Understanding nutrient allocation based on leaf nitrogen isotopes and elemental ratios in the karst region of Southwest China

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ABSTRACT

It is important to understand nutrient-use-efficiency based on the interaction between nutrient allocation and plant growth. This study utilized mulberry (*Morus* spp.), an economically important plant for farmers, as an example, to investigate the effect of nutrient allocation on plant growth and its response to environmental conditions in the karst region of Southwest China. Leaf stoichiometric analysis and isotopic measurements were applied to reveal the causes and effects of the allocation of different nutrients. The results illustrated that the N:P ratios of mulberry leaves decreased as the mean temperature increased and plant tissues grew, exhibiting a high leaf N:P ratio (21.82 ± 6.33) at the early stage of leaf expansion, and a low ratio (10.70 ± 2.66) at the late stage of leaf development. The low leaf N:P ratios indicated that leaf development was largely limited by nitrogen availability as the plant grew. The slight difference of $\delta^{15}N$ between leaf and root (leaf-root $\delta^{15}N$, −0.30 ‰) implied that the mulberry preferred ammonium-N at the early stage of leaf development, while the large difference of leaf-root $\delta^{15}N$ (1.58 ‰) implied that the mulberry preferred nitrate-N at the late stage. Although the ammonium uptake could improve P availability by increasing soil pH value deficiency at the early stage of leaf expansion, plant growth was constrained by P availability, given the relatively high P uptake from soils and the large P allocation in leaves. Moreover, the allocations of N and P were strongly correlated with other nutrients allocations. P exhibited strongly positive effects on Ca and K allocations under P deficiency at the early stage of leaf expansion, while N presented important impacts on them under N deficiency at the late stage. To summarize, this study highlighted the interactions between allocations of different nutrient components which are related to plant growth and environmental conditions in the karst system.

1. Introduction

The concentrations of phosphorus (P) and nitrogen (N) in plant leaves vary seasonally, which is related to nutrient availability, environmental conditions, annual physiological cycle, and extent of maturity (Regina et al., 1997; Kandylis et al., 2009; Zhang et al., 2015; Okada et al., 2017; Ren et al., 2017). P, as one of the most essential macronutrients for plant development and growth, is most likely insufficient for plant usage (Netzer et al., 2017; Wu et al., 2017). The uptake of P from soils and its allocation into leaves mainly occur in spring (from mid-March to late May) (Rosecrance et al., 1996), while the deficiency of P in plants may occur across growing seasons, especially for newly established vegetation (Goloran et al., 2016). Likewise, N uptake varies across chemical species (nitrate, ammonium etc.) and growing stages, depending on ion proportions and environmental conditions (Silva et al., 2013). Seasonal changes of leaf N content reflect

plant physiology and environmental impacts (Chen et al., 2015). Therefore, understanding nutrient dynamics is essential for fertiliser management, which can optimize nutrient using efficiency via fertilization according to plant uptake capacity of nutrients (Rosecrance et al., 1996; Khan et al., 2018; Sun et al., 2018).

Zinc (Zn) and Copper (Cu) are important micronutrients for plants by promoting growth and improving stress tolerance (Hasanuzzaman et al., 2017). For example, Zn acts to resist drought stress by maintaining high water use efficiency (Hasanuzzaman et al., 2017). Therefore, Zn and Cu in plant leaves could be used to indicate plant growth status and local environmental stress (Jain et al., 2010). Many studies showed that the two micronutrients were often insufficient for plant growth, leading to a massive loss of crop yield, mainly because the availability of Zn and Cu in soils was frequently constrained by environmental factors such as drought (Jain et al., 2010).

Previous studies found that the supply of nitrogen would positively

affect the Zn concentration in the plant tissue/organ (Cakmak et al., 2010), and nitrogen addition might increase the concentration of Zn in wheat grain (Kutman et al., 2010; Shi et al., 2010; Xue et al., 2014) and potatoes (Muttucumaru et al., 2013). Conversely, the supply of Zn negatively affected the uptake of P (Zhu et al., 2001). Ova et al. (2015) reported that P-induced Zn deficiency was one of the negative interactions most commonly studied in plant nutrition allocation. Meanwhile, the preferred uptake of N in plants could enhance potassium (K) allocation in leaves due to the fact that K was the counter cation for nitrate transportation through the xylem (Piao et al., 2018). Therefore, maintaining a balance among different nutrients in the soil is critical for plant growth (Tang and Luan, 2017), and the negative or positive interactions between nutrients should be considered for fertilizer treatments to increase the nutrient use efficiency for plant growth (Bindraban et al., 2015).

Mulberry (*Morus* spp.) is an important economic plant for farmers in China, especially in the karst regions of Southwest China (Piao et al., 2016), because its leaves can be used as a protein-rich forage supplement (Gulyani and Khurana, 2011). Understanding the impact of environmental factors, such as temperature and drought, on nutrient allocation of mulberry leaves is crucial for developing measures to improve the quality of leaves and their tolerance to environmental stresses (Hasanuzzaman et al., 2017). Moreover, the mulberry has helped conserve soil and water in the mountain regions with fragile ecosystems suffering from severe deforestation and soil erosion (Liu and Willison, 2013), especially in the mountainous karst terrains of Southwest China, where land degradation becomes serious and results in economic backwardness (Liu, 2007; Liu et al., 2020). Mulberry is a perennial tree widely planted in Southwest China for economic benefits and medicinal purposes. Local farmers usually plant mulberry trees on sloping grounds and sparse agricultural land abandoned for direct economic benefits and to assist in soil conservation in the hilly karst areas. However, little is known about the patterns of nutrient characteristics and allocation in mulberry trees. Therefore, it is necessary to understand the pattern for improvement of agricultural economic benefits in future. This study aims to assess how the nutrient allocations (including N, P, Ca, Mg, K, Cu and Zn) in the mulberry leaves respond to temperature, and to investigate how the different N species (nitrate and ammonium) affect P uptake in leaf growth and development.

2. Materials and methods

2.1. Study sites and sample collection

The study sites are located in the Guizhou Province of Southwest China, a hilly karst area with a fragile ecosystem. Karst landscapes are widely distributed throughout Southwest China, where economic development is hindered by thin soils in the continuous mountain terrains inhabited by large populations (Liu, 2007; Liu et al., 2016, 2020). Agriculture has long been present on the hilly slopes and on valley floors wherever soils exist, leading to serious degradation of water and soils. As a result, Mulberry were widely planted on many hill slopes to not only produce economic benefits but also prevent soil losses in the karst region of Southwest China (Piao et al., 2016).

Mulberry samples were collected at different field sites during two seasons: a) samples were collected from Yizhou, Libo and Huangping (n = 31) located in Southwest China in late March, i.e. at the early stage of leaf expansion; b) samples were collected from Libo and Huangping (n = 33) in late May, i.e. in the late stage of leaf development. The average lowest and highest temperatures, which were determined by altitudinal and latitudinal gradients, decreased from Yizhou, to Lib, to Huangping (Table 1). In this study, the below-ground parts of mulberry trees aged less than five years were used, whereas all shoots (above-ground parts) irrespective of plant age were used. Yellow Ultisols are the main soil types. Maize was grown before the mulberry plantations in some sampled soils. Soil samples were collected from a topsoil

surface of 15 cm, with six replicated plots for each sample around the canopy for measurement.

2.2. Laboratory analysis

Plant samples were dried in the oven at 60 °C for 48 h and then were ground for laboratory analysis. The total organic carbon and nitrogen content in plants and soils was analysed by a CHNS autoanalyser (PE 2400–ll., USA). Soil macronutrients were extracted using 1 M NH_4OAC for the analysis. The P was measured by the vanadomolybdate colorimetric method after digestion. The concentration of macronutrients of the plant and soil samples were determined by Atomic Absorption Spectroscopy (AAS, PE900 F, USA) after digestion. The available Cu and Zn in soils were measured with ICP-MS and ICP-OES after extraction with diethylene triamine pentacetic acid (DTPA) (Lindsay and Norvell, 1978). The content of soluble sugar was determined by a modified version of the method used by Chinnasamy and Bal (2003) after 0.25 g of air-dried material was extracted four times with distilled water at 75 °C. The N isotope, including ammonium-N and nitrate-N, of samples was analyzed using an IsoPrime isotope ratio mass spectrometer interfaced with a Euro EA 3000 elemental analyzer (Elementar Analyse System GmbH, GER) (Piao et al., 2012).

2.3. Statistical analysis

Statistical analysis in this study was conducted using SPSS software. The differences in $\delta^{15}N$ values between the leaves and the roots, i.e., $\delta^{15}N$ (leaf-root), were utilised to indicate the deviation of leaves from roots. The *t*-test was used to evaluate the difference between soil and tissue samples in terms of nutrient concentrations, leaf C:N:P stoichiometry, and $\delta^{15}N$ values. Pearson correlation coefficients and linear regression were employed to assess the correlation between soil and plant parameters. Differences were considered significant at values of $P < 0.05$ for all statistical analyses in this study.

3. Results

3.1. Soil properties

Soil pH values in Huangping significantly differed from those in Libo and Yizhou ($P < 0.05$, Table 1). Correspondingly, soil-extractable Ca concentrations were the highest in Huangping soils developed from limestone, and were the lowest in Libo and Yizhou developed from dolomite, indicating the presence of a significant positive relationship between soil Ca and soil pH ($r = 0.750$, $P < 0.001$, n = 64). The values of soil organic carbon, soil total N and P concentrations differed slightly in the Yizhou, Libo and Huangping soils. Soil extractable Mg and K concentrations in Huangping were similar to those in Yizhou (Table 1), but were higher than those in Libo (p < 0.01). In the samples collected in late March, both soil extractable Ca and Mg did significantly affect leaf Ca ($r = 0.425$, $P < 0.05$) and Mg concentrations ($r = 0.566$, $P < 0.01$), respectively. There was no significant correspondence of soil extractable Ca ($r = -0.014$) and Mg (r = −0.258) with altitude. Other nutrients in soils, including K, N, P, Cu and Zn, did not significantly affect leaf nutrient concentrations. In the samples collected in late May, neither soil extractable Ca nor Mg significantly affected leaf Ca ($r = 0.271$) and Mg concentrations ($r = 0.104$). Soil extractable Ca ($r = 0.361$, $P < 0.05$), Mg ($r = 0.639$, $P < 0.001$) and K ($r = 0.510$, $P < 0.01$) increased with altitude, while other nutrients did not. No significant correspondence was found between soil and leaf nutrients, including Ca, Mg, K, N, Zn and Cu, except for P ($r = 0.345$, $P < 0.05$).

3.2. Leaf nutrient concentrations and stoichiometry

The plant height decreased significantly with increasing altitude for samples collected at the same sampling period (Fig. 1), indicating that

Table 1
The characteristics of sampling sites.

|		Yizhou (N = 16)	Libo (N = 25)	Huangping (N = 23)
Altitude (m)		201 ± 33	591 ± 93	798 ± 69
Latitude		24°18′N-24°39′N	25°17′N-25°29′N	26°46′N-26°55′N
March	Lowest temperaturea	14 °C	13 °C	8 °C
	Highest temperatureb	20 °C	20 °C	15 °C
May	Lowest temperaturea	22 °C	21 °C	17 °C
	Highest temperatureb	29 °C	29 °C	26 °C
Soil pH	(H₂O)	5.24 ± 0.90	5.05 ± 0.91	6.01 ± 0.96
Soil C$_{org}$c	(mg g-1)	20.94 ± 8.93	27.33 ± 23.51	20.79 ± 13.20
Soil TotalN	(mg g-1)	1.37 ± 0.59	1.44 ± 0.70	1.77 ± 1.30
Soil Total P	(mg g-1)	0.44 ± 0.24	0.50 ± 0.41	0.62 ± 0.34
Soil extr. Cad	(mg g-1)	1.67 ± 1.20	1.62 ± 1.74	2.80 ± 1.35
Soil extr. Mg	(mg g-1)	0.29 ± 0.49	0.14 ± 0.15	0.39 ± 0.32
Soil extr. K	(mg g-1)	0.20 ± 0.10	0.07 ± 0.04	0.22 ± 0.22

[a]Lowest monthly mean temperature; [b]Highest monthly mean temperature; [c]Soil organic C; [d]Soil extractable Ca.
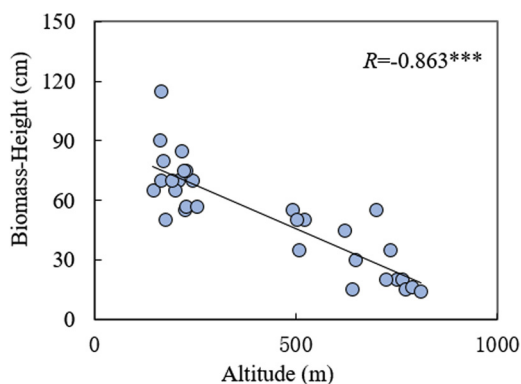
Fig. 1. Diagram showing relationships between plant height and altitude for the samples collected in late March (n = 31).

altitude largely affected plant height as well as biomass. The leaf N:P ratio of samples collected during late March (early leaf development) greatly decreased with the increasing altitude ($r = -0.829$, $P < 0.001$) (Fig. 2a), indicating a significantly positive correlation between leaf N:P ratio and plant biomass at the early stage ($r = 0.720$, $P < 0.001$). This trend was mainly driven by variations in P concentrations, given that leaf P concentrations significantly increased with increasing altitude ($r = 0.770$, $P < 0.001$) (Fig. 2c) while the leaf N concentrations rarely changed with altitude ($r = -0.160$, Fig. 2b). Similarly, leaf Ca concentrations increased with altitude ($r = 0.496$, $P < 0.01$), illustrating the consistent change of leaf P and Ca concentrations ($r = 0.750$, $P < 0.001$, Table 2). In samples collected during late May (late leaf expansion stage), the leaf N:P ratios also decreased with increasing altitude ($r = -0.493$, $P < 0.01$, Fig. 3a). However, this trend was mainly driven by variations in the leaf N concentrations ($r = -0.435$, $P < 0.05$, Fig. 3b) rather than in the leaf P concentrations ($r = 0.272$, Fig. 3c). By contrast, the Ca concentrations did not vary with altitude ($r = 0.165$), lacking mutual dependence between the change of leaf P and Ca concentrations ($r = -0.069$, Table 3). In addition, no significant relationships between leaf P and N concentrations were observed in the samples collected in either late March or May in this study (Tables 2 and 3), but the relatively high concentrations of leaf Ca induced relatively low leaf N concentrations, especially in the samples collected in late May ($r = -0.377$, $P < 0.05$) (Tables 2 and 3).

In the samples collected in late March, leaf C concentrations negatively correlated with leaf P concentrations ($r = -0.548$, $P < 0.01$), which negatively correlated with leaf sugar concentrations ($r = -0.564$, $P < 0.01$, Table 2). By contrast, both leaf C ($r = -0.602$, $P < 0.001$) and leaf sugar concentrations ($r = -0.457$, $P < 0.05$) correlated negatively with altitude. However, no significant relationship between leaf P and either leaf C or leaf sugar concentrations was
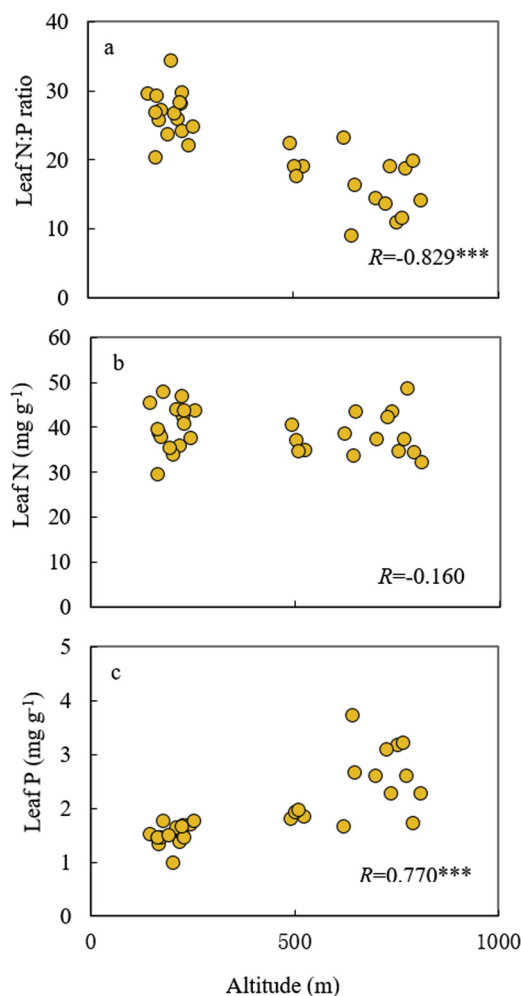
Fig. 2. Plots showing the relationship between altitude and nutrients in the samples collected in late March (n = 31). (a) Leaf N:P ratios (b) leaf N concentrations, and (c) leaf P concentrations.
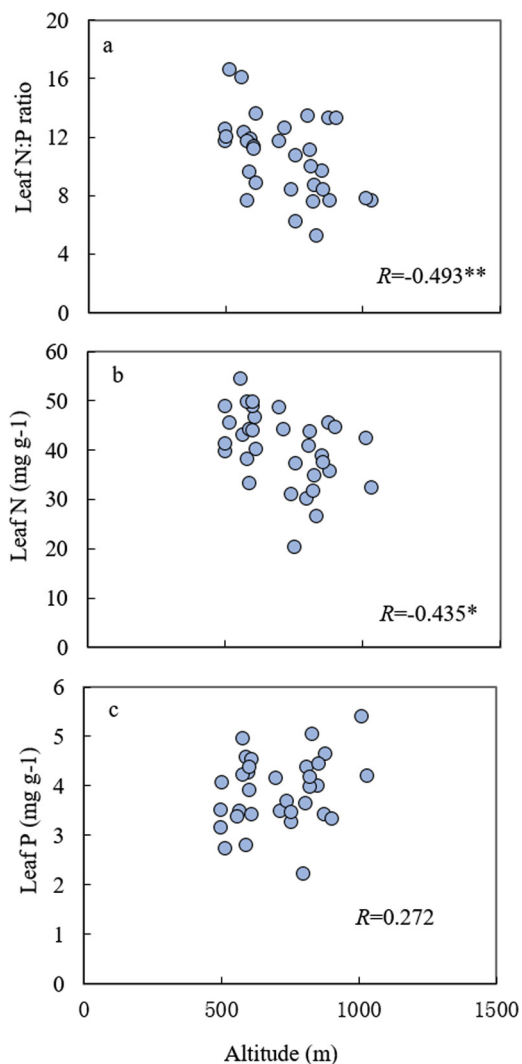
observed in the samples collected in late May (Table 3), nor was relationship between either leaf C ($r = -0.082$) or leaf sugar concentrations ($r = 0.217$).

A significant positive relationship between leaf sugar and leaf C:N ratios was observed in samples collected in late March ($r = 0.790$, $P < 0.001$, Fig. 4a), responding to the variations in both leaf C ($r = 0.596$, $P < 0.001$, Fig. 4b) and leaf N concentrations ($r = -0.393$, $P < 0.05$, Fig. 4c). Similarly, leaf sugar concentrations were positively

Table 2
Coefficients (*r*) of Pearson correlations between leaf nutrients in samples collected in late March (n = 31).

| Late of March	Leaf N	Leaf Ca	Leaf Mg	Leaf K	Leaf Al	Leaf sugar	Leaf C
Leaf P	−0.039	0.750***	−0.229	0.409*	0.526**	−0.564**	−0.548**
Leaf N		−0.178	0.055	0.204	−0.070	−0.393*	0.320
Leaf Ca			−0.225	−0.012	0.535**	−0.285	−0.309
Leaf Mg				−0.166	0.146	0.110	0.223
Leaf K					0.228	−0.505**	−0.462**
Leaf Al						−0.289	−0.262

* *P* < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Plots showing the relationship between altitude and nutrients in the samples collected in late May (n = 33). (a) Leaf N:P ratios (b) leaf N concentrations, and (c) leaf P concentrations.

Fig. 4. The variation of parameters as a function of the leaf sugar in the samples collected in late March (n = 31). (a) Leaf N:P ratios (b) leaf C concentrations, and (c) leaf N concentrations.

Table 3
Coefficients (*r*) of Pearson correlations between leaf nutrients in samples collected in late May (n = 33).

Late of May	Leaf N	Leaf Ca	Leaf Mg	Leaf K	Leaf Al	Leaf sugar	Leaf C
Leaf P	0.057	−0.069	0.158	0.180	0.088	−0.232	−0.099
Leaf N		−0.377*	0.292	0.123	−0.295	−0.718***	0.313
Leaf Ca			−0.151	0.293	0.109	0.019	−0.742***
Leaf Mg				−0.304	0.035	−0.398*	0.139
Leaf K					−0.036	−0.529**	−0.364*
Leaf Al						0.060	0.272

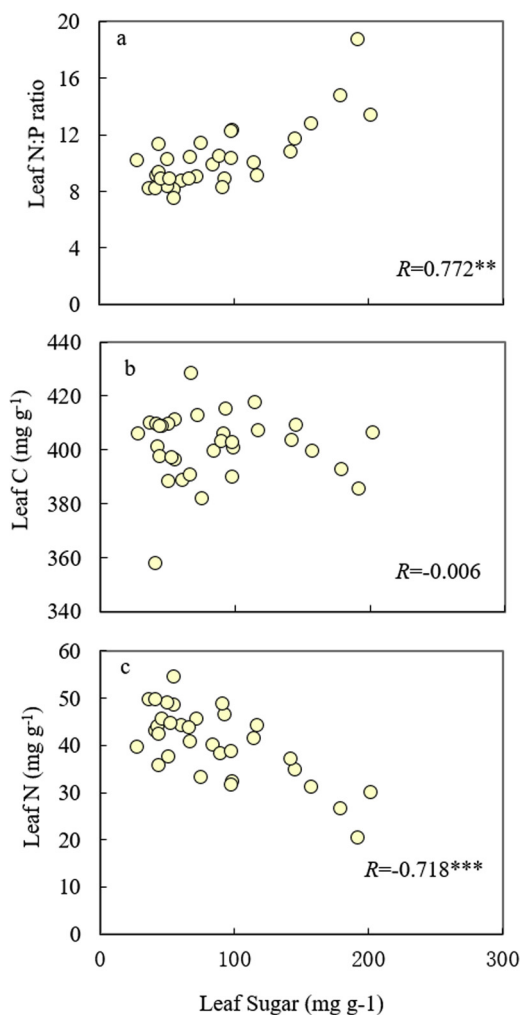* *P* < 0.05; **P < 0.01; ***P < 0.001.
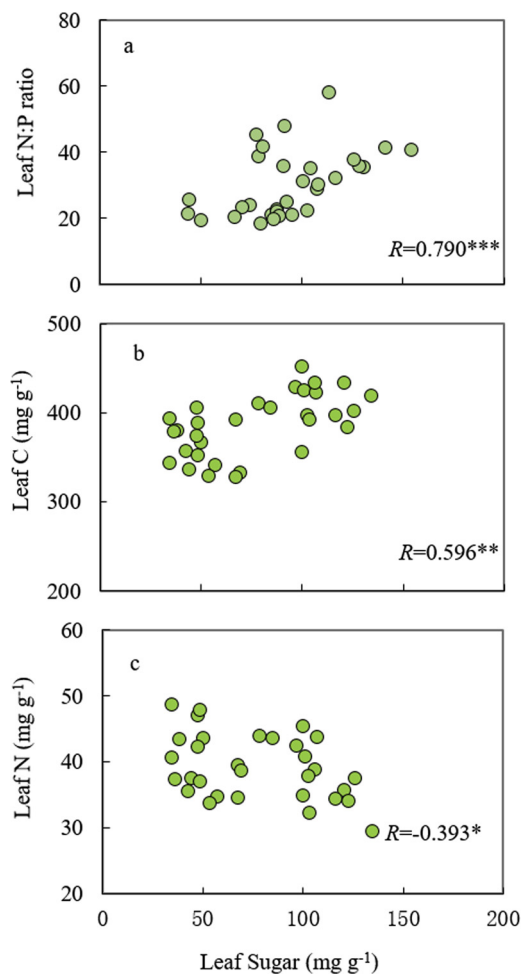
Fig. 5. The variation of parameters as a function of the leaf sugar. (a) Leaf N:P ratios (b) leaf C concentrations, and (c) leaf N concentrations in the samples collected in late May (n = 33).
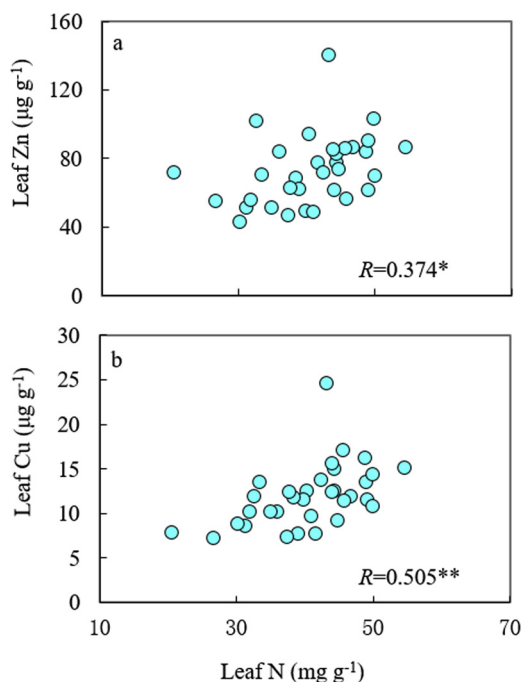

Fig. 6. The leaf metal elements vs leaf nitrogen (a) leaf Cu, (b) leaf Zn.

collected in late May were significantly higher than that in the samples collected in late March, while leaf Ca concentrations varied little between the two groups (Table 4).

There were no significant relationships between altitude and leaf sugar ($r = 0.217$), leaf Zn ($r = -0.113$) or leaf Cu ($r = -0.215$). Leaf N concentrations increased with leaf Zn ($r = 0.374$, $P < 0.05$) and leaf Cu ($r = 0.505$, $P < 0.01$) concentrations (Fig. 6), while leaf C concentrations decreased as leaf Zn ($r = -0.381$, $P < 0.05$) and leaf Cu ($r = -0.465$, $P < 0.01$) concentrations increased (Fig. 7). Therefore, leaf Zn and leaf Cu concentrations were negatively correlated with leaf C:N ratios (Fig. 8). Similarly, leaf sugar concentrations were negatively correlated with leaf Zn ($r = -0.386$, $P < 0.05$) (Fig. 9a) and leaf Cu ($r = -0.579$, $P < 0.001$) (Fig. 9b) concentrations.

3.3. Variation of $\delta^{15}N$(leaf-root) value

There was no significant correlation between soil $\delta^{15}N$ and leaf or root $\delta^{15}N$ and $\delta^{15}N$ (leaf-root). The mean value of $\delta^{15}N$ (7.75 ± 1.44 ‰) in soil samples collected in late March was higher than that (6.59 ± 1.23 ‰) collected in late May. As listed in Table 5, the mean value of $\delta^{15}N$ (2.95 ± 1.23 ‰) in leaf samples collected in late March was lower than that (5.09 ± 2.30 ‰) collected in late May. Meanwhile, the mean value of $\delta^{15}N$ (leaf-root) was −0.30 ± 1.86 ‰ in the samples collected in late March and was significantly lower than that (1.58 ± 1.20 ‰) collected in late May listed in Table 5.

correlated with leaf C:N ratios ($r = 0.772$, $P < 0.001$) (Fig. 5a), which were mainly determined by the variation in leaf N ($r = -0.718$, $P < 0.001$) (Fig. 5c, Table 3). Leaf C:N ratios were not significantly correlated with altitude in the samples collected in late March ($r = -0.259$), but were positively correlated in the samples collected in late May ($r = 0.370$, $P < 0.05$). The difference in leaf C:N ratios between the two groups (late March and late May) of the samples was not significant. However, a significant difference was found in leaf N:P ratios between the two groups. Therefore, the mean value of leaf N:P ratios (21.82 ± 6.33) in the samples collected in late March was relatively higher than that (10.70 ± 2.66) in the samples collected in late May (Table 4). The mean concentration of leaf P in the samples collected in late May was higher than that in the samples collected in late March (Table 4). The mean concentrations of leaf Mg, K and Al in the samples

Table 4
Comparison mean leaf nutrient concentrations between the samples collected at late of March and May.

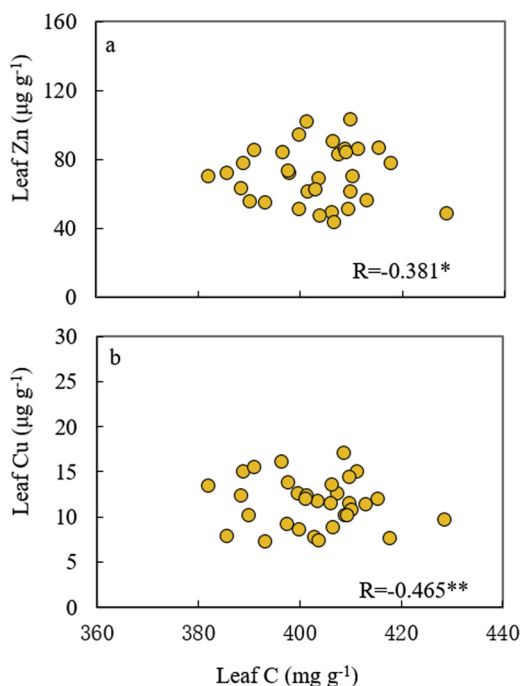|	Leaf N (mg g-1)	Leaf P (mg g-1)	Leaf Ca (mg g-1)	Leaf Mg (mg g-1)	Leaf K (mg g-1)	Leaf Al (μg g-1)	Soil pH (H$_2$O)
Late of March	39.30	1.97	16.89	3.47	19.09	3.65	5.46
N = 31	(4.87)	(0.65)	(5.06)	(0.94)	(4.94)	(2.09)	(0.84)
Late of May	40.52	3.90	18.93	4.82	22.54	5.43	5.42
N = 33	(7.52)	(0.70)	(4.71)	(1.76)	(5.83)	(3.78)	(1.16)
t test	NS	P < 0.001	NS	P < 0.001	P < 0.05	P < 0.05	NS

Fig. 7. The leaf metal elements vs leaf carbon (a) leaf Cu and (b) leaf Zn.
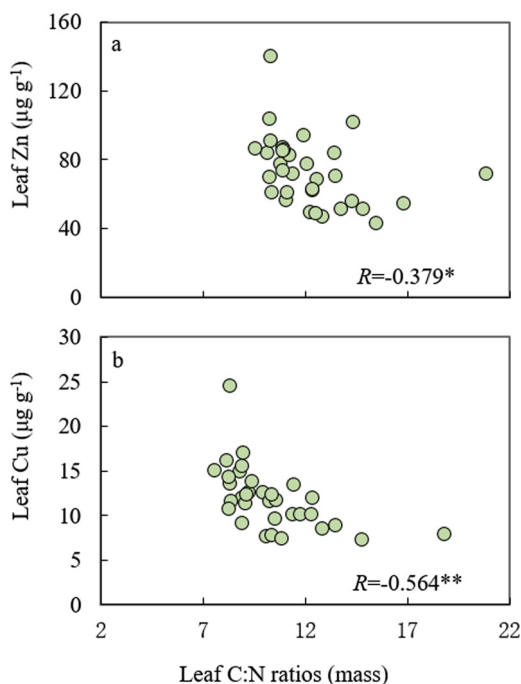
Fig. 8. Parameters (a) Leaf Cu and (b) leaf Zn vs leaf C:N ratios.

Fig. 9. The relationship between (a) leaf Cu, (b) leaf Zn and leaf Sugar.

Table 5
Comparison of mean values of $\delta^{15}N$ in mulberry collected at different seasons. Standard deviations in brackets; $\delta^{15}N$ (leaf-root), i.e., the difference of $\delta^{15}N$ between leaf and root.

	Soil $\delta^{15}N$ (‰)	Leaf $\delta^{15}N$ (‰)	Root $\delta^{15}N$ (‰)	$\delta^{15}N$ (leaf-root) (‰)
Late of March	7.75	2.95	3.25	− 0.30
N = 31	(1.44)	(1.23)	(2.06)	(1.86)
Late of May	6.59	5.09	3.50	1.58
N = 33	(1.23)	(2.30)	(2.33)	(1.20)
t-test	P < 0.01	P < 0.001	NS	P < 0.001

4. Discussion

4.1. Leaf C:N:P stoichiometry and nutrient allocation

The results in this study showed different trends between altitude and leaf nutrients at different growing status of the Mulberry (Figs. 1–3). These differences could have resulted from various environmental conditions such as temperature, which affects plant growth significantly (Piao et al., 2018; Wang et al., 2018). Previous studies showed that climatic conditions in various places could influence leaf nutrients content and ratios (Ågren, 2008; Sardans et al., 2016). Furthermore, the ratios of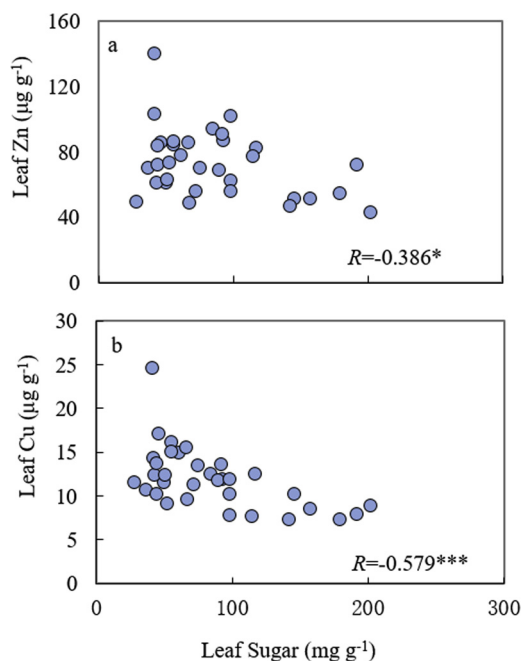 N:P were also reported to increase with mean temperature (Reich and Oleksyn, 2004; Sardans et al., 2016), and the above-ground biomass was positively correlated with the above-ground biomass N:P ratio with an average of 24.4 ± 2.9 (Ai et al., 2017). Generally, high temperature would lead to an increase in C:N ratios (Sardans et al., 2016). In this study, increasing temperature resulted in the reduction in both leaf N availability ($r = -0.952$, $P < 0.001$) and leaf C concentrations ($r = -0.194$) during the late stage of leaf expansion, but the former mainly contributed to the reduction in leaf C:N ratios. Meanwhile, various soil microbial activities under different environmental conditions could be linked to the C:N:P ratio in the plant-soil system (Ren et al., 2017), which might be responding to the nutrient allocation in the leaf.

The plant leaf C:N ratios in this study were controlled by variations in both leaf C ($r = 0.409$, $P < 0.05$) and N concentrations ($r = -0.722$, $P < 0.001$) during the early stage of leaf expansion (Fig. 4). By contrast, the leaf C:N ratios were controlled by leaf P concentrations at the late stage, and accompanied a lower ratio of N:P. These results illustrated that the variations of leaf C:N values were influenced by temperature during the early stage of leaf expansion and by changes in N availability during the late stage. The previous study suggested that a phosphorus deficiency mostly affected plant growth in the early season of grassland, with a decrease in the N:P ratio during the primary growth (Bélanger et al., 2017). However, Chen et al. (2015) reported that leaf N concentrations varied among different phenological phases, showing higher values in the early leaf expansion. Therefore, while plant growth might be limited by nitrogen during the beginning of the growing season, it is limited by phosphorus during the middle and final stages

(Rong et al., 2015). Moreover, leaf expansion was highly related to nitrogen concentrations given that the lowest nitrogen concentrations correlated with the lowest expansion rates (Malcolm et al., 2008). However, the variation of N:P ratio was reported as being mostly due to the variation in P concentration (Ågren, 2008). Overall, the present results indicated that leaf development was strongly limited by phosphorus availability during the early stage of leaf expansion (Figs. 2, 3), while the development was strongly limited by nitrogen availability during the late stage of leaf expansion. The result is in agreement with the previous study related with secondary and primary forests in the karst region (Zhang et al., 2015).

Leaf P concentrations were negatively correlated with leaf sugar concentrations ($r = -0.564$, $P < 0.01$) during the early stage of leaf expansion (Table 2), which indicated that lower leaf sugar concentrations were induced by higher leaf P concentrations (Figs. 4, 5). However, P starvation should induce the accumulation of sugar concentrations in leaves, which has immediate and direct impact on photosynthesis (Hammond and White, 2008). P deficiency might lead to an increase in leaf carbohydrate concentrations, while a good P absorption rate results in a decrease in leaf carbohydrate concentrations (Ruiz et al., 1996). In this study, it was apparent that the variations in leaf P concentrations were mainly caused by changes in mean temperature during the early stage of leaf expansion. Furthermore, the variations in leaf sugar concentrations were mainly controlled by changes in leaf N concentrations ($r = -0.718$, $P < 0.001$) rather than leaf P concentrations ($r = -0.232$), implying that relatively high concentrations of leaf N reduced the accumulation of leaf sugar during the late stage. Malcolm et al. (2008) found that leaf expansion was related to nitrogen concentration, since the leaf varieties with the lowest N concentrations also had the lowest expansion rates. Therefore, the pattern of nutrient allocations suggested that the changes in mulberry growth rate were caused by P deficiency, which responded to the variance in mean temperature during the early stage of leaf expansion. By contrast, the changes in growth rate caused by N deficiency were induced by the changes in N availability at the early stage of leaf expansion, and were gradually changed by temperature-related N demands during the late stage of leaf development.

4.2. Phosphorus availability and allocation in soil-plant system

Soil pH has significant impact on soil P availability. The solubility of phosphate salt could change with soil pH values, resulting in more available P for plant growth at soil pH 5–6 (Haneklaus and Schnug, 2016; Noyce et al., 2017). Imtiaz et al. (2017) suggested that changes in soil pH might influence the P uptake of plants, especially at pH < 6.0. Among samples collected during the late leaf expansion, Huangping exhibited a higher mean soil pH value (6.13 ± 0.71) than that (5.32 ± 0.53) in Libo. Correspondingly, the mean leaf P concentration (2.83 ± 0.67 mg g-1) in Huangping was higher than that (2.10 ± 0.38 mg g-1) in Libo. The results indicated that relatively high soil pH facilitated the uptake of P by the mulberry.

Soil pH in samples collected during the early stage of leaf development was not significantly correlated with altitude. By contrast, leaf P concentrations were significantly correlated with altitude, implying that soil pH was not the dominant factor controlling the variations of leaf P concentrations along an altitudinal gradient. Previous studies showed that ammonium-induced acidification was an important mechanism to control phosphorous availability in soils (Jing et al., 2010; Shen et al., 2011; George et al., 2016; Zhu et al., 2016; Gérard et al., 2017; Noyce et al., 2017). For example, root-induced acidification increases the efficiency of phosphorus acquisition, showing that ammonium-fed plants are more phosphorus efficient due to fast ammonium assimilation. Therefore, ammonium-induced acidification in soils would decrease soil pH and improve phosphorus uptake (Zhu et al., 2016). Besides, the result could be caused by the dissolution of Ca-phosphates in the soils (Noyce et al., 2017), in which root-induced

acidification could decrease pH by 2–3 units in the rhizosphere relative to the bulk soil (Marschner, 1995). Other studies showed that cotton and wheat have greater leaf phosphorus uptake rates in ammonium-rich soils than that in nitrate-rich soils (Goloran et al., 2016). The high calcium and alkaline environment in the karst area could immobilise phosphorus in the soil (Liu, 2007), and thus cause a phosphorus absence for plant growth. However, the soil nitrification of ammonium in this study could decrease pH value and enhance phosphorus availability for mulberry growth.

In the present study, leaf P concentrations increased slightly with altitude (Figs. 2, 3), indicating that the relatively high P concentrations in leaves occurred during the early stage of leaf development. In the karst region, Ca supply is abundant in the soil for plant growth (Liu, 2007; Liu et al., 2016). Thus, mulberry is much more efficient in acquiring P from Ca-P minerals at relatively high altitudes than at relatively low altitudes. Phosphorus retention in neutral-to-calcareous soils is dominated by precipitation reactions (Lindsay et al., 1989), producing dicalcium phosphate that is available for plant growth (Shen et al., 2011). Thus, the increasing leaf P concentrations corresponded with increasing leaf Ca concentrations along an altitudinal gradient but not with increasing leaf N concentration, indicating that leaf development in the early stage was more adversely affected by phosphorus deficiency than by nitrogen deficiency during the early growth stage. Moreover, the effect of soil P availability on leaf P concentration might be important given its active interactions with minerals, and should be investigated in future.

4.3. Uptake of nitrogen species by the plant constrained by leaf nitrogen and isotope

Plant growth in natural ecosystems underwent seasonal variances due to environmental changes, which caused seasonal changes in photosynthetic rates (Lewis et al., 1996). In seedlings of *Pinus radiata*, higher rates of photosynthesis were found to be more associated with nitrate availability than ammonium availability (Bown et al., 2010; Tsabarducas et al., 2017). However, ammonium increased the photosynthetic rate under water stress during the early developmental stage of rice (Guo et al., 2007), and photosynthetic activity was significantly higher in tomato plants provided with ammonium than in plants provided with nitrate (Horchani et al., 2010; Tsabarducas et al., 2017). Rates of change in nitrogen uptake might not be uniform across nitrogen forms, and the uptake of nitrate was more sensitive to temperature than that of ammonium during certain growth periods (Boczulak et al., 2014). Leaf N concentrations did not change along an altitudinal gradient during the early stage of leaf development in this study, while leaf N concentrations increased with increasing air temperature during the late stage of leaf expansion. This pattern could be attributed to the heterogeneity of nitrogen distribution.

The high temperature condition would increase the overall nitrogen availability in soils (Lukac et al., 2011), and the uptake of most nitrogen species might be positively correlated with temperature (Boczulak et al., 2014). The Douglas-fir showed a preferable uptake of nitrate during growth, which was common in warm areas, while the Sitka spruce showed a preferable uptake of ammonium during growth, which was abundant in cold soils (Boczulak et al., 2014). Cui et al. (2017) found that grasses preferred NH_4+ at the early stage but switched to NO_3- later, according to a field experiment using ^{15}N-labeled nitrogen. Furthermore, Hou et al. (2018) suggested that warming changed the uptake pattern of N for winter wheat by increasing the contribution of NO_3- while decreasing the contribution of NH_4+. The uptake of nitrate by spruce and beech increased with a rise in temperature at the maximum uptake occurring at 25 °C, but the uptake of ammonium decreases with a rise in temperature (Gessler et al., 1998). Warren (2009) suggested that the uptake of organic N forms was favored with cool temperatures, while nitrate was favored with warm temperatures. In addition, the higher soil temperature rapidly promoted the nitrification

of ammonium-N, increasing nitrate concentrations in the soil (Wang et al., 2016). As a result, nitrate was common in warm soils, while ammonium was abundant in cold areas (Boczulak et al., 2014). Soil NH_4+ concentration was higher than NO_3- at early stages but lower at later stages in the grass fields (Cui et al., 2017). Therefore, in this study, it seems that mulberry preferred ammonium during the early stage of leaf expansion because of the relatively low mean temperature in the spring season due to the small mean value of $\delta^{15}N$ (leaf-root) in the samples collected in late March.

Differences in $\delta^{15}N$ between leaves and roots, i.e. $\delta^{15}N$ (leaf-root), potentially gave a hint on interspecific differences in inorganic nitrogen source preferences (Kalcsits et al., 2015; Piao et al., 2018; Wu et al., 2019). The Chinese prickly ash had relatively high values of $\delta^{15}N$ (leaf-root), about 2.6‰, suggesting that Chinese prickly ash preferred nitrate in the previous study (Piao et al., 2018). However, for the Chinese fir, the value of $\delta^{15}N$ (leaf-root), -0.4‰, was relatively low (Piao et al., 2012), reflecting that the Chinese fir preferred ammonium. In the present study, the mean value of $\delta^{15}N$ (leaf-root) was -0.30‰ during the early stage of leaf development, but reached 1.58 ‰ during the late stage of leaf development.

A significant proportion of NO_3- assimilation occurred in shoots under various conditions due to the smaller amount of energy required for the assimilation processes during growth (Wang et al., 2012; Cui et al., 2017). Since nitrate reductases generally preferred the light isotopes, any residue root nitrate should be enriched in heavy nitrogen isotopes related with assimilated nitrate. The leaf $\delta^{15}N$ should become more enriched relative to the root if this enriched pool was transported to the leaf by assimilation processes (Kolb and Evans, 2002), resulting in higher leaf $\delta^{15}N$ than that in root. However, little difference in $\delta^{15}N$ between leaves and roots was found when ammonium was the nitrogen source during the experiment in the previous studies (Evans et al., 1996; Kolb and Evans, 2002). For example, aspen had a high value of $\delta^{15}N$ (leaf-root), reaching up to 3.0‰, when nitrate was supplied (Kalcsits et al., 2015). Plants need more energy to absorb and assimilate NO_3- compared to NH_4+ during growth, which might lead to a large isotopic difference. Therefore, it can be concluded that mulberry preferred ammonium during the early stage of leaf development, and nitrate partially during the late stage of leaf development.

4.4. Interactions of chemical compositions in leaf and impacting factors

Nutrient use efficiency could be impacted by the interaction of different nutrient components and environmental factors, such as moisture content, which influences the nutrient uptake (Bindraban et al., 2015). In this study, significant positive correlations were found between leaf N and Zn concentrations ($r = 0.374$, $P < 0.05$) as well as Cu concentrations ($r = 0.505$, $P < 0.01$). Contrarily, leaf Cu ($r = -0.465$, $P < 0.01$) and Zn concentrations ($r = -0.381$, $P < 0.05$) were significantly correlated negatively with leaf C content (Figs. 6, 7). The positive effect of improved N nutrition on leaf Zn in plants has also been documented in recent reports (Cakmak et al., 2010; Kutman et al., 2010; Shi et al., 2010). Low leaf Zn and Cu might reflect more active plant photosynthesis, showing good growing status. Thus, the negative correlation between these metals and C:N ratio (Fig. 8) suggested fast growth during the early stage for mulberry.

Sugar concentration in leaf was found to be negatively correlated with leaf nitrogen ($r = -0.718$, $P < 0.001$), Mg ($r = -0.398$, $P < 0.05$), and K ($r = -0.529$, $P < 0.01$), while it was shown not to be significantly related with leaf Ca and P concentrations. Therefore, increasing leaf N and P concentrations induced a decrease in leaf sugar concentrations under N deficiency at the early stage and P deficiency at the late stage, respectively, of leaf expansion. Verbruggen and Hermans (2013) found that the accumulation of sugar in leaves was a major consequence of decreased Mg. Like N and Mg, the increases in the concentrations of Cu and Zn in leaves were also associated with the decrease in leaf sugar concentrations (Fig. 9). Therefore, under N

deficiency in the late stage of leaf development, positive effects of N on Cu and Zn were more apparent than those of P. Like N, increasing leaf Zn and Cu concentrations were favorable for normal plant growth and metabolism (Cambrollé et al., 2011), potentially through decreasing leaf sugar concentrations.

5. Conclusion

This study investigated the nutrient allocations in the leaves of Mulberry (*Morus spp.*) and their responses to environmental conditions in the karst ecosystem of Southwest China. The Mulberry species preferred to take up ammonium rather than nitrate during the early stage of leaf expansion, and developed physiological strategies by enhancing ammonium-induced acidification in the high-Ca karst region for absorbing sufficient P. A strong relationship between leaf sugar and N concentrations suggested that leaf development was mainly determined by N availability, mainly because high temperature stimulated soil nitrification and plant uptake of nitrate in the late stage of leaf development. The isotopic analysis of $\delta^{15}N$ (leaf-root) further demonstrated that the mulberry preferred ammonium at the early stage of leaf development, but preferred nitrate at the late stage. Moreover, a strong correlation between leaf sugar and P concentrations reflected the fact that increased leaf P could inhibit the accumulation of leaf sugar, and thus facilitated photosynthesis in the early stage of leaf expansion with a relatively low air temperature. Besides, P exhibited more positive effects on Ca and K allocations under P deficiency at the early stage, but N presented more important impacts under N deficiency at the late stage. Furthermore, the concentrations of P and N affected the allocations of K、Ca、Zn and Cu, depending on the availability of P and N. For example, the allocations of Ca and K were controlled by P when its availability was limited at the early stage of leaf expansion, but were controlled by N when its availability was limited at the late stage. To summarise, this study illustrated that the concentrations and allocations of nutrients in mulberry leaves changed with growing stages, and were regulated by environmental conditions such as temperature, and the allocations of different nutrients interacted integrally. These results could facilitate managing the karst systems in pursuit of economic benefits and agricultural environmental conservation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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