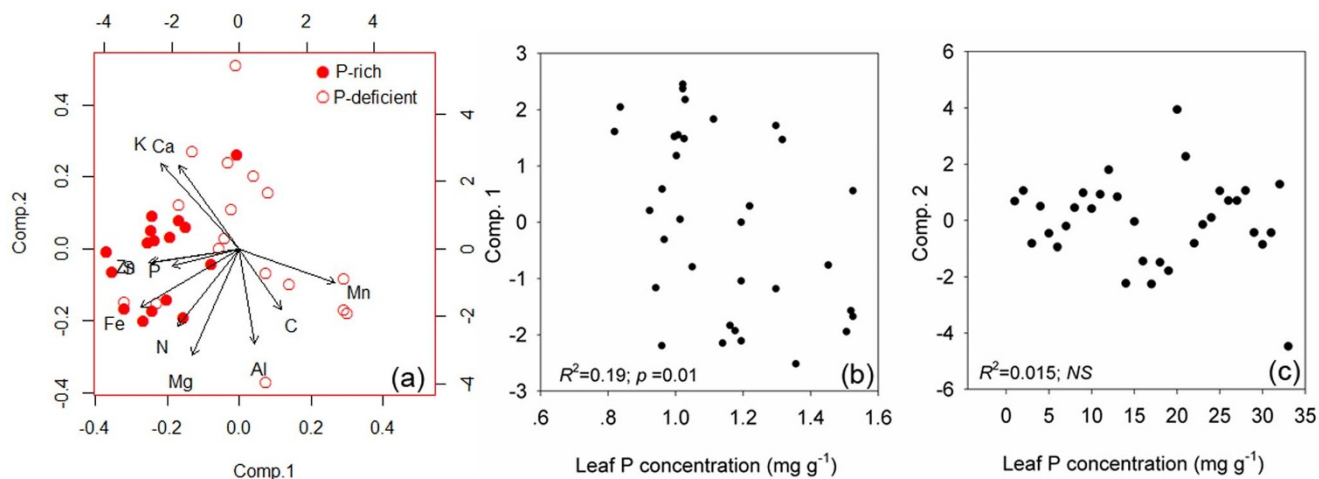
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Figure 3 | Ordination (PCA) of sampling sites by 11 elements (a) and relationships of *Q. variabilis* leaf P concentration with 11 elements by PCA coefficients of Comp.1 (component 1) (b) and Comp. 2 (component 2) (c) scores.

ratios (e.g. Mn : P (6.21), Al : P (9.09), and Zn : P (12.89)) (Fig. 4, Supplementary Table S4).

Variation in leaf element concentrations. The variation in element concentrations decreased with the increase of element concentrations for both site types (regression *slope* $= -2.2$ and $p = 0.017$ for P-rich sites and regression *slope* $= -2.1$ and $p = 0.015$ for P-deficient sites, Fig. 5). This trend (regression slope) was statistically similar between the two site types (ANCOVA, $F = 0.008$, $p = 0.93$) (Fig. 5).

Element function categorization. The Comp.1 (explaining 22% of the variation) of PCA, mainly loaded with leaf P, N, S, Fe, Mn, and Zn, was negatively correlated with leaf P concentration (Fig. 3b), while the Comp.2 (explaining 18% of the variation), mainly loaded with leaf C, K, Ca, Mg, Al, and Mn, had no significant relationship with leaf P (Fig. 3c). These elements (except for Mn) were positively correlated with leaf P (negative loading coefficients on Comp.1) (Supplementary Table S5).

Discussion

Species growing in diverse nutritional conditions can develop nutrient-based ecotypes (populations)[33,34]. This likely is the case in *Q. variabilis* trees where leaf N, P, Fe, Na, and Zn concentrations were higher at P-rich sites than those at P-deficient sites (Table 1). These differences in leaf element concentrations likely reflect adaptation of *Q. variabilis* to the variation of soil elements in the local environment in order to optimize growth and biological functions[35]. Comparatively, variation was much less and homeostasis was higher in element ratios than in element concentrations, suggesting that leaf ratios were more tightly regulated than individual element concentrations in *Q. variabilis* leaves. This is in line with Yu et al. (2011) who also found a consistently higher H of N : P than H of N and P in vascular plants of Inner Mongolia, China. The likely reason that increasing P concentration is coupled with the increases of other elements is that elements in plants are required in certain proportions in order to have normal growth and functioning[21,35]. These results together support our expectation that organisms need nutrients of certain proportions to conduct normal and orderly functions[36].

Interestingly, leaf C : P ratio differed between the two site types, suggesting that increasing P leads to a decreasing proportion of C in *Q. variabilis*. The C : P ratio at P-rich sites was similar to that of temperate broadleaf trees (922 : 1)[37], but much lower than that of P-deficient sites and typical subtropical forests[38,39]. The C : P ratio

at P-deficient sites, however, was close to that of subtropical broadleaf trees (1449 : 1)[37]. Comparatively, other element : P ratios are much more stable, indicating that local P-rich geochemistry alters the stoichiometry of plant C : P ratio that are generally controlled by zonal climate[40], but not stoichiometry of other element : P ratios.

Early ecological stoichiometry indicates a strict homeostasis and low variability for macroelements (e.g. N and P) and a flexible stoichiometry for microelements (e.g. Cu, Pb, and Se)[7,32]. However, the situation is more complicated as more data have emerged[8,11]. Our results did not support the hypothesis that homeostasis regulation on macroelements (N, P, and Ca) is greater than that on microelements (Mn, Zn, and Al) (Fig. 4). This may be due to the fact that N and P, generally limiting elements for growth and reproduction[41] and required in large quantities, readily increase with the increases of soil nutrient supplies. Comparatively, microelements are low in plant requirements and environment availability and therefore need to be strongly regulated in order to have adequate but not excess uptake. This suggests that the degree of element homeostasis regulation depends more on plant demands and element restriction in the environment than on the quantities of these elements in organisms in order to maximize uptake for optimal growth and at the same time to avoid toxic damage.

The weakest homeostasis in Fe (Fig. 4) is likely due to the fact that Fe is an essential element for most energy-transducing biological reactions[23] and, in most cases, is a limiting element in the soil. At
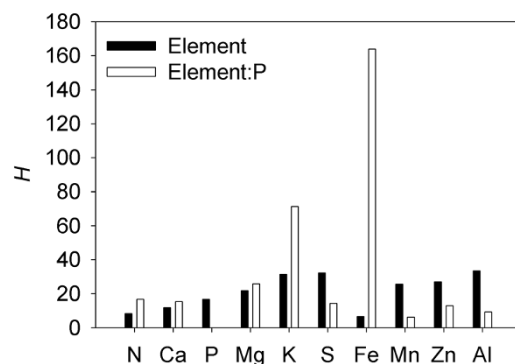

Figure 4 | H values of 10 elements and 9 element:P ratios in *Q. variabilis* leaves. Elements on the x-axis are grouped by macronutrients (N, P, S, K, Ca, and Mg), micronutrients (Fe, Mn, and Zn), and non-essential micronutrients (Al). The black bars represent individual elements while the white bars represent element:P ratios.
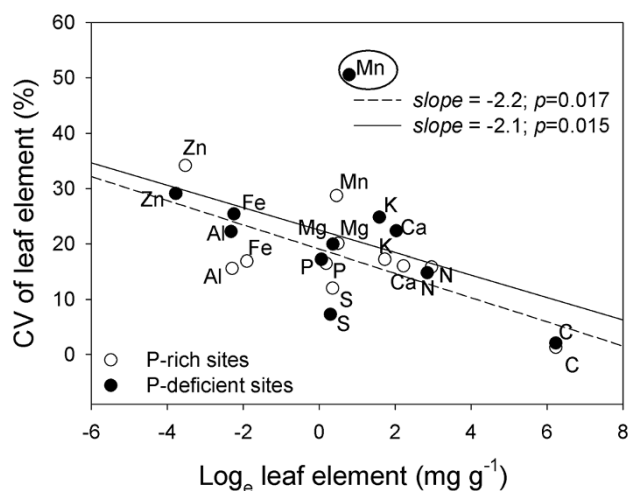
Figure 5 | Relationships between element concentrations and their coefficients of variation (CV) in *Q. variabilis* leaves across the study area in Yunnan Province, southwestern China. The data of Mn circled are excluded from the analysis.

insufficient level, Fe uptake increases with the increase of soil supply, showing low homeostasis. In contrast, leaf Mn in *Q. variabilis* was not significantly correlated with the soil supply (Fig. 2) and showed a stronger homeostasis regulation than Fe. This is due to the antagonistic interaction of Mn with Fe or P in organisms where Mn concentration decreases with the increase of Fe concentration (Supplementary Fig. S1)[1,42]. Therefore, leaf Mn concentration is less influenced by soil supply, but more by other associated elements in plants.

As found in previous studies[43–45], the PCA analysis showed two different groups of elements, N, P, S, Fe, and Zn associated with the

first component and C, Ca, K, and Mg with the second component (Fig 3a, Supplementary Table S5). This grouping reflects different biological functions of these elements[43]. N, P, and S are major elements for nucleic acids and proteins[22,24], while Fe and Zn are important for enzyme activities and Ca, K and Mg are associated with plant structure[4,22,46,47]. The significant correlation of leaf P with the protein-related elements (Fig. 3b) suggests that protein synthesis for biomass growth and development is coupled with RNA content (strong relationship with body % P), consistent with Growth Rate Hypothesis (GRH)[48].

Our results are consistent with the findings by Han *et al.* (2011) and Karimi & Folt (2006) that the variability of element concentrations increases from macronutrients to micronutrients and to non-essential metals. Karimi & Folt (2006) attribute this increasing variability to decreasing homeostasis regulation. Our results seem to be contradicted in the variability of element concentrations and homeostasis regulation between macroelements and microelements. We believe that this relates to the calculation of homeostasis coefficient (H)[4] that only indicates a general trend of plant nutrient variation with the environment. Depending on how well homeostasis regression line is fit, high homeostasis can be either associated with low variability (small residuals or good regression fit) or high variability (large residuals or poor regression fit). In this study, macroelements had low homeostasis and low variability (small residuals), while microelements showed high homeostasis and high variability (large residuals). This suggests that data distribution affects the relationship between element variability and homeostasis due to the way how homeostasis is defined.

In summary, our study provided a more complete picture of plant stoichiometry for both macro- and micro-elements. Our conclusions are (i) element ratios are more stable than individual element concentrations, (ii) stoichiometric homeostasis of macroelements is weaker than that of microelements, and (iii) varying P concentration has greater impacts on protein-related elements than on non-protein
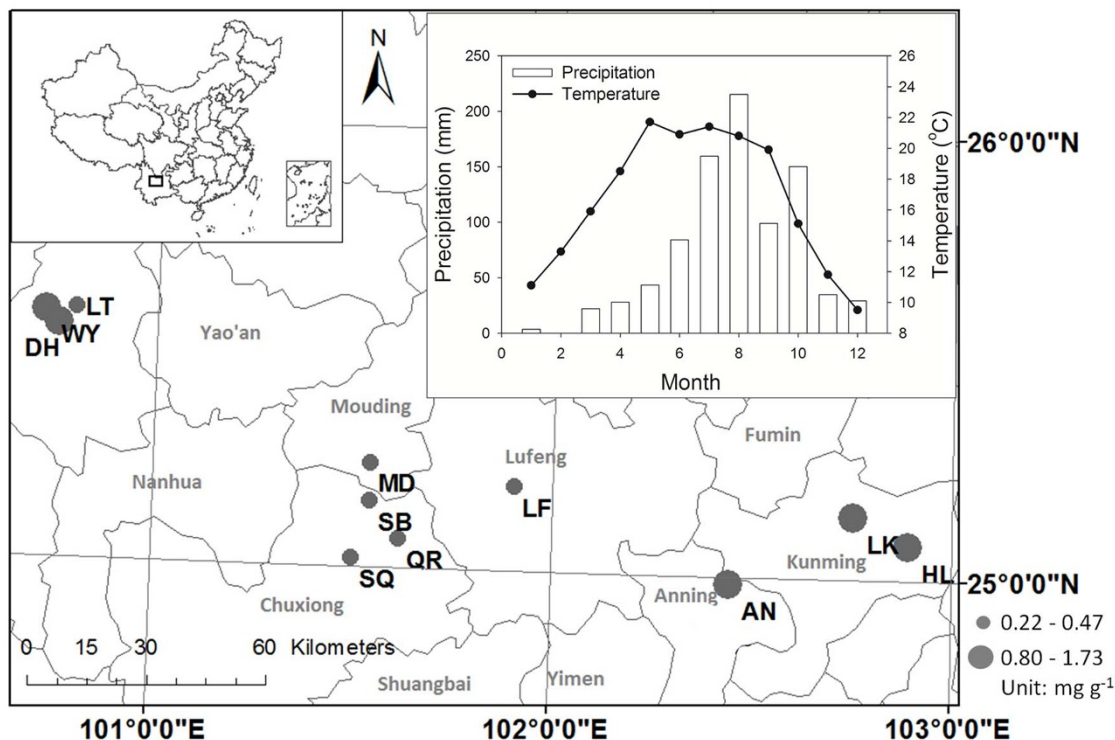

Figure 6 | Distribution, temperature, and precipitation of sampling sites across the study area in Yunnan Province, southwestern China. Circles in different sizes represent different soil P concentrations. Bar plot and line chart show precipitation and temperature in Kunming city. The complete name of each site is given in Supplementary Table S1. The map was drawn by the author of Xuan Zhou with Arcgis 10.0 by ESRI.

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elements in *Q. variabilis* growing on a range of P concentrations from P-rich to P-deficient soils.

Methods

Study area. The study area is located in Kunming city and the surrounding areas, in Yunnan Province, southwestern China (21°8′32″–29°15′8″ N, 97°31′39″–106°11′47″ E) where natural phosporite is distributed in mosaics (Fig. 6). The area belongs to the central Yunnan–Guizhou Plateau (about 2000 m a.s.l.). The mean annual temperature is 15°C and the mean annual precipitation is 1050 mm (Fig. 6). The main soil types are ferralic cambisol and orthic acrisol, both of which are acidic (Supplementary Table S6). The zonal vegetation is evergreen and deciduous broadleaf forests. In the study area, *Q. variabilis* occurs in single species stands or in mixtures with other broadleaf trees.

Sampling. A total of 11 sites were selected across the study area (Fig. 6; Supplementary Table S6). At each site, three 20 m × 20 m plots were established and a total of nine *Q. variabilis* trees (3 × 3, three trees each plot) were selected. The exception was the Anning site where soil P is extremely high and four plots were established. All trees were estimated to be more than 30 years old at the time of sampling.

The leaf and soil sampling was conducted in August, 2011. The leaves that were collected in the upper middle part of the south-facing crown, from the three trees of each plot, were mixed to make a composite leaf sample. Thus, a total of 34 leaf samples (3 plots × 10 sites + 4 × 1 Anning site) were obtained. The leaf samples were dried at 105°C for 0.5 h before 60°C for 48 h. The sample leaves were ground and sieved through a 60 mesh sieve (0.25 mm diameter) for chemical analysis. Soil cores were systematically extracted with a cylindrical auger at 5 locations within each plot. The soil sample of each location was divided into three layers (0 cm to 10 cm, 10 cm to 20 cm, and 20 cm to 30 cm) and the samples of the same layer were mixed, making 3 composite soil samples for each plot. Soil samples were sieved after air drying through a 60 mesh sieve (0.25 mm diameter) for chemical analysis.

Chemical analysis. Soil pH was determined in a 1 : 5 soil: 0.01 M KCl solution. The soil and leaf samples were digested using trace metal-grade nitric acid and diluted in 100 mL of distilled water. Total C and N (mg g^{-1}) were determined with elemental analysis-stable isotope ratio mass spectrometer (Vario ELIII, Elementar, Germany) and total P, S, K, Ca, Mg, Al, Fe, Mn, and Zn with plasma optical emission spectrometer (ICP-OES) (Iris Advantage 1000, Thermo Jarrell Ash, Franklin, MA) at the Analytical and Testing Centre, Shanghai Jiao Tong University. All the element concentrations were expressed in milligrams per gram (mg g^{-1}) dry weight.

Calculation of homeostasis coefficient. The degree of stoichiometric homeostasis of a leaf element was characterized by the homeostasis coefficient H[4]:

$$H = \frac{\log_e(x)}{\log_e(y) + \log_e(c)} \quad (1)$$

where y is the element concentration or ratio in plants, x is the same element concentration or ratio in the environment[4] and c is a constant[4]. The values of H were obtained from the relationships between y and x through linear regression analysis with SigmaPlot 10.0 (Systat software Inc.). A higher H represents a stricter homeostasis regulation, while a lower H indicates a weaker homeostasis[4].

Statistical analysis. A cluster analysis was run to group sample sites with different soil P concentrations. An 80% dissimilarity was chosen in the resulting hierarchical tree to distinguish sites of high and low soil P. Based on this grouping, ANOVA was used to test the differences of soil element concentrations and analysis of covariance (ANCOVA) was utilized to test the differences of regression lines between the two site types. Major axis regression (MA regression) analysis was used to determine a line of best fit for a bivariate relationship between leaf and soil element concentrations (data were log$_e$ transformed so as to fit linear regressions). We also conducted principal component analysis (PCA) to determine whether the element concentrations between P-rich and P-deficient sites could be discriminated and to examine which elements influence the separation of plant stoichiometry between the two soil site types. PCA was done with leaf data (C, N, P, S, K, Ca, Mg, Al, Fe, Mn, and Zn) that included 34 plots from both P-rich and P-deficient sites. Then generalized linear models (GLM) were used with PCA scores of first and second components as dependent variables and the concentrations of leaf P as independent variables. All these analyses were carried out with R version 2.15.0 (The R Foundation of Statistical Computing, 2012) and SigmaPlot 10.0 (Systat software, Inc., 2006). Map was drawn with Arcgis 10.0 by ESRI.

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Author contributions

C.L. designed the study; X.Z., X.S., B.D. and C.L. collected field samples and data; X.Z. analyzed the data; X.Z. and C.L. wrote the manuscript; and S.Y. provided editorial advice.

Additional information

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