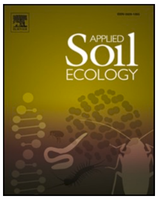
Applied Soil Ecology 169 (2022) 104253

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Soil specific enzyme stoichiometry reflects nitrogen limitation of microorganisms under different types of vegetation restoration in the karst areas

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ARTICLE INFO

Keywords:
Extracellular enzyme activity
Ecological stoichiometry
Vegetation restoration
Resource limitation
Karst area

ABSTRACT

Knowledge about resource limitations faced by soil microorganisms is crucial for understanding ecosystem functions and processes. In recent decades, vegetation restoration has been carried out in the degraded karst areas, leading to the alteration in the status of microbial resource limitation (MRL). However, mechanisms underlying MRL in different karst ecosystems remain poorly understood. Here we investigated MRL based on the theory of soil extracellular enzyme stoichiometry. Soil carbon (C), nitrogen (N), and phosphorus (P) acquiring enzyme activity (glucosidase, cellobiohydrolase, leucine aminopeptidase, urease and alkaline phosphatase) per unit microbial biomass carbon (MBCE) and per unit soil organic carbon (SOCE) of four main vegetation types (natural community, NC; ecological forest, EF; abandoned cropland, AC and economic plantation, EP) at 0–20 cm depth were measured in the karst areas in southwestern China. Significantly higher MBCE levels were found in EP than the other three vegetation types ($p < 0.05$), with EF having significantly higher SOCE than NC ($p < 0.05$), suggesting the highest metabolic activity and soil organic carbon (SOC) utilisation efficiency in EP and EF, respectively. C- and N-specific enzyme activity (MBCE and SOCE) were strongly negatively correlated with microbial biomass and soil C, N contents ($p < 0.05$), suggesting microbial resource requirement promotes the production of specific enzyme in resource-deficient ecosystems. Homeostasis analysis of microbial biomass C:N and resources C:N for all communities showed no homeostasis, indicating the microorganisms may be autotrophic to meet their N demands. In addition, the specific enzyme C:N ratios were less than 1, the N:P ratios were greater than 1, and vector angles were all less than 45° in all four types of vegetation restoration, indicating clear N limitation. A homeostasis analysis, as well as extracellular enzymatic stoichiometry and vector analysis, all suggested that soil microorganisms in the four vegetation types were nitrogen-limited, with NC most severely affected. Overall, we suggest that nitrogenous fertilisers should be added to restore the balance of elements while recovering the karst ecosystems.

1. Introduction

Human activities that lead to vegetation change can have a critical impact on the carbon (C), nitrogen (N) and phosphorus (P) cycles, as well as on soil-microbial community composition and structure (de Paula et al., 2020; Guo et al., 2020; Vazquez et al., 2020). In turn, soil microorganisms regulate soil fertility, plant growth and succession of plant communities by producing various extracellular enzymes that decompose dead organic matter and other large organic polymers to their usable constitutive elements via nutrient cycles, as the mediators and catalysts of biochemical processes in soil (Mooshammer et al., 2014; Schmidt et al., 2016; Zhang et al., 2020). Soil extracellular enzyme activity (EEA) represents the interface between nutrient demand by microorganisms and nutrient supply by the environment, which effectively links ecological stoichiometric theory with the concept of threshold elemental ratios (Hill et al., 2012). C-, N- and P-acquiring enzymes have attracted much attention as they target key substrates, and their activity makes a significant contribution to energy flow and nutrient release

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https://doi.org/10.1016/j.apsoil.2021.104253
Received 10 November 2020; Received in revised form 29 August 2021; Accepted 2 October 2021

(Aponte et al., 2020; Ding et al., 2020; Y.X. Fan et al., 2020; Z.W. Xu et al., 2020; Yan et al., 2020; Yang et al., 2020). Specifically, among soil hydrolytic enzymes, glycosidases, including β-1, 4-glucosidase (BG) and cellobiohydrolase (CBH), are responsible for C acquisition by hydrolysing cellulose and sugar into dissolved organic C (Sinsabaugh et al., 2005); leucine amino peptidase (LAP) and urease are N-acquiring enzymes that target protein and urea, respectively (Jian et al., 2016) and alkaline phosphatase (ALP) is P-acquiring enzymes that can liberate phosphate from organic molecules (such as phosphomonoesters, phosphodiesters and phosphate) (Jia et al., 2020). Based on the assumption that these five enzymes regulate C, N and P acquisition, and based on microbial physiology that nutrient demand drives the production of relevant enzymes, microbial C, N and P limitation can be assessed using enzymatic stoichiometry, such as enzymatic ratios and vector variables (Chen et al., 2018a; Chen et al., 2019). Therefore, EEAs and their stoichiometries (EESs) can be used to characterise the nutrient requirements and physiological processes of microorganisms (Burns and Dick, 2002), and EESs could also reflect microbial nutrient availability from limiting resources (J. Li et al., 2019; Xiao et al., 2020; Zhang et al., 2020; Zheng et al., 2020b). Microorganisms generally possess a competitive advantage over plants when it comes to obtaining nutrients from soil (Lipson et al., 1999; Hodge et al., 2000). Thus, if microorganisms are limited by a certain nutrient, plants should also be limited by that nutrient (Zheng et al., 2020b). Consequently, the investigation of soil-microbial resource limitation (MRL) is of great importance to identify plant nutrient strategies and use this information to improve the soil C pool or vegetation restoration efficiency.

EEAs and EESs are highly dependent on microbial biomass, microbial composition (Elser et al., 2000; Zungu et al., 2020) and environmental conditions, such as climate (Deltedesco et al., 2020) and edaphic factors (Zheng et al., 2019), especially SOC, total nitrogen (TN) and soil pH, which are the most commonly explored edaphic factors (Kooch and Noghre, 2020; L.X. Wang et al., 2020; Zungu et al., 2020). Changes in vegetation types can initiate changes in fine roots (L.X. Wang et al., 2020), litter quality (Zheng et al., 2020a), microbial biomass carbon (MBC) (H.Y. Wang et al., 2020) and soil physiochemical characteristics (Luo et al., 2020; Sun et al., 2020), which can subsequently affect soil-microbial enzyme activities. However, different factors were found to have inconsistent impacts on soil EEAs and EESs across different ecosystems. Therefore, it is necessary to elucidate the dominant driving factors that result in MRL in communities with different vegetation types, with more studies needed to address these issues on a regional scale.

Soil specific enzyme activity, a product of the enzyme activity in soil-microbial biomass or SOC per unit of C, is a more appropriate and clearer indicator than absolute enzyme activity for identifying differences under various vegetation types (H.W. Xu et al., 2020). Soil specific enzyme activity has the following advantages: (1) it eliminates strong covariances with SOC and MBC (Raiesi and Beheshti, 2014; H.W. Xu et al., 2020); (2) it standardises differences in SOC and MBC content (H.W. Xu et al., 2020); (3) it represents the metabolic status of a microbial community based on organic substances (Raiesi and Beheshti, 2014; Silva et al., 2019) and (4) it is more sensitive to short-term land use conversion than absolute enzyme activity (Y. Li et al., 2019; Silva et al., 2019; Yu et al., 2019). Thus, these variables would be a good indicator of microbial change in various vegetation restoration communities.

Karst regions have been experiencing vegetation degradation, soil erosion and even rocky desertification (Wang et al., 2019). In recent decades, various vegetation types, such as protected natural forests, artificial plantations, abandoned land, cropland, were found in karst fragile ecosystem after a series of vegetation restoration and reconstruction projects have been carried out in this region (Qiao et al., 2020). However, soil microbes are still limited by C and P in most karst ecosystems due to low primary productivity and soil erosion (Chen et al., 2019). We therefore hypothesized that soil-microbial communities would suffer from C and P limitation in current study (Hypothesis I). Additionally, previous studies confirmed that land use or vegetation type is the major factor regulating EEAs and EESs in the karst areas (Z.Z. Fan et al., 2020; Y. Wang et al., 2020). Accordingly, we hypothesized that EEAs and EESs, as well as microbial nutrient limitations, would differ under different vegetation restoration strategies (Hypothesis II).

2. Materials and methods

2.1. Site description

This study was carried out in Guizhou Province, southwestern China (24°37′–29°13′N, 103°36′–109°35′E), spanning an elevation range of 153 to 2885 m. The climax vegetation in the area is evergreen broadleaf forest the mean annual temperature is 6.8 °C–20.5 °C and the mean accumulated temperature (>0 °C) is 2814.1 °C–7483.8 °C. The mean annual precipitation ranges between 761.6 and 1507.0 mm, most of which occurs between April and August. The terrain is dominated by plateaus and mountains. The soil type of all 20 plots is a Calcisol formed from limestone, and the soil has a similar texture, dominated by silty loam (Table 1). Severe soil erosion in this region has resulted in shallow soil depth and poor ecosystem stability. Various types of vegetation restoration have been implemented in recent decades, which alleviated karst rocky desertification, which is about $2.47 \times 10^6 \text{hm}^2$ in 2016, comprising 14.03% of the total land area of Guizhou Province.

2.2. Experimental design and soil sampling

Our field survey was conducted during July 2019. Twenty sites were selected for the treatments (Table 1), including three natural communities (undisturbed natural communities, NC); six ecological forests (artificial forests protected for more than 20 years, EF); five abandoned croplands (abandoned for between 3 and 8 years, AC) and six economic plantations (subject to human interference, such as cultivation, fertilization and harvesting, EP). For each site, between four and six plots for the woody community (10 × 10 m) and ten plots for the herb community (1 × 1 m) were selected. We collected and homogenised at least six soil samples from a depth of 0–20 cm using a soil auger (5 cm inner diameter stainless-steel corer) from three random plots at each site. Samples for soil EEA analysis were placed in ice boxes after removal of the roots and any debris and rapidly transported to the laboratory. Another portion of samples was air dried and sieved through 2- and 0.15-mm mesh to collect samples for testing soil properties. Three soil corners (100 cm^3 each) were collected at each site for determination of water content.

2.3. Soil analysis, microbial biomass and soil EEA assays

At each plot, the following parameters were measured according to the method of Lu (1999). Briefly, soil water content (SWC) was determined from the loss of mass after drying soil samples at 105 °C for 48 h. Soil pH was measured using a pH electrode (Mettler Toledo, Switzerland) after shaking the dry soil - water at a ratio of 1:2.5 (w:v) suspension for 30 min. SOC was measured by wet oxidation with KCr_2O_7 and H_2SO_4, phenanthroline was used as indicator, and titration with $FeSO_4$. Soil TN was measured by the Kjeldahl method with H_2SO_4, methyl red - bromocresol green was used as indicator, and titration with HCl. Soil total phosphorus (soil TP) was measured by a spectrophotometer after HF–$HClO_4$ resolution. Dissolved organic carbon (DOC) and soil available N (soil AN) was extracted from 10 g fresh soils in 50 mL of 0.5 M K_2SO_4, then measured using a TOC analyser (liquid TOC II, elementar, Germany). Soil available phosphorus (soil AP) was extracted from 5 g fresh soils with 100 mL of 0.5 M $NaHCO_3$ and analyzed colorimetrically.

Soil-MBC, microbial biomass nitrogen (MBN) and phosphorus (MBP) were measured by chloroform fumigation. MBC and MBN were determined using a TOC analyser (liquid TOC II, elementar, Germany), at an extraction coefficient of 0.38 and 0.45 for MBC and MBN, respectively;

Table 1
Sampling sites information.

Community type	Community subtype	Plantation time/year	Location	Longitude and latitude	Elevation/m	Aspect/°	Slope Position	Slope/°	Current Management	Soil texture
Natural community	Natural grassland	–	Zhenfeng county	105.64, 25.63	1175.06	N 3	Middle	5	None	Sandy loam
	Natural shrub tussock	–	Luodian county	106.86, 25.57	610	SE 125	Middle	29	None	Silty loam
	Natural secondary forest	–	Puding county	105.77, 26.31	1370	S 192	Low	27	None	Sandy loam
Ecological forest	*Catalpa ovata*	21	Qingzhen county	106.35, 26.56	1250	SW 246	Foot	5.7	None	Silty loam
	Cryptomeria japonica var. sinensis	21	Qingzhen county	106.39, 26.57	1240	SW 233	Middle	36	None	Silty loam
	Cupressus funebris	21	Puding county	105.80, 26.32	1400	SE 135	Middle	37	None	Sandy loam
	Toona sinensis	30	Zhenning county	105.64, 26.05	1077.83	SW 310	Low	14	None	Silty loam
	Liquidambar formosana	22	Wangmo county	106.46, 25.25	550	S 184	Low	20	None	Silty loam
	Pinus massoniana	21	Huishui county	106.70, 26.26	1020	E 91	Low	29	None	Silty loam
Abandoned cropland	Abandoned grassland 1	3	Puding county	105.79, 26.32	1330	N 342	Low	23	None	Silty loam
	Abandoned grassland 2	8	Zhenning county	105.74, 26.09	1273.67	–	Top	0	None	Silty loam
	Abandoned grassland 3	8	Zhenning count	105.63, 26.06	1077.71	SW 134	Low	14	None	Silty loam
	Abandoned grassland 4	6	Wangmo county	106.47, 25.24	500	W 286	Foot	26	None	Silty loam
	Abandoned grassland 5	7	Guiyang city	106.66, 26.35	1130	SE 151	Foot	15	None	Silty loam
Economic plantation	*Vitis vinifera*	5	Zhenning county	105.63, 26.05	1040.18	–	Flat	0	Fertilize, weed, harvest	Silty loam
	Prunus salicina var.	17	Guanling county	105.61, 25.72	1157.47	–	Flat	0	Fertilize, weed, harvest	Loam
	Hylocereus undatus	15	Guanling county	105.66, 25.68	575.19	SW 139	Middle	16	Fertilize, weed, harvest	Silty loam
	Zanthoxylum bungeanum	12	Zhenfeng county	105.67, 25.67	531.43	N 2	Middle	19	Fertilize, weed, harvest	Sandy loam
	Prunus salicina var.	8	Zhenfeng county	105.68, 25.39	980	W 280	Low	5	Fertilize, weed, harvest	Silty loam
	Eucalyptus robusta	16	Luodian county	106.46, 25.21	470	SW 236	Upper	30	Deforestation	Silty loam

Note: N, S, W, E, SW and SE indicates north, south, west, east, southwest and southeast, respectively.
Information about 21 sample sites, including the description of sites community type, community subtype, location, elevation, aspect, slope position, slope and the current management in each site.

MBP was measured by molybdenum–antimony colorimetry, at an extraction coefficient of 0.4.

Soil EEA of five soil enzymes, including C-acquiring enzymes (β-1,4-glucosidase, BG; cellobiohydrolase, CBH), N-acquiring enzymes (leucine aminopeptidase, LAP; urease) and a P-acquiring enzyme (alkaline phosphatase, ALP) were quantified using the double antibody sandwich method. First, purified enzyme antibodies were used with microplates to produce solid phase antibodies. Second, the corresponding enzyme was added to the microplates coated with monoclonal antibodies. Then, the same enzyme that marked by horseradish peroxidase (HRP) was added, formed an 'antibody-antigen-labelled enzyme' antibody compound. For example, purified BG antibody-BG enzyme-HRP labelled BG enzyme. After thorough washing, tetramethylbenzidine (TMB) was added to provide colour; TMB was turned blue by the HRP enzyme and to a final yellow colour by the acid. The darkness of the colour was positively related with the enzyme content of the sample. Fluorescence was measured using a fluorometer set at 450 nm emission. Finally, enzyme activity concentration in a sample was calculated using a standard curve and presented in units of μmol·min^{-1}·g^{-1} soil.

2.4. Enzymatic stoichiometry and stoichiometric homeostasis

According to the definition of the geometric mean of enzyme activity (GMEA) (Hinojosa et al., 2004), we calculated GMEA as follows:

$$GMEA = \sqrt[5]{BG \times CBH \times LAP \times Urease \times ALP} \tag{1}$$

Before analysing the enzymatic stoichiometry, enzyme activity was normalised to MBC (μmol·min^{-1}·g^{-1}MBC) and SOC (μmol·min^{-1}·g^{-1}MBC) by dividing absolute enzyme activity by MBC or SOC (H.W. Xu et al., 2020). Three methods were selected to assess MRL, as previously described (Zheng et al., 2020b). 1) MRL was estimated based on a scatter plot between (LAP+urease)/ALP and (BG+CBH)/(LAP+urease), with the four parts in the plot indicating C and P limitation, C and N limitation, N limitation and P limitation (Hill et al., 2012). The four parts were partitioned according to the deviation from the expected enzyme activity ratios of C:N:P (1:1:1) (Sinsabaugh et al., 2009; Zheng et al., 2020b). 2) The ratios of enzymatic activities, including C:N [(BG+CBH)/(LAP+urease)], C:P [(BG+CBH)/ALP] and N:P [(LAP+urease)/ALP], reflected the MRL situation, with higher C:N, C:P and N:P indicating lower N, P and P limitation, respectively (Waring et al., 2014). 3) A vector analysis of enzymatic stoichiometry can also be used to assess MRL (Moorhead et al., 2013). Vector length and vector angle were calculated as follows (Moorhead et al., 2016):

$$Vector\ length = \sqrt{X^2 + Y^2} \tag{2}$$

$$Vector\ angle = Degrees[ATAN2(Y, X)] \tag{3}$$

where X is ln(BG + CBH)/ln(LAP+urease), Y is ln(BG + CBH)/lnALP. A longer vector length represents greater C limitation, and a vector angle <45° or >45° indicate N or P limitation, respectively (Moorhead et al., 2016).

To evaluate the response of microbial C:N (or C:P or N:P) homeostasis (H) of microbial communities to vegetation restoration, we used following equation:

$$H = \frac{log_{10}(x)}{log_{10}(y) - log_{10}(c)} \tag{4}$$

where x is the resource nutrient stoichiometry (resource C:N, C:P or N:P), y is the microbial nutrient stoichiometry (soil-microbial biomass C:N, C:P or N:P) and c is a constant. Therefore, 1/H is the slope of the regression between $\log_{10}C:N_{microbes}$ vs. $\log_{10}C:N_{resource}$ and should be between 0 and 1. Data showing significant regressions and $1/H \geq 1$ indicates weak or no homeostasis; data with significant regressions and $0 < 1/H < 1$ were classified as homeostatic (0–0.25), weakly homeostatic (0.25–0.5), weakly plastic (0.5–0.75) or plastic ($1/H > 0.75$) (Sterner and Elser, 2002). Cases with the least squared regression slope were not significant ($p > 0.05$) and were classified as strictly homeostatic (J.Y. Zhang et al., 2019).

2.5. Statistical analyses

We used soil EESs to analyse microbial nutrient limitations by applying Tukey's HSD test at 0.05 with one-way ANOVA (analysis of variance). Pearson's correlation coefficient was used to explore correlations between EEAs and EESs and biotic and abiotic factors. Redundancy analysis (RDA) was performed in R version 3.61 (https://www.r-project.org/) according to the axis lengths value (less than 3.0) in *decorana* (species).

3. Results

3.1. Content and ratios of C, N, and P in the soils and microbial biomass

SOC, soil TN and soil AN content was higher in NC compared to the other communities, while soil TP and soil AP was higher in EP than in other communities ($p < 0.05$) (Table. 2). In addition, soil C:N, C:P and N:P were higher in NC compared to EP ($p < 0.05$). Similarly, MBC and MBN both had significant higher values in NC compared to the other communities, and minimum numerical values for EP. MBC:MBN and MBC:MBP both had minimum numerical values at NC, while MBN:MBP showed no significant difference among these communities.

3.2. Soil extracellular enzyme activities and their stoichiometries with vegetation restoration

One-way ANOVA showed that soil EEAs (BG, CBH, LAP, urease and ALP) of both soil specific enzyme activity per unit soil-microbial biomass carbon (MBCE) and per unit soil organic carbon (SOCE) differed among the vegetation restoration types ($p < 0.05$, Fig. 1a–j). MBCE in EP was the highest, being higher than that found at other sites ($p < 0.001$). SOCE was highest in EF, but only significantly higher than NC, and parts of SOCE (ALP and urease) in EP were also significantly higher than NC ($p < 0.05$). GMEA can show overall enzyme activities as well as soil microbiological activities [Eq. (1)], could sufficiently show the responses of soil specific enzyme activities to environmental changes (Yu et al., 2017; Raiesi and Salek-Gilani, 2018; Yu et al., 2019). Our results showed that GMEA had a similarly changing trend to single MBCE and SOCE (Fig. 1k, l).

The enzyme C:N ratio calculated by ln(BG + CBH)/ln(LAP+urease) per MBC and per SOC showed the same result, that the enzyme C:N ratio in NC was significantly lower than that at other sites (Fig. 2a, f). The enzyme C:P ratio calculated by ln(BG + CBH)/ln(ALP) showed no significant differences among the four sites (Fig. 2b, g). However, the enzyme N:P ratio calculated by ln(LAP+urease)/ln(ALP), both per MBC and per SOC, was the highest in NC and significantly higher than those at other sites; no significant differences were found among the other three communities (Fig. 2c, h). The enzyme C:N ratios at all sites were less than 1, while the C:P and N:P ratios at all sites were higher than 1. The natural logarithm of soil C-, N- and P-acquiring enzyme ratios per MBC and per SOC were 1:1.33:0.92 and 1:1.62:0.83, respectively, which deviated from 1:1:1.

Table 2
Soil properties in four types of vegetation restoration.

Soil properties	NC	EF	AC	EP
pH	$7.64 \pm 0.0.08^{a}$	$6.59 \pm 0.0.18^{ab}$	6.34 ± 0.33^{b}	6.99 ± 0.28^{ab}
SWC	44.25 ± 2.42^{a}	32.04 ± 1.72^{bc}	34.37 ± 1.74^{b}	26.33 ± 1.81^{c}
SOC	49.60 ± 3.12^{a}	24.86 ± 4.18^{b}	26.30 ± 2.51^{b}	28.49 ± 2.54^{b}
Soil TN	4.71 ± 0.23^{a}	2.69 ± 0.30^{b}	2.60 ± 0.15^{b}	3.21 ± 0.23^{b}
Soil TP	0.85 ± 0.07^{b}	0.60 ± 0.05^{b}	0.65 ± 0.05^{b}	1.37 ± 0.16^{a}
$Soil_{C:N}$	10.48 ± 0.23^{a}	8.61 ± 0.45^{b}	9.85 ± 0.35^{ab}	8.62 ± 0.29^{b}
$Soil_{C:P}$	61.01 ± 5.36^{a}	42.22 ± 6.18^{a}	41.86 ± 3.65^{a}	22.66 ± 1.64^{b}
$Soil_{N:P}$	5.77 ± 0.43^{a}	4.69 ± 0.45^{ab}	4.21 ± 0.28^{b}	2.68 ± 0.22^{c}
DOC	66.66 ± 6.23	47.95 ± 2.87	75.89 ± 13.90	72.26 ± 8.38
AN	61.54 ± 3.86^{a}	33.09 ± 3.23^{b}	29.41 ± 2.05^{b}	33.14 ± 1.88^{b}
AP	1.37 ± 0.13^{b}	1.27 ± 0.14^{b}	1.30 ± 0.14^{b}	10.76 ± 1.55^{a}
$R_{C:N}$	1.15 ± 0.15^{b}	1.58 ± 0.11^{b}	2.56 ± 0.41^{a}	2.18 ± 0.22^{ab}
$R_{C:P}$	49.49 ± 2.67^{a}	43.59 ± 4.02^{a}	59.11 ± 8.54^{a}	14.75 ± 4.55^{b}
$R_{N:P}$	50.78 ± 8.44^{a}	30.50 ± 4.05^{b}	27.64 ± 4.45^{b}	5.70 ± 1.26^{c}
MBC	1880.55 ± 107.32^{a}	1363.83 ± 154.82^{b}	1152.87 ± 101.03^{b}	652.76 ± 67.84^{c}
MBN	121.24 ± 13.56^{a}	53.32 ± 13.45^{b}	32.07 ± 6.70^{b}	30.07 ± 3.66^{b}
MBP	16.56 ± 0.78^{a}	8.57 ± 2.18^{b}	5.21 ± 1.30^{b}	8.84 ± 1.95^{ab}
MBC: MBN	17.05 ± 1.97^{b}	38.38 ± 5.16^{ab}	55.22 ± 10.30^{a}	27.70 ± 5.14^{b}
MBC:MBP	115.44 ± 7.98^{b}	239.97 ± 31.28^{ab}	406.26 ± 66.23^{a}	198.11 ± 52.25^{b}
MBN:MBP	7.46 ± 0.95	6.86 ± 0.81	9.07 ± 1.51	7.05 ± 1.49
GMEA	1.21 ± 0.04	1.21 ± 0.03	1.18 ± 0.02	1.23 ± 0.02

Note: SWC: soil water content; SOC: soil organic carbon; soil TN: soil total nitrogen; soil TP: soil total phosphorus; $Soil_{C:N}$, $Soil_{C:P}$, $Soil_{N:P}$ indicate the ratio of SOC to soil TN, the ratio of SOC to soil TP, the ratio of soil TN to soil TP, respectively. DOC: dissolved organic carbon; soil AN: available nitrogen; AP, available phosphorus; $R_{C:N}$, $R_{C:P}$, $R_{N:P}$ indicate the ratio of DOC to AN, the ratio of DOC to AP, the ratio of AN to AP, respectively. MBC, MBN, MBP indicate soil microbial biomass carbon, nitrogen, and phosphorus, respectively. GMEA: the geometric mean of enzyme activity. Different lowercases indicate significant difference ($p < 0.05$) among vegetation restoration types of the same index.

Vector analysis (including vector length and vector angle) of soil EEAs was used to evaluate microbial nutrient limitation (Fig. 2d, e, i and j). No significant differences were found among the four sites for vector length. The vector angles in NC were lowest for both vector angle-MBC and vector angle-SOC; no significant differences were found among the other three communities ($p < 0.01$). Specifically, the vector angles at the four sites were all less than 45°, indicating that soil microorganisms suffer N limitation in these four restoration types (Moorhead et al., 2013). Similarly, the soil enzymatic stoichiometry scatter plot (associations between (BG + CBH)/(LAP+urease) and (LAP+urease)/ALP) showed that all data points fell within the N-limitation group (Fig. 3).

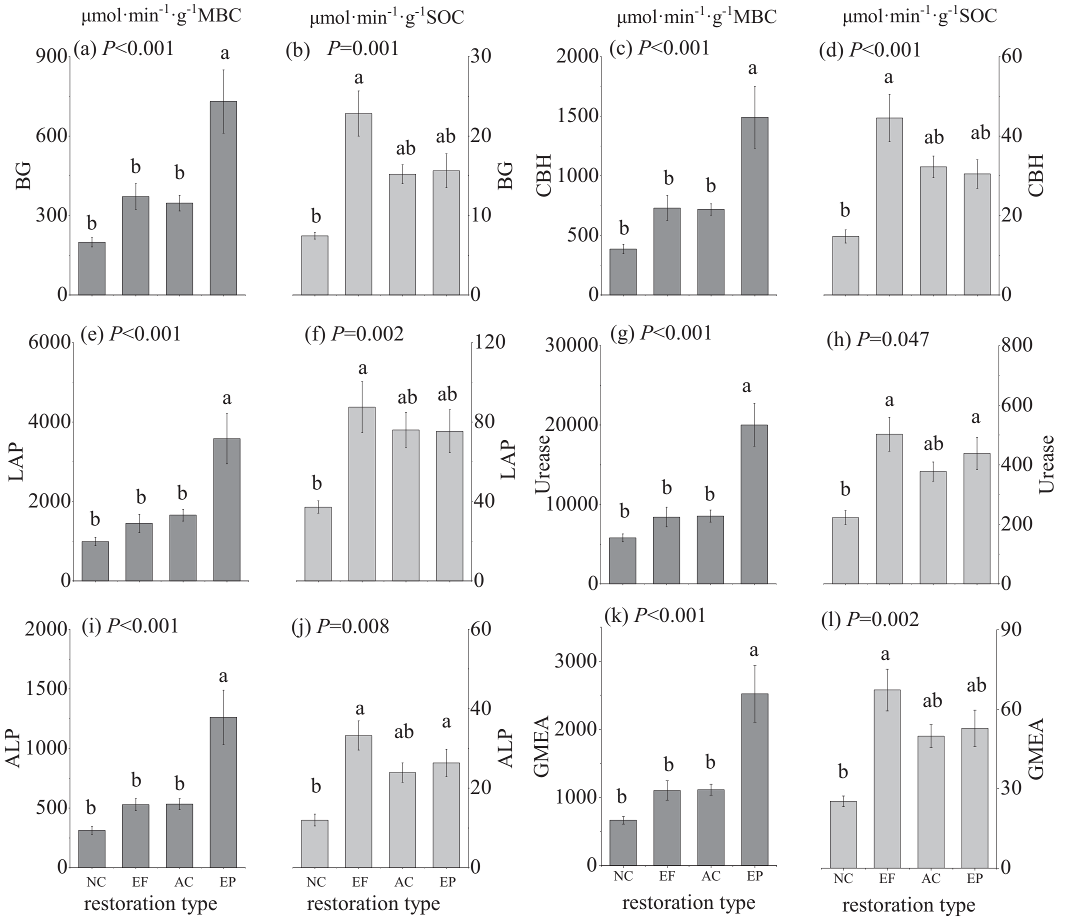

Fig. 1. Soil enzyme activity responses to four types of vegetation restoration.

Note: a, c, e, g, i, k with dark grey column: enzyme activity per MBC; b, d, f, h, j, l with light grey column: enzyme activity per SOC. BG: β-1,4-glucosidase; CBH: cellobiohydrolase; LAP: leucine aminopeptidase; ALP: alkaline phosphatase. NC: natural community, n = 3; EF: ecological forest, n = 6; AC: abandoned cropland, n = 5; EP: economic plantation, n = 7. The bars are standard errors, the different letters indicate significant difference between restoration types according to the Tukey test, *p* values are given according to one-way ANOVA. The same below.

3.3. *Stoichiometric homeostasis*

Associations between the microbial biomass stoichiometric ratios (C:N and C:P) and those for soil resources were analyzed to test the strength of the stoichiometric homeostasis (Fig. 4). The slope of $\log_{10}B_{C:N}$ vs $\log_{10}R_{C:N}$ indicated no homeostasis ($1/H > 1$) for microorganisms, while the slope of $\log_{10}B_{C:P}$ vs $\log_{10}R_{C:P}$ indicated a weakly homeostatic interaction ($0.25 < 1/H < 0.5$) between microbial C:P and soil C:P. when using the data from all twenty sites (Fig. 4a, b). In addition, the separate slopes of both $\log_{10}B_{C:N}$ vs $\log_{10}R_{C:N}$ and $\log_{10}B_{C:P}$ vs $\log_{10}R_{C:P}$ of each community varied widely among these four restoration types, indicating no homeostasis for microorganisms in EF and EP (Fig. 4c, d, Table S1).

3.4. *Soil extracellular enzyme activities and their stoichiometries linked to abiotic and biotic factors*

MBCE and SOCE showed a clearly decreasing trend with the increases in SOC and MBC, soil TN and MBN and soil TP and MBP, respectively. Significantly negative correlations were found between specific C-acquiring enzyme activity and MBC or SOC. Similar findings were seen for specific N-acquiring enzyme activity and MBN or soil TN and specific P-acquiring enzyme activity per SOC and MBP or soil TP ($p < 0.05$) (Fig. 5; Table S2). However, enzyme C:N, enzyme C:P and enzyme N:P showed a weak response to soil-microbial biomass cooperative changes (Fig. 5; Table S2).

Pearson's correlation coefficient analysis revealed significant relationships between abiotic, biotic factors and soil EEAs (Table S2) and EESs (Table S3). All the MBCE and SOCE showed a similar negatively relationship tendency with abiotic and biotic factors, including SWC, SOC soil TN, soil C:N, soil C:P, AN, resource N:P ratio, MBC and MBN ($p < 0.05$). Similarly, the vector degrees were negatively correlated with soil TN, AN and MBN, and enzyme C:N ratios of both per MBC and per SOC were negatively correlated with soil C:N ratio ($p < 0.05$). Although enzyme C:N and N:P ratio per MBC were negatively correlated with MBC, MBN and MBP, no significant relationships were found with microbial stoichiometry.

Redundancy analysis (RDA) revealed that abiotic and biotic factors together explained 77.61% (axis 1, 75.62%; axis 2, 0.73%) of the total variation in MBCE and EESs per MBC (Fig. 6a), and explained 83.90% (axis 1, 80.19%; axis 2, 1.34%) of the total variation in SOCE and EESs per SOC (Fig. 6b). Axis 1 explained the majority of variances, indicating that these abiotic and biotic factors were highly correlated with soil EEAs and EESs.

4. Discussion

4.1. *Soil specific enzyme activities per unit of MBC (MBCE) and per unit of SOC (SOCE) in four types of vegetation restoration area*

Soil specific enzyme activity is a sensitive and reliable indicator of short-term vegetation restoration (Yu et al., 2019). MBCE represents the

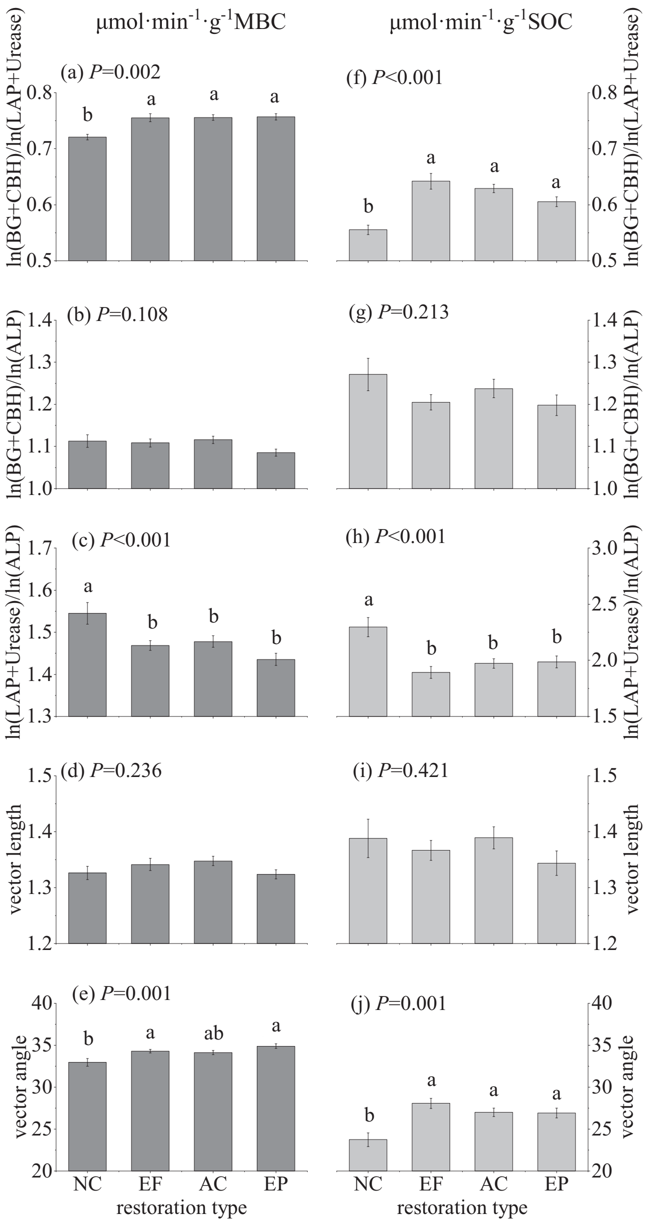

Fig. 2. Responses of soil enzymatic stoichiometries (a–c, f-h), vector length (d, i) and vector angle (e, j) to four types of vegetation restoration.

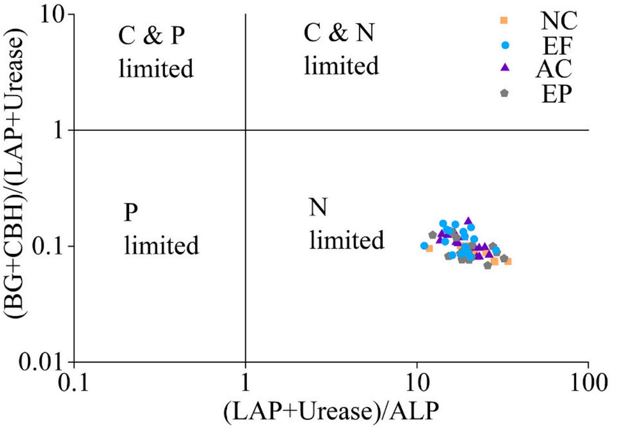

Fig. 3. A scatter plot of soil enzymatic stoichiometries showing the pattern of microbial resource limitation.

metabolic status of the microbial community and fluctuations in stabilised EEA (Silva et al., 2019). In our study, MBCE, including C-, N- and P-acquiring enzyme activities (BG, CBH, LAP, urease, ALP and their GEMA), was greater in EP, indicating a higher physiological efficiency of the microorganisms present (Lagomarsino et al., 2011). It should be noted this was the only community currently subject to frequent human interference. These findings are in line with previous results. H.L. Zhang et al. (2019) reported that a managed eucalyptus plantation had higher MBCE than bare land, restored secondary forest and undisturbed forest in a tropical region of China. Érica de Oliveira Silva et al. (2019) found that MBCE of agricultural lands that suffered environmental disturbance were more metabolically active than undisturbed forest. Absolute EEAs increase when resources are sufficient and decrease when they are deficient (Luo et al., 2017; Xu et al., 2017; Li et al., 2018; Cui et al., 2019; H.Y. Wang et al., 2020); which is a survival strategy of soil microorganisms according to microbial metabolism theory (Sinsabaugh et al., 2008). Several studies have confirmed that MBCE and absolute enzyme activity show opposite trends, and MBCE is negatively related with soil nutrients (Raiesi and Salek-Gilani, 2018; H.L. Zhang et al., 2019). Our results (Fig. 6, Table S2) support the finding that most abiotic factors are negatively corrected with the MBCE. Thus, the poor soil condition in EP (Table 2), with relatively low SWC, soil C:N, C:P and N:P ratios, resulted in higher specific enzyme activities in this site. Further analysis was performed to explore the mechanism of change in MBCE. Two direct mechanisms have been suggested as the cause of the high MBCE seen in EP. One is that the enzyme activity in EP decreases at a lower rate than the microbial biomass, the second is that the production and release of enzymes by soil microorganisms in EP is higher (Raiesi and Beheshti, 2014). In this study, both phenomena occurred when EP was compared with NC, with a higher content of absolute GMEA (not statistically significant) but lower MBC in EP (only 34.71% of that seen in NC) (Table 2). On the other hand, based on microbial physiology, nutrient demand-driven enzymes are produced by microorganisms when nutrients are scarce; this could mobilise resources from more complex sources and those accumulated by facilitated diffusion against a concentration gradient (Y. Wang et al., 2020). Thus, lower MBC and relatively higher enzyme activity occurred in EP, with the microbial community of EP considered to be more metabolically active than the other three types of vegetation restoration.

SOCE accounts for changes in soil properties other than SOC and responded more clearly than the absolute enzymatic activities to environmental differences (Liu et al., 2017; Xiao et al., 2019). In our study, soil specific enzyme activity (BG, CBH, LAP, urease, ALP and their GEMA) per SOC in NC were lowest and significantly lower than in EF, while urease and ALP in NC were also significantly lower than in the EP. Our results were consistent with those of previous studies. Luo et al. (2019) reported that natural broadleaf forest had lower specific C hydrolase than plantation forests; de Medeiros et al. (2015) verified that SOCE in preserved natural forests was just one-third of that found in cultivated areas. Bhattacharyya et al. (2020) also found that SOCE (*N*-acetyl beta-glucosaminidase, alpha-glucosidase and phenol oxidase) were lower in undisturbed rainforest than in pasture. One explanation may be that microorganisms could drive the production of more enzymes when there is nutrient deficiency, as mentioned above (Y. Wang et al., 2020). The other may be that, compared with the situation in the undisturbed natural communities, soil aggregates in plantation areas may be destroyed following disturbance, which not only decomposes SOC but also releases enzymes that had been trapped or immobilised (Raiesi and Beheshti, 2014, 2015; Luo et al., 2019). This was supported by our result that significantly higher SOC content was found in NC (Table 2). In our study, both EP and EF had higher content of SOCE than NC. However, EF were under protection for more than 20 years. A possible reason may be that SOC accumulation in EF was very slow, supported by our result that no significant difference was found between

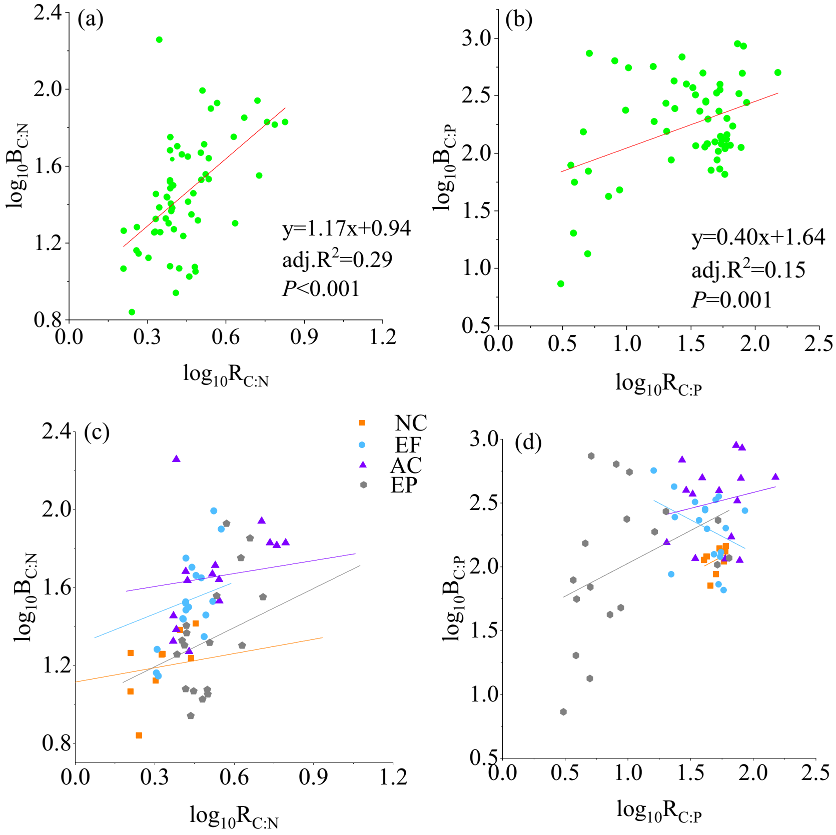

Fig. 4. Soil microbial community homeostasis correlated with N (a) and P (b) acquisition, and homeostasis correlated with N (c) and P (d) in every type of vegetation restoration.

Note: $\log_{10}R_{C:N}$, $\log_{10}R_{C:P}$ represent the natural logarithm of soil resource C:N and C:P ratios; $\log_{10}B_{C:N}$, $\log_{10}B_{C:P}$ represent the natural logarithm of microbial biomass C:N and C:P ratios.

AC, EP and EF (Table 2). Additionally, C-, N- and P-acquiring enzymes are produced via microbial growth supported by a C supply, which could easily lead to SOC decomposition if nutrient inputs are too low (Wang et al., 2012; Bowles et al., 2014; Yang et al., 2018; Luo et al., 2019). Therefore, we speculated that the formation rate of soil large aggregates in EF was slow, thus, soil enzymes could make full use of SOC in EF.

Based on these results, we can conclude that specific enzyme activity increases in nutrient-deficient communities. In addition, higher specific enzyme activity would exacerbate the loss of soil organic material, attributable to the negative correlations found between specific enzyme activity and MBC and SOC content (Silva et al., 2019), which we also found (Table S2).

4.2. *Microbial N limitation induced by soil extracellular enzyme stoichiometries in four types of vegetation restoration*

EESs link microbial decomposition with nutrient mineralisation, and soil-microbial nutrient limitation can be judged by the homeostasis between microbial biomass and resource. In our study, the slopes of the relationship between $\log_{10}R_{C:N}$ and $\log_{10}B_{C:N}$ for all study sites were significantly greater than 1, indicating no homeostasis; the microorganisms may therefore have been autotrophic. However, the relationship between $\log_{10}R_{C:P}$ and $\log_{10}B_{C:P}$ for all four communities was weakly homeostatic, suggesting the microorganisms were heterotrophic, which would be inconsistent with the above results. A similar discrepancy was found in an earlier study (Y.S. Zhang et al., 2019). This phenomenon suggests that P content in all sites may be abundant, while N content may be deficient; therefore, microorganisms must be autotrophic to meet their N demands.

The soil enzyme C:N:P ratio, represents the biochemical equilibrium between soil-microbial nutrient allocation and environmental resource availability (Xiao et al., 2018). A meta-analysis based on data from 40 ecosystems globally found that the ratios of soil C, N and P hydrolytic enzyme activities was close to 1:1:1 (Sinsabaugh et al., 2008), showing that soil microorganisms play an extensive role in the stoichiometric homeostasis of elements. However, recent studies have shown that this equilibrium pattern could easily be disrupted due to the wide variation in ecosystems and regional environmental conditions (Xiao et al., 2020; Zhou et al., 2020). In the present study, the ratios of the natural logarithm of C-, N- and P-acquiring enzymes per MBC and per SOC were 1:1.33:0.91 and 1:1.62:0.83, respectively, which all deviated from 1:1:1, indicating that soil-microbial investment in C-, N- and P-acquiring enzymes varied distinctly among the vegetation restoration types. This phenomenon could be explained by differences in soil properties, vegetation types and anthropic interference intensity between karst areas and other ecosystems (Chen et al., 2018b; Yang et al., 2020). Additionally, the large proportion of N enzymes indicated that increased N was demanded by microorganisms relative to C and P in this area, which also suggests the karst ecosystem was more N-limited.

We quantified the metabolic limitation of microorganisms by extracellular enzymatic stoichiometry (per MBC and per SOC), further supporting the notion that soil microorganisms in all four types of vegetation restoration were limited by N. This result was supported by several lines of evidence. First, the enzyme activity of the C:N ratio, using data from both MBCE and SOCE, was less than 1 (Fig. 2a, b), while the N:P ratio of MBCE and SOCE was greater than 1 (Fig. 2c, h), suggesting that soil microorganisms were limited by N across all types of vegetation restoration. Second, vector angles were all less than 45° in all four types of vegetation restoration (Fig. 2e, j), indicating clear N limitation. In addition, the soil enzymatic stoichiometry scatter plot provided intuitive evidence that soil microorganisms in all four types of vegetation restoration were limited by N (Fig. 3). This finding is in disagreement with previous studies that suggested soil microorganisms in karst ecosystems covering cropland, grassland, shrubland and forest were more co-limited by C and P rather than N (Chen et al., 2018a; Chen et al., 2019; Zheng et al., 2020b) and contrast to our hypothesis I.

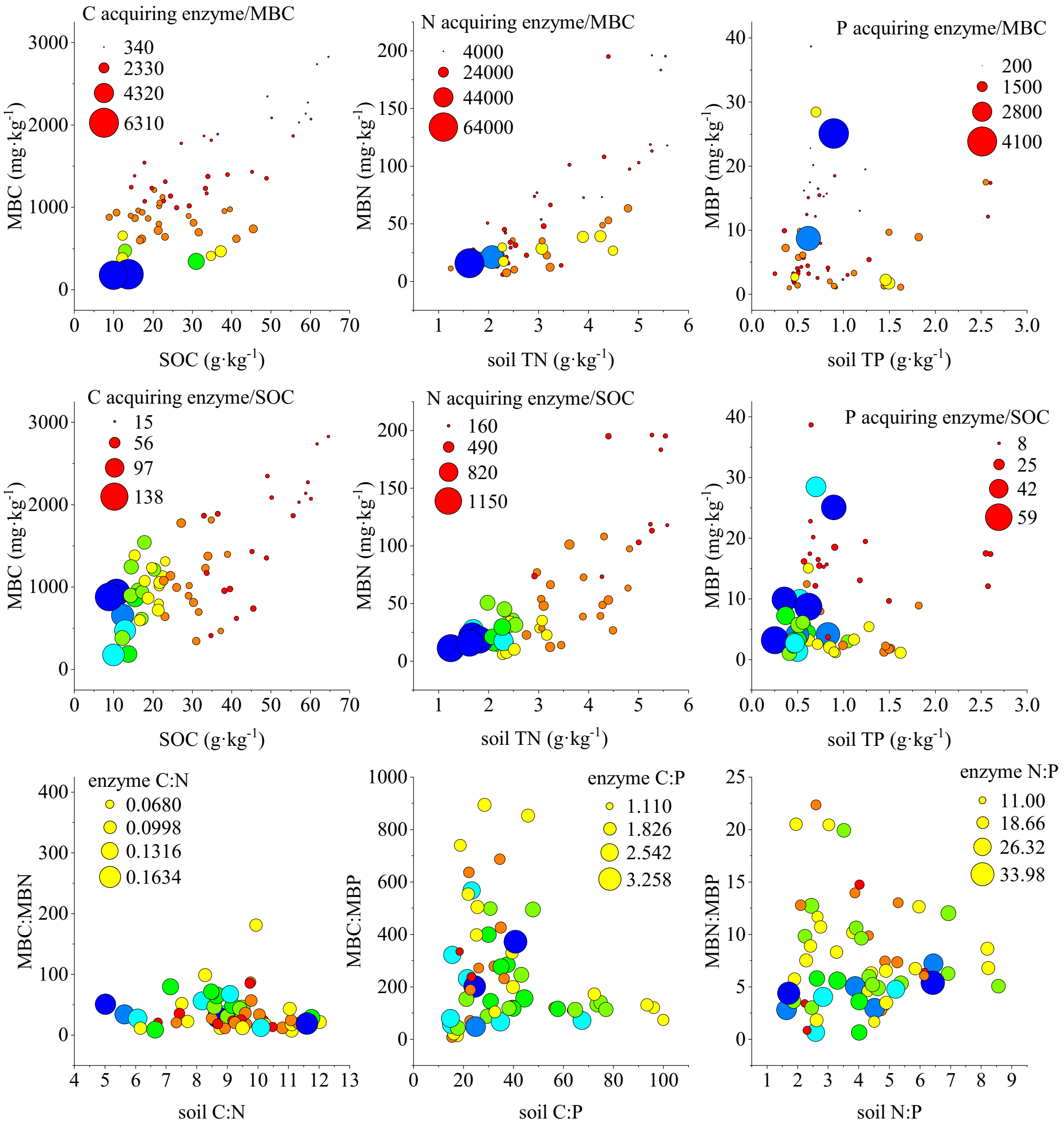

Fig. 5. Responses of soil enzyme activity and enzymatic stoichiometry to the collaborative recovery of soil – microbial biomass
Note: SOC: soil organic carbon; soil TN: soil total nitrogen; soil TP: soil total phosphorus; soil C:N, C:P and N:P: the stoichiometries of SOC, soil TN and soil TP. MBC, MBN and MBP: soil microbial biomass carbon, nitrogen and phosphorus.

However, other studies claimed that N limitation is globally distributed (LeBauer and Treseder, 2008; Kou et al., 2020; Meyerholt et al., 2020); thus, it was not surprising we found N limitation in our study. In addition, N:P stoichiometry of soil nutrients and soil-microbial biomass at the global scale were found to be 17:1 and 6:1, respectively (Xu et al., 2013). Lower soil N:P (2.68–5.77) and higher microbial biomass N:P (6.86–9.07) (Table 2) were observed in our study, suggesting a greater demand by soil microorganisms for N and more severe N limitation in karst regions than the global average. Lower soil N:P can be explained by the following: a large proportion of N is stabilised by becoming directly bound to calcium minerals or to numerous calcium bridges in soil organic matter in karst soils (Pan et al., 2016), leading to low N availability. Moreover, major loss of nutrients can occur via the underground drainage networks resulting from the shallow soil cover and the highly developed epikarst system in karst regions (Zeng et al., 2018; Liu et al., 2020), which rapidly leach away any newly dissolved available N. Conversely, P is mainly supplied by the weathering of rock in the natural environment (Ren et al., 2017). Rock outcrops in karst areas would be subject to weathering, which ensures a higher P content in this area relative to other areas around the globe.

N limitation was very severe in NC, with the significantly lowest enzyme C:N ratio, the greatest enzyme N:P ratio and the smallest vector angle (Fig. 2). This was consistent with our hypothesis II that EESs and nutrient limitation would differ in different communities. Microbial N limitation is a relative concept, depending on the quantity of other nutrients, such as C and P (Chen et al., 2018a). Previous studies confirmed that the addition of one nutrient can cause the restriction of another one. For example, N addition can enhance C limitation (Yuan et al., 2020; Zhu et al., 2020) or P limitation (Gao et al., 2019) of the soil-microbial community. The addition of P increased microbial N demand (R.Z. Wang et al., 2020). In the present study, NC had the most abundant SOC and the highest soil C:N, which greatly increases the demand for N by soil microorganisms. In other words, microbial N limitation would limit the decomposition of organic matter, thus contributing to SOC accumulation (Sistla et al., 2012). These findings advance our understanding on the soil microbial metabolic limitation and nutrient cycling in karst ecosystems. However, our enzyme activity measurements represent a single point in time, which may not accurately reflect the enzyme activity in other seasons or vegetation restoration stages. In addition, our assumption that EES could reflect resource status is based on a limited

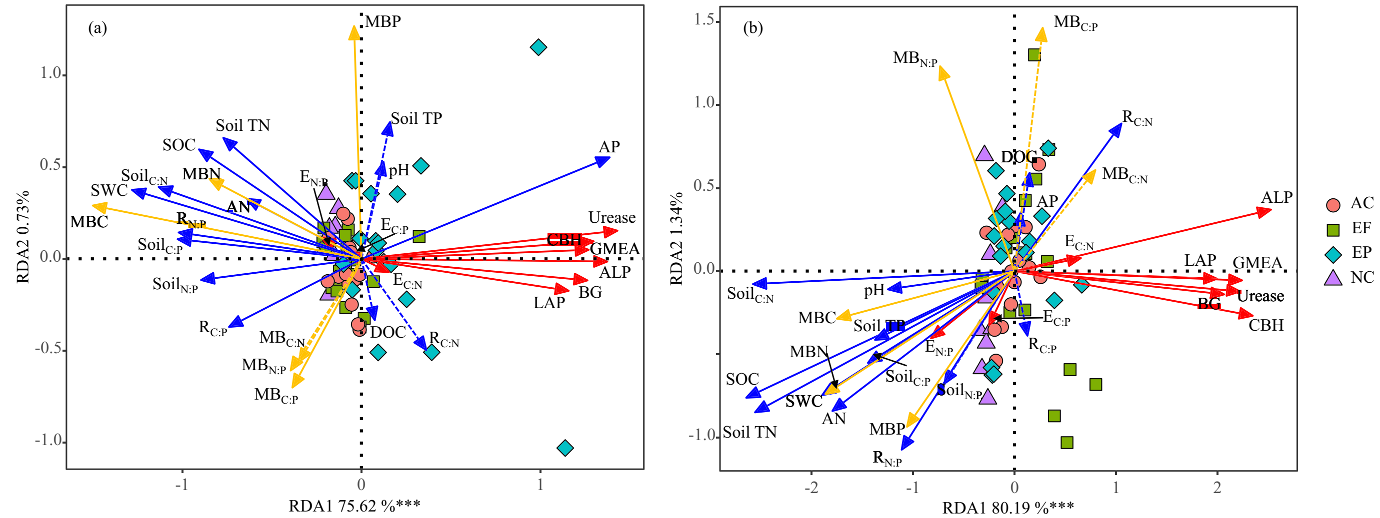

Fig. 6. Redundancy analysis (RDA) of soil specific enzyme activity per MBC and its stoichiometries (a), and soil specific enzyme activity per SOC and its stoichiometries (b), factors including soil, resource, microbial biomass C, N, P content and their stoichiometries.
Note: Blue line indicates abiotic factors, yellow line indicates biotic factors, solid line indicates that the factor has a significant influence on the index ($p < 0.05$), while the dotted line shows no significance. ***indicates RDA model was significant at $p = 0.001$ level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number, though widely reported, EEAs. They may be the most representable to C, N and P EEAs, but not equivalent to their total amount. These data gaps may weaken the linkage between EES and soil resource.

5. Conclusion

We used specific enzyme activity and enzymatic stoichiometry to study microbial resource limitations under different vegetation restoration types in the karst areas. We found that the microbial community in EP had the highest enzyme activity per unit microbial biomass, and the microbial community in EF had the highest utilisation rate of SOC. The specific enzyme activity (MBCE and SOCE) was generally negatively correlated with microbial biomass and soil C, N, P contents, and this was especially significant for C and N, indicating microbial resource requirement will drive much more specific enzyme production in resource-deficient ecosystems. The findings from enzymatic stoichiometry suggested that these karst ecosystems were N-limited rather than P-limited. NC had more severe N limitation than other types of vegetation restoration due to its greater organic C accumulation. Future studies should focus on the long-term observations of enzyme activity and microbial communities, and further exploration into the community composition and gene function of soil microbial communities is required to identify which groups of microbes respond to resource change during vegetation restoration.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104253.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Acknowledgements

We are grateful to the land owners of economic plantations who allowed access to their property. We also thank Qin Tian and Rongrong Hu of Guizhou university and Xinyuan Zhang of Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences for their great help in plant species identification and field sampling. We thank Haiyang Zhao of Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences for his guidance in soil microbial biomass C, N, P test.

Funding

This work was supported by the National Key Research and Development Program (Grant No. 2017YFC0506505) and the National Natural Science Foundation of China (Grant No. 41922056).

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