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Responses of soil microbial resource limitation to multiple fertilization strategies

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ABSTRACT

Fertilization is a key management practice for maintaining or improving soil fertility in agro-ecosystems. Nevertheless, how fertilization strategies impact the status of soil microbial resource limitation is poorly understood. Here, we investigated the effects of long-term (11 years) fertilization on microbial resource limitation in a karst cropland under maize–soybean rotation. Soil microbial resource limitation was assessed using enzymatic stoichiometry. Six fertilization strategies were included, i.e., i) no fertilization (control), ii) inorganic fertilizers only (NPK), iii) inorganic fertilizers plus a low amount of straw (LSNPK), iv) inorganic fertilizers plus a low amount of manure (LMNPK), v) inorganic fertilizers plus a high amount of straw (HSNPK), and vi) inorganic fertilizers plus a high amount of manure (HMNPK). Overall, soil microbes were not limited by nitrogen, but co-limited by carbon and phosphorus across the six fertilization strategies. However, the degrees of microbial resource limitations were different between the control and fertilizer treatments. Application with inorganic fertilizers only aggravated microbial carbon limitation, but combined application of inorganic fertilizers and organic matters did not change the status of carbon limitation relative to the control. None of the fertilizer treatments changed the status of microbial nitrogen limitation. The treatments of NPK, LSNPK and LMNPK alleviated microbial phosphorus limitation, but HSNPK and HMNPK had no significant effects on phosphorus limitation relative to the control. By contrast, the crop production had no significant difference among all fertilizer treatments in the current study. Together, our results indicate that fertilizations can change microbial resource limitation status, which might be a more sensitive indicator to identify effective fertilization strategies relative to the crop production. Here we suggest that karst croplands do not need too much nitrogen fertilizer due to the nitrogen-rich characteristic, and that combined inorganic and organic fertilization strategies are better than single fertilization strategy in karst croplands.

1. Introduction

Microbes regulate many soil processes including carbon (C), nitrogen (N) and phosphorus (P) transformations, and thus play a key role in energy flow and nutrients release (Chen et al., 2018b). However, these microbial-controlled processes are strongly dependent on microbial biomass or activity, which is mostly limited by energy and nutrient availability. This is called microbial resource limitation or substrate limitation (Chen et al., 2018a). The status of microbial resource limitation, however, can be altered in agro-ecosystems due to application of inorganic fertilizers and organic fertilizers (e.g. straw or manure) alone or in combination (Zhang et al., 2017), because fertilizer inputs may change the relative availability of soil C, N and P (Dong et al., 2012; Tian et al., 2015; Zhu et al., 2015; Hu et al., 2018).

Studying how soil microbial resource limitation changes under different fertilization strategies is important for identifying effective fertilization strategies to improve soil C sequestration and crop productivity in agro-ecosystems. First of all, microbes are an important C source in soil (Kallenbach et al., 2016). It has been suggested that the microbial residues can contribute over 50% of C to soil C pool (Simpson et al., 2007; Liang et al., 2011), and the microbial residue C is relatively stable (Craig et al., 2018). Microbial residues are strongly related to

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microbial growth, which is controlled by microbial resource limitation status, so that adopting effective fertilization strategies to relief microbial resource limitation and thus increase microbial growth is important in soil carbon accumulation in agro-ecosystems. Moreover, it is traditionally proposed that microbes possess competitive advantage over plants in the acquisition of nutrients from soil (Lipson et al., 1999; Hodge et al., 2000). Therefore, if microbes are limited by a nutrient, plants should also be limited by that nutrient. Consequently, investigation of soil microbial resource limitation is useful for identifying fertilization strategies in order to improve soil C pool or crop productivity. Although the effects of fertilization on crop growth or production have been commonly studied to identify suitable fertilization strategies (Cai and Qin, 2006; Steiner et al., 2007), to our knowledge, few studies have directly assessed how microbial resource limitation status changes under different fertilization strategies in agro-ecosystems.

Different methodologies may be used to assess the alteration of microbial substrate limitation by fertilization. For example, microbial substrate limitation is traditionally assessed by investigating the changes of substrate-induced respiration or microbial biomass following substrate (C compounds or nutrients) addition to soils under different fertilization strategies (Traoré et al., 2016). However, this approach is relatively time consuming, and is criticized since the added "non-natural" nutrients may be bound to soil matrix and have side effects (Sullivan et al., 2014; Sayer and Banin, 2016). Additionally, C is usually added as glucose, which is not representative of soil available C, so that may under- or over-estimate microbial C limitation (Fanin et al., 2012). Alternatively, enzymatic stoichiometry has been suggested as a useful approach for assessing microbial resource limitation (Chen et al., 2018a). This approach assumes that the acquisitions of organic C, N and P can be represented by four enzymes, i.e., β-D-glucosidase (BG), L-leucine aminopeptidase (LAP) and β-*N*-acetylglucosaminidase (NAG), and phosphatase (alkaline or acid, AP), respectively (Sinsabaugh and Shah, 2012). BG is a cellulase associated with acquisition of organic C (Jian et al., 2016). LAP and NAG are two N-acquisition enzymes that depolymerize protein and chitin, respectively (Chen et al., 2018c). Phosphatase is an enzyme associated with organic P acquisition (Marklein and Houlton, 2012). Based on the assumption that these four enzymes regulate C, N and P acquisition, respectively, relative microbial C, N, and P limitation can be assessed using enzymatic stoichiometry, including enzymatic ratios, vector variables and Threshold Elemental Ratios (TER) (Chen et al., 2018a, b). Since enzymatic stoichiometry is more effective than the traditional methods described above in assessing microbial resource limitation, it has received considerable attention (Hill et al., 2014; Waring et al., 2014; Fanin et al., 2016; Moorhead et al., 2016).

In the current study, the responses of soil microbial resource limitation to multiple fertilization strategies were assessed in a karst cropland under maize–soybean rotation. In theory, application with inorganic fertilizers only may alleviate soil microbial nutrient limitation, but aggravate microbial C limitation (Aber et al., 1998; Chen et al., 2018d). In contrast, application with organic fertilizers provides additional C sources and therefore can alleviate microbial C limitation, but may lead to microbial nutrient limitation (Kamble and Bååth, 2014). Accordingly, we hypothesized that microbial C limitation will be aggravated by inorganic NPK fertilization only, but will be alleviated by organic fertilizer application (**Hypothesis I**). Additionally, our previous studies show that enzymatic stoichiometry is a useful tool for measuring microbial resource limitation in karst soils, and soil microbes are limited by C and P but not N in most karst ecosystems (Chen et al., 2018a, b). We therefore hypothesized that fertilization may not affect microbial N limitation status since soil microbes are already N-saturated (**Hypothesis II**), and that microbial P limitation will be relieved under the treatments with P fertilizers (**Hypothesis III**). Finally, given that microbes are more competitive than plants in nutrient uptake, we hypothesized that the changes in microbial nutrient limitation status may be more sensitive than that in plant productions (**Hypothesis IV**).

2. Materials and methods

2.1. Study site and experimental design

The study was conducted at the Huanjiang Observation and Research Station for Karst Ecosystems, located in Huangjiang County, Guangxi Province (Hu et al., 2018). This area has a subtropical monsoon climate. The mean annual temperature is 18.5 °C, and the mean annual precipitation is 1389 mm. The wet season is from April to August, and the dry season is from September to March. The soil is leptosols based on the FAO World Reference Base for Soil Resources (IUSS-Working-Group, 2006).

The experiment was established in a maize-soybean rotation field with a randomized complete block design with four blocks and six treatments (Hu et al., 2018). In each block, there were six plots (4 m × 7.5 m each) corresponding to the six treatments. Concrete walls were built around each plot to avoid interference between the treatments. The treatments were:

(i) control: no fertilizers were applied.
(ii) N, P and K fertilizers (NPK): urea, superphosphate and potassium chloride were applied at a rate of 200 kg N ha^{-1}, 90 kg P_2O_5 ha^{-1} and 120 kg K_2O ha^{-1} during the maize cropping season, and 22.5 kg N ha^{-1}, 60 kg P_2O_5 ha^{-1} and 67.5 kg K_2O ha^{-1} during the soybean cropping season;
(iii) low amount of straw and inorganic NPK (LSNPK): 2700 kg ha^{-1} soybean straw (equaling about 46.5 kg N ha^{-1}, 11 kg P_2O_5 ha^{-1} and 36 kg K_2O ha^{-1}) was added in the maize season, and 740 kg ha^{-1} maize straw (equaling about 6.75 kg N ha^{-1}, 1.7 kg P_2O_5 ha^{-1} and 2.15 kg K_2O ha^{-1}) was added during the soybean season. The N, P and K fertilizer was applied at a rate of 153.5 kg N ha^{-1}, 79 kg P_2O_5 ha^{-1} and 84 kg K_2O ha^{-1} during the maize season, and 15.75 kg N ha^{-1}, 58.3 kg P_2O_5 ha^{-1} and 65.35 kg K_2O ha^{-1} during the soybean season;
(iv) low amount of cow manure and inorganic NPK (LMNPK): 3730 kg ha^{-1} cow manure (about 60 kg N ha^{-1}, 33 kg P_2O_5 ha^{-1} and 43 kg K_2O ha^{-1}) was added during the maize season, and 420 kg ha^{-1} cow manure (about 6.8 kg N ha^{-1}, 3.8 kg P_2O_5 ha^{-1} and 4.9 kg K_2O ha^{-1}) was added in the soybean season. The N, P and K fertilizer was applied at a rate of 140 kg N ha^{-1}, 57 kg P_2O_5 ha^{-1} and 77 kg K_2O ha^{-1} during the maize season, and 15.7 kg N ha^{-1}, 56.2 kg P_2O_5 ha^{-1} and 62.6 kg K_2O ha^{-1} during the soybean season;
(v) high amount of straw and inorganic NPK (HMNPK): 5400 kg ha^{-1} soybean straw (about 93 kg N ha^{-1}, 22 kg P_2O_5 ha^{-1} and 72 kg K_2O ha^{-1}) during the maize season, and 1480 kg ha^{-1} maize straw (about 13.5 kg N ha^{-1}, 3.4 kg P_2O_5 ha^{-1} and 4.3 kg K_2O ha^{-1}) during the soybean season. The N, P and K fertilizer was applied at a rate of 107 kg N ha^{-1}, 68 kg P_2O_5 ha^{-1} and 48 kg K_2O ha^{-1} during the maize season, and 9 kg N ha^{-1}, 56.6 kg P_2O_5 ha^{-1} and 63.2 kg K_2O ha^{-1} during the soybean season;
(vi) high amount of cow manure and NPK (HMNPK): 7430 kg ha^{-1} cow manure (about 120 kg N ha^{-1}, 68 kg P_2O_5 ha^{-1} and 87 kg K_2O ha^{-1}) was added in the maize season, and 840 kg ha^{-1} cow manure (about 13.6 kg N ha^{-1}, 7.7 kg P_2O_5 ha^{-1} and 9.8 kg K_2O ha^{-1}) was added in the soybean season. The N, P and K fertilizer was applied at a rate of 60 kg N ha^{-1}, 22 kg P_2O_5 ha^{-1} and 33 kg K_2O ha^{-1} during the maize season, and 8.9 kg N ha^{-1}, 52.3 kg P_2O_5 ha^{-1} and 57.7 kg K_2O ha^{-1} during the soybean season.

The experiment was initiated in April 2006. The fertilizers were applied three times in the maize season every year (before maize planting, at maize shoot growth stage and at the reproductive growth stage), and two times in the soybean season (before soybean planting and at the pre-flowering stage). Equal amounts of inorganic and organic

N, P and K were added during each fertilization event.

2.2. Soil sampling and physicochemical assay

Soil sampling was conducted in May 2017. In each plot, five soil cores (15 cm depth) were randomly collected and mixed to create a composite sample. Soil samples were passed through a 2–mm sieve and then thoroughly homogenized and divided into two portions. One portion was air-dried at room temperature for analyzing soil pH, soil organic C (SOC), total nitrogen (TN), total phosphorus (TP), dissolved organic C (DOC), total dissolved N (TDN), available P (AVP) and available K (AVK) using the methods described in a previous study (Chen et al., 2018a). Another portion was used for analyses of microbial biomass C (MBC), N (MBN) and P (MBP) and soil enzyme activities. Microbial biomass C, N and P were measured using a chloroform fumigation extraction method (Vance et al., 1987). The activities of four extracellular enzymes, β-$_{\text{D}}$-glucosidase (BG), $_{\text{L}}$-leucine aminopeptidase (LAP), β-*N*-acetylglucosaminidase (NAG) and acid phosphatase (AP), were assayed using a microplate protocol. Detailed methods for enzyme activity assays have been described in a previous study (Chen et al., 2017). The enzyme activity was expressed as nmol g^{-1}soil h^{-1}.

2.3. Enzymatic stoichiometry

Before analyzing enzymatic stoichiometry, enzyme activity was normalized to SOC (nmol g^{-1} SOC h^{-1}). Four methods were used to assess microbial resource limitation as described previously (Chen et al., 2018a). In the first method, microbial resource limitation was judged based on the scatter plot between (LAP + NAG)/AP and BG/(LAP + NAG) with the four parts in the plot representing N limitation, P limitation, C & N limitation and C & P limitation, respectively (Hill et al., 2012). The partitioning of the four parts is based on the deviation from the expected enzyme activity ratios of C:N (1:1) or N:P (1:1) (Sinsabaugh et al., 2009).

In the second method, microbial resource limitation was assessed based on ratios of enzymatic activities, specifically BG/(LAP + NAG) and BG/AP. Higher BG/(LAP + NAG) and BG/AP indicate lower N and P limitation, respectively (Waring et al., 2014).

In the third method, a vector analysis of enzymatic stoichiometry was used to assess microbial resource limitation (Moorhead et al., 2013). Vector length (unitless) and vector angle (degree) were calculated as follows:

$$Vector\,\text{length} = \sqrt{(\ln\text{BG}/ln\,[NAG + LAP])^2 + (\ln\text{BG}/lnAP)^2} \tag{1}$$

$$\text{Vector angle} = \text{Degrees}(ATAN2((\ln BG/lnAP), (\ln\text{BG}/ln\,[NAG + LAP]))) \tag{2}$$

A longer vector length indicates greater C limitation, and the vector angles of < 45° and > 45° indicate N and P limitation, respectively (Moorhead et al., 2013).

Lastly, comparisons between Threshold Elemental Ratios (TER) for C:N and C:P ($\text{TER}_{\text{C:N}}$ and $\text{TER}_{\text{C:P}}$, respectively) and the C:N and C:P of available resources (DOC/TDN ($\text{R}_{\text{C:N}}$) and DOC/AVP ($\text{R}_{\text{C:P}}$), respectively) were performed to assess microbial resource limitation. The $\text{TER}_{\text{C:N}}$ and $\text{TER}_{\text{C:P}}$ were calculated using the following equations (Sinsabaugh et al., 2009):

$$\text{TER}_{\text{C:N}} = (\text{BG}/(\text{NAG} + \text{LAP})) \times \text{B}_{\text{C:N}}/\text{n}_0 \tag{3}$$

$$\text{TER}_{\text{C:P}} = (\text{BG}/\text{AP}) \times \text{B}_{\text{C:P}}/\text{p}_0 \tag{4}$$

where $\text{B}_{\text{C:N}}$ and $\text{B}_{\text{C:P}}$ are microbial biomass C:N and C:P ratios, and n_0 *and* p_0 are the intercepts calculated from regressions of ln(BG) vs ln (NAG + LAP) and ln(BG) vs ln(AP), respectively. When the $\text{R}_{\text{C:N}}$ $-\text{TER}_{\text{C:N}}$ or $\text{R}_{\text{C:P}}$ $-\text{TER}_{\text{C:P}}$ is less than zero, soil microbes are not limited by N or P. When the $\text{R}_{\text{C:N}}$–$\text{TER}_{\text{C:N}}$ or $\text{R}_{\text{C:P}}$–$\text{TER}_{\text{C:P}}$ is greater than zero, microbes are N or P limited. In the latter case, higher $\text{R}_{\text{C:N}}$–$\text{TER}_{\text{C:N}}$ or $\text{R}_{\text{C:P}}$ $-\text{TER}_{\text{C:P}}$ indicate higher N or P limitation (Sterner and Elser, 2002).

2.4. Crop production measurements

The data about productions of maize and soybean was provided by Huanjiang Observation and Research Station for Karst Ecosystems, Chinese Academy of Sciences. Harvest was conducted in July (for maize) and October (for soybean) each year starting from 2011 to 2016. To avoid edge effects, the plants were harvested from the center (a 2 m × 3 m subplot) of each plot. The plant numbers were counted and the grain weight of maize and soybean were weighted after drying at 75 °C for 72 h. Then, the crop production was calculated as g plant^{-1}.

2.5. Statistical analysis

Normality of the data was tested using the Shapiro-Wilk test and homogeneity of variances was tested by Levene's test. The effects of fertilizer treatments on soil properties, enzyme activities, and enzymatic stoichiometry variables were examined using one-way analysis of variance (ANOVA). When the *F* test was significant, means were compared using the Tukey test. To compare the differences in productions of maize and soybean, a repeated measure ANOVA was used. To test the difference between $\text{R}_{\text{C:N}}$ $-\text{TER}_{\text{C:N}}$ (or $\text{R}_{\text{C:P}}$ $-\text{TER}_{\text{C:P}}$) and zero, a one-sample *t*-test was used. In addition, correlation and regression analyses were conducted to analyze the relationships between the studied variables. All statistical analyses were conducted using SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA). All reported significant differences in the current study are at $p < 0.05$.

3. Results

3.1. Soil enzyme activity

In the control, average enzyme activities (per g soil) for BG, NAG, LAP, and AP were 24.3 ± 2.5 (mean ± standard error), 11.4 ± 1.1, 0.5 ± 0.1 and 183.7 ± 14.1 nmol g^{-1} soil h^{-1}, respectively (Fig. 1). The average enzyme activities (per g SOC) for BG, NAG, LAP, and AP were 1149 ± 94, 546 ± 70, 23 ± 4 and 8671 ± 443 nmol g^{-1} SOC h^{-1}, respectively (Fig. 1). The soil based and SOC normalized enzyme activities showed similar responses to fertilizer treatments (Fig. 1). BG activity significantly increased under NPK treatment compared to the control, but not for other fertilizer treatments (Fig. 1a, e). NAG activity significantly increased in all the fertilizer treatments relative to the control (Fig. 1b, f). There was no significant difference in LAP activity between the six treatments (Fig. 1c, g). As for AP, the activity significantly decreased under LSNPK relative to control, but was not altered by the other fertilizer treatments (Fig. 1d, h).

3.2. Enzymatic stoichiometry

The soil enzymatic stoichiometry scatter plot showed that all the data points fell within the C and P co-limitation group (Fig. 2). As for the vector analyses, the vector length significantly increased under NPK treatment relative to control, but showed no significant change under the other fertilizer treatments (Fig. 3a). The vector angles were greater than 45° in the six treatments with the values in NPK, LSNPK and LMNPK significantly lowered relative to the control (Fig. 3b). The ratio of BG/(LAP + NAG) significantly increased under NPK treatment relative to control, but showed no significant change under the other fertilizer treatments (Fig. 3c). The ratio of BG/AP significantly increased under NPK, LSNPK and LMNPK, but showed no significant change under HSNPK and HMNPK compared to control (Fig. 3d). With regards to the comparison between TER and available nutrient ratios, there was no significant difference between $\text{R}_{\text{C:N}}$– $\text{TER}_{\text{C:N}}$ and zero for all the six treatments, and there was no significant difference in $\text{R}_{\text{C:N}}$–$\text{TER}_{\text{C:N}}$ among the six treatments (Fig. 3e). The $\text{R}_{\text{C:P}}$– $\text{TER}_{\text{C:P}}$ was

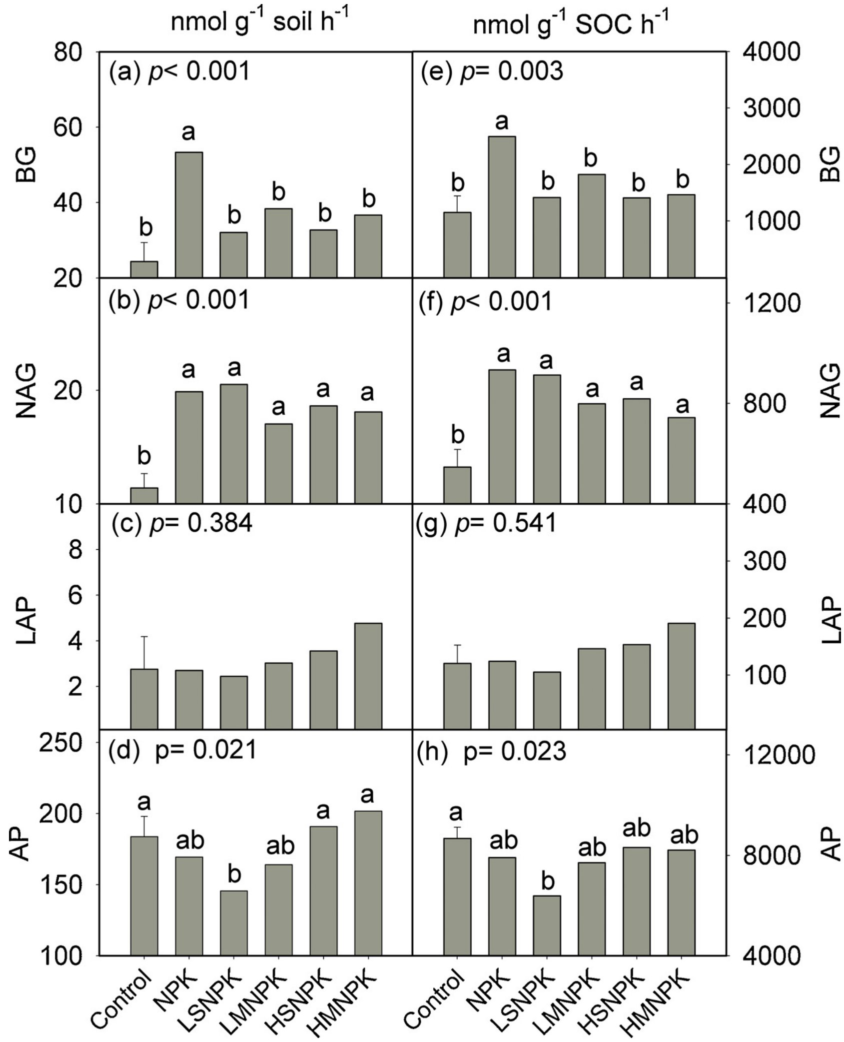

Fig. 1. Soil enzyme activity responses (a– d: activity per soil; e– h: activity per SOC) to fertilization strategies. The bars in the control treatments are standard errors; *p* values are given according to one-way ANOVA, and the different letters indicate significant difference between treatments according to the Tukey test. Information about the abbreviations can be found in the methods.

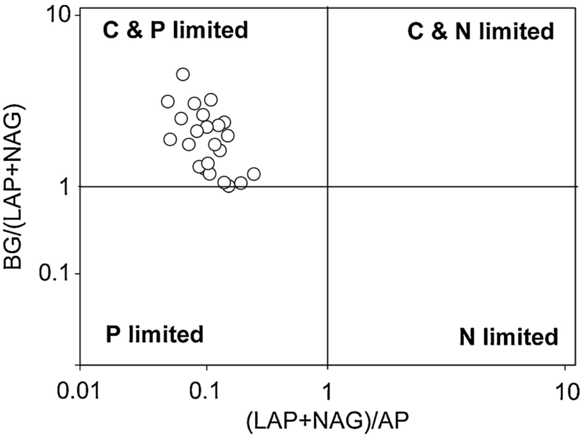

Fig. 2. A scatter plot of soil enzymatic stoichiometry showing the general pattern of microbial resource limitation.

significantly greater than zero in all the six treatments, and the values were significantly lowered under the five fertilizer treatments relative to the control (Fig. 3f).

3.3. Soil properties

Long-term fertilizer application altered some soil properties (Table 1). Soil organic C significantly increased under HMNPK treatment compared to the control, but showed no significant change under the other fertilizer treatments. Similarly, total N increased significantly under HSNPK and HMNPK, but showed no change under the other fertilizer treatments. Total P increased significantly in all fertilizer treatments compared to the control. There was no significant difference in soil pH, C/N, C/P, and N/P among the six treatments.

Microbial biomass C and N increased significantly under HSNPK and HMNPK compared to the control, but not for the other fertilizer treatments. Microbial biomass P was significantly higher under the fertilizer treatments than in the control. Dissolved organic C and total dissolved N showed no significant change, but available P significantly increased under all the fertilizer treatments compared to the control. As a result, there was no significant difference in $M_{C:N}$ and $R_{C:N}$ among the six treatments. The ratios of $M_{C:P}$ and $M_{N:P}$ were significantly higher in NPK, LSNPK and LMNPK, but showed no significant change under HSNPK and HMNPK compared to the control. The ratios of $R_{C:P}$ and $R_{N:P}$ were significantly lower under all the fertilizer treatments than under the control.

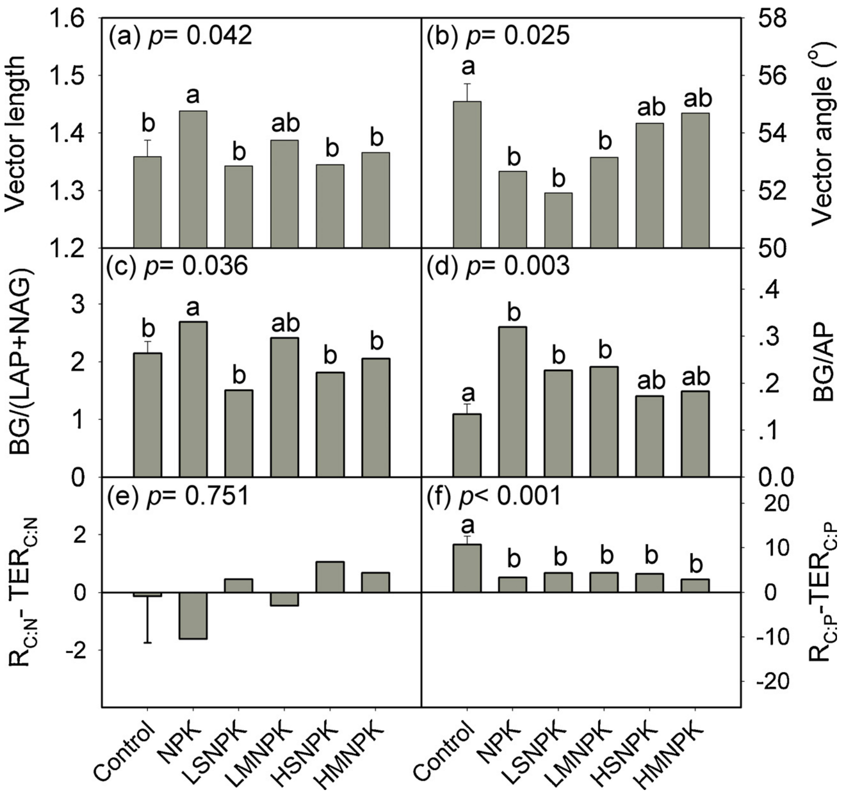

Fig. 3. Responses of vector length (a), vector angle (b), BG/(LAP + NAG) (c), BG/AP, $R_{C:N}$–$TER_{C:N}$ (e) and $R_{C:P}$–$TER_{C:P}$ (f) to fertilization strategies. The bars in the control treatments are standard errors; *p* values are given according to one-way ANOVA, and the different letters indicate significant difference between treatments according to the Tukey test. Information about the abbreviations can be found in the methods.

Table 1
Effects of fertilization strategies on soil properties.

	Treatments						One-way ANOVA	
Variables	Control	NPK	LSNPK	LMNPK	HSNPK	HMNPK	SE	*p*-values
pH	6.71	6.58	6.63	6.57	6.68	6.62	0.16	0.938
SOC (g kg^{-1})	21.12 b	21.41 b	22.55 ab	21.24 b	23.06 ab	24.76 a	1.11	0.029
TN (g kg^{-1})	2.43 c	2.54 bc	2.59 bc	2.51bc	2.81 ab	2.91 a	0.11	0.037
TP (g kg^{-1})	0.97 c	1.21 ab	1.23 a	1.19 ab	1.11 b	1.17 ab	0.06	0.001
C/N	8.69	8.43	8.73	8.47	8.19	8.51	0.31	0.610
C/P	21.87	17.75	18.47	17.88	20.90	21.29	1.62	0.060
N/P	1.80	1.51	1.52	1.51	1.82	1.78	0.14	0.079
MBC (mg kg^{-1})	222.02 b	202.36b	220.83 b	221.50 b	294.79 a	315.44 a	28.6	0.004
MBN (mg kg^{-1})	37.03 b	33.09 b	35.45 b	35.45 b	57.56 a	56.54 a	6.20	0.001
MBP (mg kg^{-1})	18.87 c	31.65 ab	33.98 a	30.90 ab	28.19 b	30.88 ab	2.44	0.001
$M_{C:N}$	6.18	6.35	6.63	6.46	5.12	5.68	1.05	0.714
$M_{C:P}$	12.02 a	6.53 b	6.52 b	7.28 b	10.63 ab	10.23 ab	1.48	0.004
$M_{N:P}$	1.97 a	1.07 b	1.04 b	1.17 b	2.07 a	1.84 a	0.24	0.001
DOC (mg kg^{-1})	160.84	184.47	196.97	203.19	182.07	176.53	16.3	0.185
TDN (mg kg^{-1})	35.22	39.48	49.37	38.83	39.66	39.54	4.67	0.120
AVP (mg kg^{-1})	14.86 b	45.63 a	39.27 a	41.80 a	40.45 a	52.22 a	6.77	0.001
AVK (mg kg^{-1})	91.58 c	348.86 a	323.90 a	215.42 b	341.18 a	357.69 a	19.7	0.001
$R_{C:N}$	4.82	4.69	4.00	5.27	4.65	4.63	0.61	0.503
$R_{C:P}$	11.39 a	4.22 b	5.02 b	5.05 b	4.73 b	3.53 b	1.35	0.001
$R_{N:P}$	2.46 a	0.89 b	1.27 b	0.98 b	1.05 b	0.79 b	0.33	0.001

Notes: SE is the standard error. *p*-values are given according to one-way ANOVA, and the different letters indicate significant difference between treatments according to the Tukey test. Information about the abbreviations can be found in the methods.

3.4. Relationships between soil properties and enzyme activities or enzymatic stoichiometry

Soil properties had strong relationships with soil enzyme activities (Table 2) or enzymatic stoichiometry (Table 3). NAG activity had significantly positive relationships with TP, MBP, DOC and AVP, and significantly negative relationships with soil pH, C/P, N/P, $M_{C:P}$ and $M_{N:P}$. AP activity had significantly positive relationships with soil pH, N/P, MBC, $M_{C:P}$ and $M_{N:P}$, but significantly negative relationships with TP, C/N and MBC. By contrast, there were no significant relationships between soil properties and BG or LAP activity.

As for enzymatic stoichiometry, the three indicators of microbial P limitation, i.e., vector angle, BG/AP and $R_{C:P}$– $TER_{C:P}$, had strong relationships with soil properties (Table 3 and Fig. 4). Vector angle had significantly positive relationships with C/P, N/P, $M_{C:P}$, $M_{N:P}$, $R_{C:P}$ and $R_{N:P}$, but showed significantly negative relationships with TP, MBP, DOC, AVP and AVK. The ratio of BG/(NAG + LAP) had a significantly positive relationship with MBP, but a negative relationship with $M_{C/P}$. $R_{C:P}$– $TER_{C:P}$ had significantly positive relationships with $M_{C/P}$, $R_{C/P}$ and $R_{N/P}$, but had significantly negative relationships with TP, MBP,

Table 2
Results (r values) from correlation analysis showing the relationships between soil enzyme activity (nmol g^{-1}soil h^{-1}) and soil properties.

Variable	BG	NAG	LAP	AP
pH	0.019	**−0.468***	0.181	**0.408***
SOC (g kg^{-1})	−0.109	−0.304	0.006	0.022
TN (g kg^{-1})	−0.032	−0.198	0.052	0.347
TP (g kg^{-1})	0.187	**0.682***	−0.051	**−0.474***
C/N	−0.136	−0.104	−0.113	**−0.668***
C/P	−0.214	**−0.682***	0.024	0.322
N/P	−0.162	**−0.593***	0.057	**0.510***
MBC (mg kg^{-1})	−0.184	−0.236	−0.037	**0.406***
MBN (mg kg^{-1})	−0.239	−0.076	0.059	0.200
MBP (mg kg^{-1})	0.225	**0.726***	0.128	**−0.491***
$M_{C:N}$	0.171	−0.132	−0.079	0.152
$M_{C:P}$	−0.296	**−0.696***	−0.149	**0.594***
$M_{N:P}$	−0.332	**−0.523***	−0.079	**0.406***
DOC (mg kg^{-1})	0.182	**0.489***	0.015	−0.400
TDN (mg kg^{-1})	0.032	0.264	0.171	−0.306
AVP (mg kg^{-1})	0.208	**0.584***	0.090	−0.286
AVK (mg kg^{-1})	−0.037	0.214	−0.249	−0.207
$R_{C:N}$	0.035	0.061	−0.166	0.005
$R_{C:P}$	−0.236	**−0.556***	−0.215	0.274
$R_{N:P}$	*−0.259*	***−0.547****	*−0.140*	*0.281*

Note: Bolded values with asterisks indicate that the relationships are significant (*p* value < 0.05). Information about the abbreviations can be found in the methods.

AVP and AVK. There were no significant relationships between the measured soil properties and vector length, BG/(NAG + LAP) or $R_{C:N}$–$TER_{C:N}$.

3.5. Crop productions

The production of maize increased significantly in five fertilizer treatments relative to control across 2011 to 2016. Similarly, the production of soybean increased significantly in five fertilizer treatments, except in 2015. However, there was no significant difference in productions among the five fertilizer treatments for both maize and soybean (Fig. 5).

4. Discussion

4.1. Effects of fertilization on individual enzyme activity

The activities of soil enzymes (BG, NAG, LAP and AP) measured in the current study are similar to those measured in nearby karst ecosystems (Chen et al., 2018a, b). We find that BG activity increased under NPK fertilization, but did not change under the other fertilizer treatments relative to the control. Elevated BG activity under NPK fertilization may be due to the increased available N, P and K (Table 1) which improved microbial demand in available C to maintain stoichiometry balance. The unchanged BG activity in the other fertilizer treatments may be attributed to organic C inputs (straw or manure) which balance microbial substrate demand. We also find that all the fertilizer treatments increased NAG activity relative to the control. One reason for this may be due to the change in soil pH in the fertilizer treatments, since it has been demonstrated that NAG activity has a negative relationship with soil pH (Sinsabaugh et al., 2008). In support of this, we did find a significantly negative relationship between soil pH and NAG activity in the current study (Table 2). Meanwhile, organic C inputs in the treatments of combined organic and inorganic fertilizer application may have stimulated NAG activity, because NAG is also involved in organic C mineralization (Stone et al., 2012). Decrease of AP activity in LSNPK treatment may be attributed to the negative effect of P addition on AP, which has been reported in previous studies (Rejmankova and Snyder, 2008; Marklein and Houlton, 2012).

However, changes of individual enzyme activities cannot provide information of microbial resource limitation because resource limitation is not determined by a single nutrient but by the relative availability of multiple nutrients (Chen et al., 2018b). For instance, in the current study, the increased BG in NPK treatment may not indicate an aggravated microbial C limitation because NAG also increased. Therefore, a further enzymatic stoichiometry analysis is needed to explore the patterns of microbial resource limitation and the responses of microbial resource limitation to fertilization.

4.2. Effects of fertilization on microbial resource limitation

Our results suggest that soil microbes in the six treatments were not limited by N, but co-limited by C and P. This finding is supported by several lines of evidence. Firstly, vector angles were greater than 45°, and the values of $R_{C:P}$–$TER_{C:P}$ were greater than zero in all the six

Table 3
Results (r values) from correlation analysis showing the relationships between soil properties and the indicators of microbial resource limitation.

Variable	Vector length	Vector angle	BG/ (NAG + LAP)	BG/AP	$R_{C:N}$ – $TER_{C:N}$	$R_{C:P}$ – $TER_{C:P}$
pH	0.203	0.203	0.203	0.203	−0.338	0.360
SOC (g kg^{-1})	0.101	0.234	0.070	−0.105	−0.109	−0.247
TN (g kg^{-1})	0.040	0.279	0.061	−0.206	−0.040	−0.291
TP (g kg^{-1})	−0.124	**−0.753***	−0.244	0.402	0.188	**−0.676***
C/N	0.074	−0.183	−0.032	0.229	−0.066	0.123
C/P	0.155	**0.676***	0.217	−0.344	−0.210	0.352
N/P	0.113	**0.678***	0.201	−0.395	−0.161	0.278
MBC (mg kg^{-1})	−0.034	0.354	0.084	−0.328	−0.096	−0.009
MBN (mg kg^{-1})	−0.158	0.134	−0.116	−0.291	0.207	−0.160
MBP (mg kg^{-1})	−0.104	**−0.788***	−0.247	**0.439***	0.165	**−0.716***
$M_{C:N}$	0.199	0.176	0.266	0.085	−0.422	0.157
$M_{C:P}$	0.075	**0.814***	0.248	**−0.520***	−0.236	**0.617***
$M_{N:P}$	−0.033	**0.590***	0.073	−0.478	0.068	0.376
DOC (mg kg^{-1})	0.001	**−0.516***	−0.055	0.338	−0.105	−0.133
TDN (mg kg^{-1})	−0.015	−0.320	−0.079	0.174	−0.261	−0.116
AVP (mg kg^{-1})	−0.069	**−0.607***	−0.197	0.338	0.237	**−0.834***
AVK (mg kg^{-1})	−0.053	**−0.613***	−0.190	0.344	0.245	**−0.809***
$R_{C:N}$	−0.034	−0.035	−0.009	0.009	0.296	0.050
$R_{C:P}$	0.083	**0.610***	0.213	−0.348	−0.255	**0.997***
$R_{N:P}$	0.057	**0.596***	0.183	−0.366	−0.330	**0.945***

Note: Bolded values with asterisks indicate that the relationships are significant (*p* value < 0.05). Information about the abbreviations can be found in the methods.

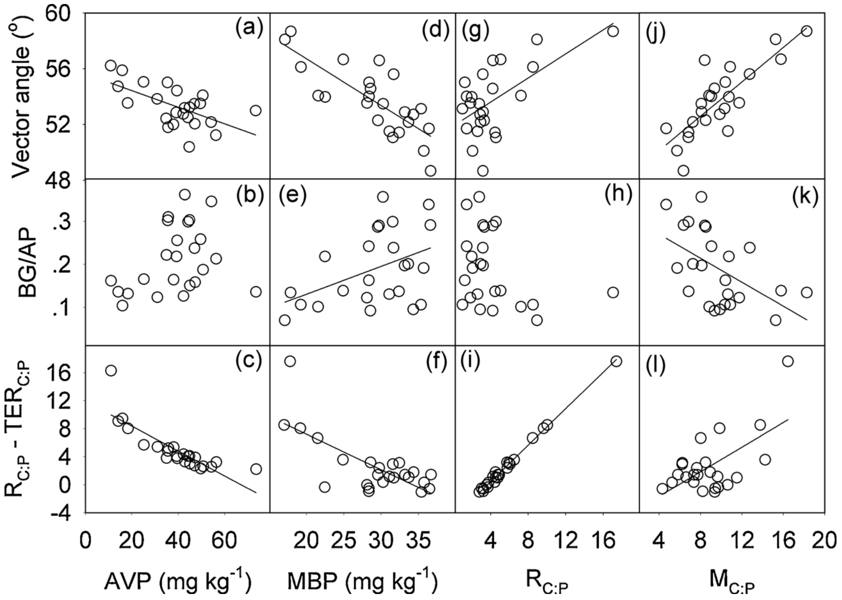

Fig. 4. Relationships between three indicators of microbial P limitation (i.e. vector angle, BG/AP and $R_{C:P}$ –$TER_{C:P}$) and (a–c) available P (AVP), (d–f) microbial biomass P, (g–i) C:P ratio of available resource, or (j–l) C:P ratio of microbial biomass. All *P* values are lower than 0.05 except (b) and (h); the *r* values are listed in Table 3.

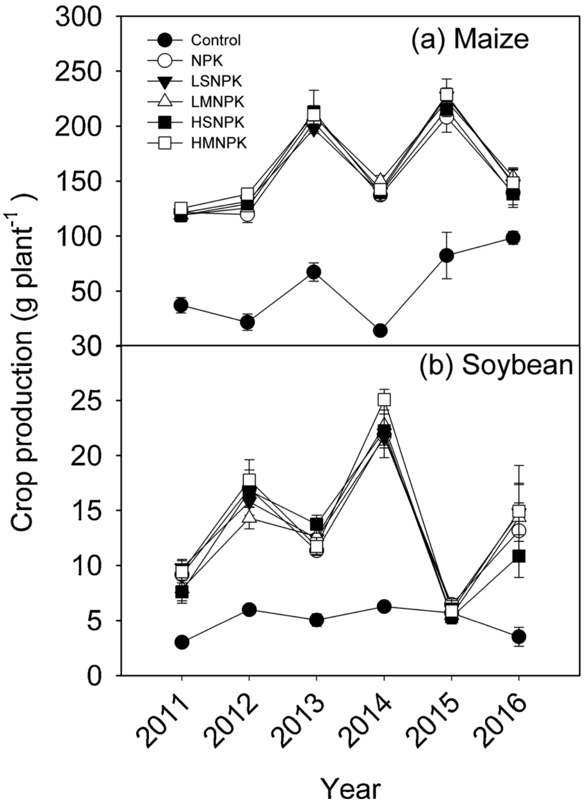

Fig. 5. Comparisons of the productions of maize and soybean among different fertilization treatments from 2011 to 2016. Information about the abbreviations can be found in the methods.

treatments (Fig. 3b, f), suggesting that soil microbes are more limited by P than by N across all the treatments. Soil microbes might even be N-saturated according to the $R_{C:N}$–$TER_{C:N}$ results which were less than zero in some treatments (Fig. 3e). The soil enzymatic stoichiometry scatter plot provided a more intuitive evidence for that the soil microbes under all the six treatments were co-limited by C and P (Fig. 2). These findings agree with the results from two previous studies conducted in neighboring karst ecosystems covering cropland, grassland, shrubland and forest (Chen et al., 2018a, b). In these studies, the co-limitation by C and P has been attributed to high N but low P contents in karst soils. Similarly, relatively higher total N (2.43 ± 0.09 g N kg^{-1}) but relatively lower available P contents (14.86 ± 1.54 g P kg^{-1}) were observed in the current study (Table 1). The higher soil TN in karst ecosystems might be related to the higher soil calcium content, which increases the stabilization of soil organic N(Wen et al., 2016). Also, the high calcium content is a reason for the lower soil P availability, because soil calcium can react with available P to form insoluble precipitates (Chen et al., 2018b). Fertilization practices did not change this general pattern of microbial resource limitation in the studied agoecosystem (Fig. 2), but the magnitudes of microbial resource limitation were altered after long-term fertilization (Fig. 3).

Consistent with the hypothesis I, we find that microbial C limitation was aggravated by NPK fertilization alone, but had no changes under combined application of inorganic and organic fertilizers. Vector length is a useful index reflecting whether soil microbes are limited by the availability of C with a greater value implying more C limitation (Moorhead et al., 2013). In the current study, vector length under NPK treatment was significantly greater than under the control, suggesting microbial C limitation was aggravated by NPK fertilization alone. Meanwhile, no significant change of vector length in the other four fertilization strategies relative to the control suggests similar status of microbial C limitation among these treatments and the control (Fig. 2a). Aggravated microbial C limitation by inorganic nutrient addition alone is also observed in some N-addition experiments (Treseder, 2008). In a recent meta-analysis, data from 36 published N-addition experiments show that N addition increases vector length, indicating that N addition aggravated microbial C limitation (Chen et al., 2018d). However, this may be not the case in the current study, because TN, TDN, C/N, and $R_{C:N}$ did not change significantly after NPK addition (Table 1). Instead, P and K additions also aggravate microbial C limitation according to the theory of element stoichiometry, which may be a more important reason. In support of this, we find that NPK treatment elevated soil available P and K concentrations but decreased $R_{C:P}$ (i.e. DOC/AVP), suggesting that C availability was lowered relative to the control (Table 1). In contrast, no change in microbial C limitation under combined application of inorganic and organic fertilizers may be attributed to addition of C sources from the organic materials which in turn balance microbial C demands. Similarly, a previous study reported that fertilization with farmyard manure prevented microbial C limitation (Kamble and Bååth, 2014). In the current study, we did find a slight increase in DOC in all treatments with manure or straw addition and a significant increase in MBC in treatments with high straw or manure addition (Table 1), which supports the above explanation.

Our results demonstrate that the fertilizer treatments tended to alleviate microbial P limitation, which is consistent with the hypothesis III. This conclusion is based on the results of $R_{C:P}$–$TER_{C:P}$, which in all the five fertilizer treatments was lower relative to the control (Fig. 3f). The alleviated P limitation is most likely due to the P addition in the fertilizer treatments. In addition, the aggravated C limitation (only for NPK treatment) might be another reason for the alleviated P limitation due to the stoichiometry relationships of C and P. Based on vector angle and BG/AP, however, the high manure or high straw addition (HSNPK or HMNPK) had only minor effects on microbial P limitation as the vector angle and BG/AP did not change under the HSNPK or HMNPK treatments compared to control (Fig. 3b and d). The differences among these indicators are acceptable because of their different calculations. We infer that the vector angle and BG/AP results may be more realistic since the amount of P added with the HSNPK and HMNPK treatments was lower than those under other fertilizer treatments. In addition, the high amount straw or manure input may increase available C levels resulting in an increase in C/P ratio, thus aggravating the microbial P limitation. We have found a higher microbial biomass C/P ratio in HSNPK and HMNPK compared to the other fertilizer treatments, which is consistent with the above explanation. This result indicates that too much organic fertilization is not always helpful.

The variations in these P-limitation indicators can be related to the changes in soil available P, microbial biomass P, and the C: P ratio of the available resources ($R_{C:P}$ and $M_{C:P}$) (Fig. 4). This phenomenon is interesting because it indicates that these soil variables can also be used as indicators for the microbial P limitation. By contrast, we did not find strong relationships between soil properties and indicators of C or N limitation (Table 3). This may indirectly indicate that soil microbes are more sensitive to changes in P or that soil microbes are more limited by P compared to other nutrients.

Fertilizer treatments did not change the status of N saturation in karst soil as $R_{C:N}$–$TER_{C:N}$ were not significantly different between the control and fertilizer treatments. This supports the hypothesis II. Another indicator of N limitation, BG/(LAP + NAG), even suggests that NPK fertilization increases soil microbe N-saturation because BG/(LAP + NAG) was higher in the NPK treatment than in the control. These results together indicate that karst soil N is so high that soil microbes are not sensitive to the addition of exogenous N.

4.3. Effects of fertilization on crop productions

We find that the crop production increased under all five fertilizer treatments, but the increased degrees had no significant difference among all fertilizer treatments. Such no difference among fertilizer treatments is not surprising due to two possible reasons. First, nutrients are easy to lose in ago-ecosystems, so plants may have no significant difference in growths despite some nutrient-addition treatments is excessive. Second, microbes is more competitive than plants in nutrient uptake (Lipson et al., 1999; Hodge et al., 2000). As a result, plants may be more insensitive than microbes in responding to nutrient additions. The later explanation is supported by the results of microbial resource limitation. Our findings lend supports to the hypothesis IV, which suggests the changes in microbial nutrient limitation status may be more sensitive than that in crop productions.

5. Conclusions and implications

In the current study, we used enzymatic stoichiometry to study the changes of microbial resource limitation under fertilizer treatments in a karst cropland. Overall, the studied cropland was C-and-P limited and not N-limited. The NPK fertilization aggravated microbial C limitation, but combined inorganic and organic fertilization had no effects on microbial C limitation. All fertilizer treatments tended to alleviate microbial P limitation, but treatments that combined NPK and high straw or manure additions had minor effects. Fertilizer treatments did not change the status of microbial N saturation in the studied cropland.

Since crop productions have failed to identify which fertilization strategy is better in the current study, our findings suggest that the microbial resource limitation investigation may provide an important auxiliary tool. According to our results, a large amount of N fertilization is not necessary in karst croplands because soils in karst croplands are not N-limited. Instead, combined organic and inorganic fertilization should be used because they provide extra sources of C and P which will help alleviate soil C or P limitation. We also highlight that a proper proportion of inorganic and organic fertilizers is important. Adding too much organic fertilizer may lessen the effects of inorganic fertilizers on microbial growth. These suggestions have important implications for the management of karst agro-ecosystems.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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