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Dynamics of Enzyme Activities during the Decomposition of *Castanopsis carlesii* Leaf Litter in the Forest Canopy and Forest Floor in a Mid-Subtropical Area

Ling Zhu [1], Wentao Wei [1], Ruobing Wu [1], Xiaoyue Zhang [1], Hongrong Guo [1], Dingyi Wang [1,2,*] and Fuzhong Wu [1,2]

[1] Key Laboratory for Humid Subtropical Eco-Geographical Processes of the Ministry of Education, Institute of Geography, Fujian Normal University, Fuzhou 350007, China
[2] Fujian Sanming Forest Ecosystem National Observation and Research Station, Fujian Normal University, Fuzhou 350007, China
* Correspondence: albertwdy@fjnu.edu.cn; Tel.: +86-159-0667-3147

Abstract: Enzyme activity plays a pivotal role in leaf litter decomposition, but the variations have not been well addressed in the forest canopy with amounts of leaf litter. Therefore, eight enzymes related to carbon, nitrogen, and phosphorus mineralization were checked during *Castanopsis carlesii* leaf litter decomposition in the forest canopy and on the forest floor from April 2021 to February 2022. The results displayed that most enzyme activities were lower in the forest canopy compared to the forest floor during litter decomposition, except for acid phosphatase, polyphenol oxidase, and peroxidase activities. Moreover, enzyme stoichiometry and enzyme vector features indicated that the microbes in both habitats were limited by carbon and phosphorus during litter decomposition. Much stronger carbon limitation was detected on the forest floor, while phosphorus limitation was higher in the forest canopy. Phosphorus limitation was weakened, but carbon limitation was strengthened in the forest canopy with leaf litter decomposition. Additionally, the redundancy analysis revealed that air temperature dominated the variations in enzyme activities during litter decomposition in the forest canopy, and litter mass-loss rate in each period explained much more dynamics on the forest floor compared with those in the forest canopy. These results provide new insight into a comprehensive understanding of litter decomposition in subtropical forests.

Keywords: microbial nutrient limitation; enzyme stoichiometry; forest canopy; forest floor; litter decomposition; enzyme activity

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1. Introduction

A large proportion of leaf litter is often retained in the forest canopy, which makes it an integral part of the material cycling of the forest ecosystem [1,2]. Enzymes can explain nearly 85%–90% of litter degradation in various ecosystems [3], making them vital in leaf litter decomposition [4–6]. Though the dynamic patterns of enzyme activity could provide critical information for litter decomposition in the forest ecosystem [7,8], it is often ignored in the forest canopy.

The forest canopy experiences a colossal temperature change, frequent wet and dry cycles [9], and lower humidity [10] than the forest floor. These harsh conditions could suppress microbial metabolic activity and affect enzyme activities. Moreover, unlike the forest floor, the forest canopy represents arid habitats [11], in which fungi and bacteria differ in density, diversity, and structure, and invertebrate decomposers can be lower [12–14]. Moreover, the nutrient and carbon released from the litter significantly differed in the forest canopy and floor [15,16]. Due to the comprehensive effect of harsh conditions, low nutrition sources [17], and diverse decomposer communities [18], enzyme activities are expected to differ between the forest floor and canopy during leaf litter decomposition.

However, the related processes are unclear, which dramatically limits the understanding of the decomposition process of forest litter.

The enzyme vector model [19,20] and enzyme stoichiometry [21,22] could estimate microbial resource limitations. Environmental facets such as temperature [22], rainfall [23] and moisture, substrate organic matter and nutrient content, and pH [7,24] have been documented to be vital in regulating resource availability during the growth of microorganisms and thus affect microbial resource limitation [25]. The spatial and temporal fluctuations in different forest microenvironments may significantly modulate microbial availability of resources through litter decomposition rates and fluxes of nutrients. For instance, the forest floor is often characterized by frequent microbial–plant interactions and active nutrient flows. On the contrary, the forest canopy generally has relatively oligotrophic conditions, with low nutrient transformation [2,26] and microbial activities [18]. In addition, the microbial community structures differ between the forest canopy and forest floor due to habitat differences, which may affect microbial metabolic patterns. These differences indicated significant differences in the metabolism of microbial communities between the forest canopy and forest floor. Herein, we hypothesized that carbon and nutrient limitation of forest canopy microbial community during litter decomposition was higher than those on the forest floor.

The subtropical region of China, known as "oases" in desert areas of the same latitude on the Earth, covers a large forest area with rich biodiversity [27]. In such ecosystems, litter-trapping litter in the canopy can capture nearly 50% of total litterfall [28] and usually play a disproportionately important role in nutrient cycling [29]. The complex vertical structure of the forest canopy changes the community diversity and affects the environment through biological and nonbiological processes. Enzyme activities may exhibit various patterns due to the sensitivity of different environmental conditions, subsequently regulating litter decomposition and material cycling. Hence, leaf litter was collected from the dominant tree species *Castanopsis carlesii*. The litterbags method was used to investigate the activities of eight enzymes related to nutrition degradation during litter decomposition in the forest canopy and forest floor over one year. We aim to not only elucidate the dynamics of enzyme activities during the decomposition of *Castanopsis carlesii* leaf litter in the forest canopy and forest floor but also to reveal the variations of microbial resource limitation. The results would provide in-depth insight into material cycling and energy flow of the forest ecosystem.

2. Materials and Methods

2.1. Study Region

The experiments were carried out at Sanming Forest Ecosystem National Observation and Research Station (Fujian Province, China, 26°19′ N, 117°36′ E). Low mountains and hills are the primary landscape, with a slope of 25–45° and an average elevation of 300 m. The dominant natural vegetation in this area is subtropical evergreen broadleaved forest. The study area belongs to the maritime subtropical monsoon climate, with annual mean precipitation of 1610 mm and an annual average temperature of 19.3 °C [30]. The rainfall mainly occurs between February and June (Figure 1). The soils were developed from granite and can be classified as Hapludults under the Ultisols order according to the United States Department of Agriculture Soil Taxonomy, with a pH of 4.38 [31,32]. Epiphytes include Araceae, Asclepiadaceae, and Rubiaceae, among which the common species are *Dischidia chinensis*, *Poths chinensis*, and *Microsorium fortune* [33].

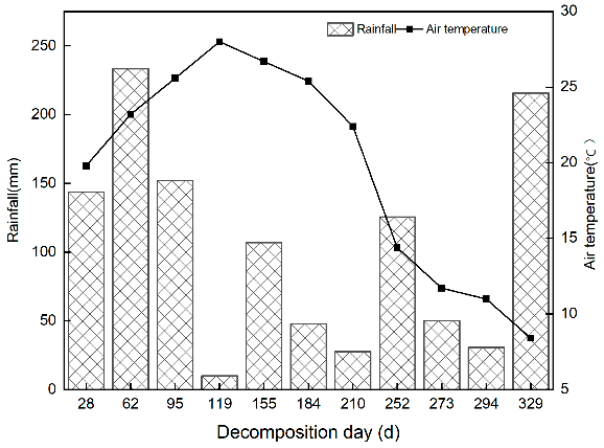

Figure 1. Dynamics of rainfall and temperature in the sampling site from April 2021 to February 2022. The numbers on the x-axis indicate the decomposition time in days after litterbag placement at each sampling time.

2.2. Experimental Design and Field Sampling

In January 2021, freshly senesced *Castanopsis carlesii* leaf litter was collected with litter boxes and then air-dried in the laboratory. Before experimenting, we first surveyed the yearly cumulative amount of litter per unit area and then loaded the required litter leaves weight according to the area of litterbags. According to the calculation, 10 g of air-dried leaf litter was placed in a 20 × 20 cm nylon bag with 1 mm mesh. In each of the three *Castanopsis carlesii* plantation forest plots (about 100 m apart), three 3 m × 3 m homogeneous quadrats were set as repeated plots. On 28 March 2021, litterbags were placed on the forest floor and canopy following standard rules. Firstly, each litterbag should be placed at least a 2 cm interval to avoid mutual disturbance during collection. Secondly, three sampling sites with uniform tree height and diameter at breast height and similar growth states were chosen. Thirdly, litterbags were laid 2–3 m above the ground, which is right in the forest canopy. As a result, a total of 72 litterbags (2 plots × 12 sampling dates (a whole year) × 3 replicates) were prepared in each habitat.

From April 2021 to February 2022, three individual litterbags in each plot were randomly retrieved per month. After retrieval, litter leaves were mixed, placed in plastic ice bags, and transported to the laboratory. The leaf sample was divided into two parts: partially dry at 70 °C for 48 h to measure leaf mass loss and analyze chemical properties; the other part was used to investigate the enzyme activities. At the beginning of the experiment, we set up rainfall buckets and thermohygrometers in the sample plots to measure rainfall and temperature.

2.3. Measurements of Enzyme Activities

In this paper, the enzyme activities related to carbon, nitrogen, and phosphorus cycle were studied. For the C cycle, the enzyme activities of β-1,4-glucosidase (βG), β-D-cellobiosidase (CBH), peroxidase (PER), and polyphenol oxidase (PHO) were evaluated; for the N cycle, the enzyme activities of L-leucine aminopeptidase (LAP), β-1,4-N-acetylglucosaminidase (NAG), and urease (URE) were measured, and acid phosphatase (AP) activity was assessed for the P cycle [34,35]. Briefly, litter samples were chopped into smaller pieces and passed through a 2 mm screen. Phenoloxidase and peroxidase were measured spectrophotometrically using L-dihydroxyphenylalanine (DOPA) as the substrate. For phenoloxidase assays, 0.1 g of litter and 5 mM L-DOPA solution (1.2 mL of acetate buffer for control groups) were mixed in 2 mL centrifuge tubes. For peroxidase activity, samples and controls were prepared similarly, with the addition of 0.6 mL of 0.3% hydrogen peroxide. All reaction mixtures were incubated at 37 °C for 1 h and centrifuged for 2 min at 5000 rpm. Absorbance was measured at 460 nm using a spectrophotometer [36]. Urease activity assays were based on the NH_4^+ released when the

litter homogenization was incubated with 0.5 mL of 10 % urea solution and 1 mL of citric acid buffer (pH 6.7) at 37 °C for 24 h. The released NH_4^+ was determined using the indophenol blue method [37]. Activities of β-D-cellobiosidase, β-1,4-glucosidase, β-1,4-N-acetylglucosaminidase, and acid phosphatase were determined based on p-nitrophenol concentration released when the buffer solution was co-cultured with the corresponding substrate (p-Nitrophenyl-β-D-cellobioside, p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-glucopyranoside, and p-nitrophenol-phosphate, respectively). A total of 0.1 g of litter leaf with buffer solution (pH = 5.5) and substrate was incubated for 1 h at 37 °C; then, 0.5 M $CaCl_2$ solution and 0.5 M NaOH were added into the mixed incubation liquids, followed by being thoroughly shaken and filtered; finally, the paranitrophenol concentration was measured at 410 nm with a spectrophotometer [38–41]. LAP was evaluated by using L-leucine-4-nitroanilide as substrate. The reaction mixture containing 60 μL L-leucine-p-nitroanilide, and 0.1 g leaf litter was incubated at 30 °C for 10 min to measure at 405 nm [42].

2.4. Analytical Methods and Calculations

Here, we used three methods to study microbial resource limitations. The first was based on the method of Hill, ln(LAP + NAG):lnAP as the x-axis and lnβG:ln(LAP + NAG) as the y-axis [43]. This picture can show four different sets of resource limitation, which is based on the deviation from the expected enzyme ratio of C:N (1:1) or N:P (1:1) [44]. In the second approach, an lnβG:ln(NAG + LAP) higher than lnβG:lnAP points toward P limitation or characterizes nitrogen limitation [45]. Vector analysis of enzyme stoichiometry is the third method [46]. The relatively long vector length represents a stronger carbon limitation; the angles <45° and >45° represent the relative degrees of N and P limitation, respectively [44,45]. The vector length and angle were calculated as follows:

$$\text{Vector length} = \sqrt{\left[\frac{\ln \beta G}{\ln(NAG + lAP)}\right]^2 + \left(\frac{\ln \beta G}{lnAP}\right)^2} \quad (1)$$

$$\text{Vector angle } (^\circ) = \text{Degrees}\left(\text{Atan2}\left(\frac{\ln \beta G}{lnAP}\right), \left(\frac{\ln \beta G}{\ln(NAG + LAP)}\right)\right) \quad (2)$$

2.5. Data Analysis

All data were analyzed by software SPSS 26.0 (SPSS Inc., Chicago, IL, USA). Logarithmic transformation of non-normal data to obtain a normal distribution for analysis. The significant difference in enzyme activity in different habitats at the same sampling time was examined by the independent-sample *t*-test. A two-tailed Pearson correlation test was used to analyze the correlation between enzyme activity, vector length and angle, enzyme stoichiometry, environmental factors, and mass-loss rate in each period. In order to further quantify the relative contribution of environmental factors and mass-loss rate on the enzyme activity, vector length, and angle and enzyme stoichiometry, a redundancy analysis (RDA) was used for this purpose. The RDA was measured using CANOCO software (version 5.0, Microcomputer Power, Inc., Ithaca, NY, USA). All the charts were drawn using OriginPro2021 (Origin Lab Corporation) and GraphPad Prism software version 8.

3. Results

3.1. Dynamics of Enzyme Activities during Litter Decomposition

There were significant differences in enzyme activities between the forest canopy and forest floor. Compared with the forest floor, the AP activity was significantly higher in the forest canopy at 119 d and 329 d incubation. AP activities reached a maximum at 155 d incubation at both the forest canopy and forest floor. The activities of βG and NAG in the forest canopy were significantly lower compared with the forest floor. βG activities reached a minimum at 28 d incubation, while NAG activities reached a maximum at 252 d incubation. During the incubation of 62 d and 119 d, the activity of URE in the forest

canopy was significantly higher than those on the forest floor. LAP activities in the forest canopy were markedly higher than that on the forest floor at 119, 294, and 329 days of incubation. The activity of PHO and PER at 252 d and 329 d incubation in the forest canopy was significantly higher compared to the forest floor (Figure 2).

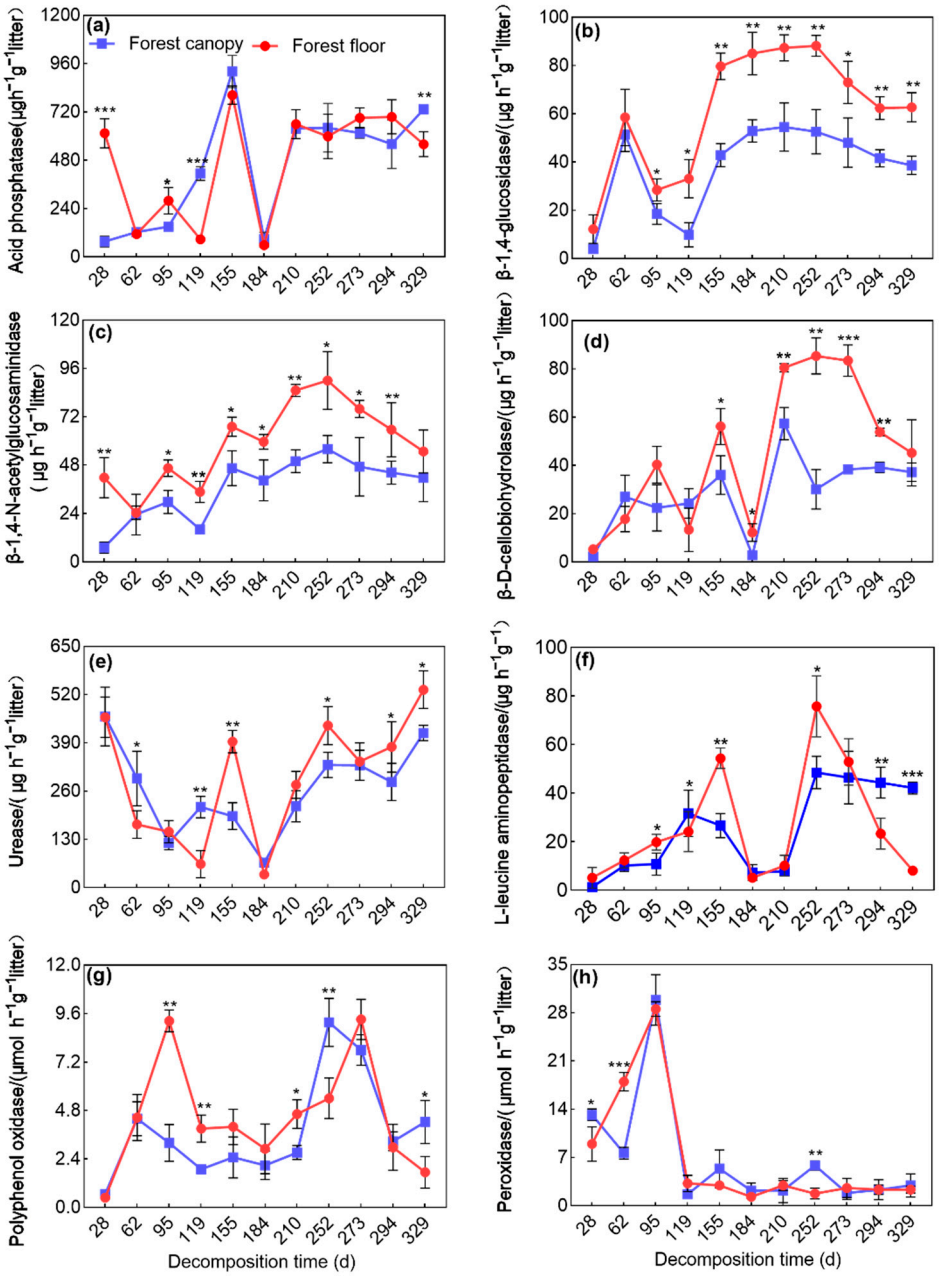

Figure 2. Dynamics of enzyme activities during leaf litter decomposition in the forest canopy and forest floor. (**a**) acid phosphatase activity, (**b**) β-1,4-glucosidase activity, (**c**) β-1,4-N-acetylglucosaminidase activity, (**d**) β-D-cellobiosidase activity, (**e**) urease activity, (**f**) L-leucine aminopeptidase activity, (**g**) polyphenol oxidase activity, (**h**) peroxidase activity. Notes: * indicate significant differences in enzyme activities at each sampling time in different habitats. ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

3.2. Indicators of Microbial Resource Limitation during Litter Decomposition

The lnβG:ln (NAG + LAP), lnβG:ln AP, and ln(NAG + LAP):lnAP ratios deviated significantly from 1, indicating microbial investments in C, N, and P are different between the forest canopy and forest floor. Both forest canopy and forest floor of lnβG:ln(NAG

+ LAP) ratio reached a maximum at 62 d incubation, but the lnβG:lnAP and ln(NAG + LAP):lnAP ratio reached a maximum at 184 d incubation (Figure 3). The lnβG:ln(NAG + LAP) ratio was significantly higher at both the forest canopy and forest floor compared with lnβG:lnAP, which denoted that microbes at the forest canopy and forest floor were limited by phosphorus during litter decomposition. Microbes in the forest canopy and forest floor were limited by phosphorus and carbon during litter decomposition (Figure 4).

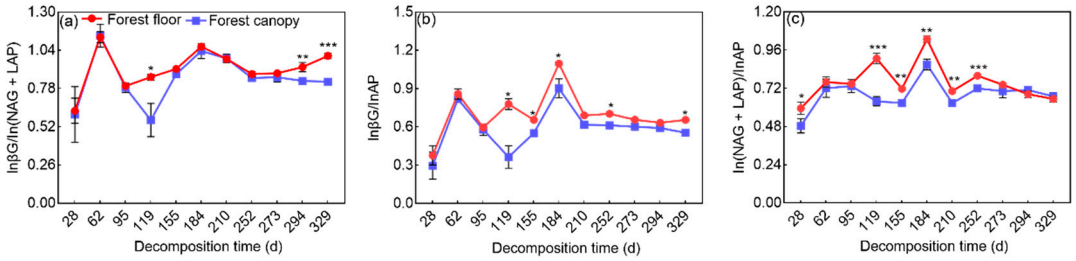

Figure 3. Dynamics of enzyme stoichiometry during the decomposition of leaf litter in the forest canopy and floor. These were the enzyme C:N, C:P and N:P ratios obtained by calculating ln βG/ln LAP + NAG (**a**), ln βG/ln AP (**b**) and ln(LAP + NAG)/ln AP (**c**) activity ratios, respectively. Notes: * indicate significant differences in enzyme stoichiometry at each sampling time in different habitats. ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

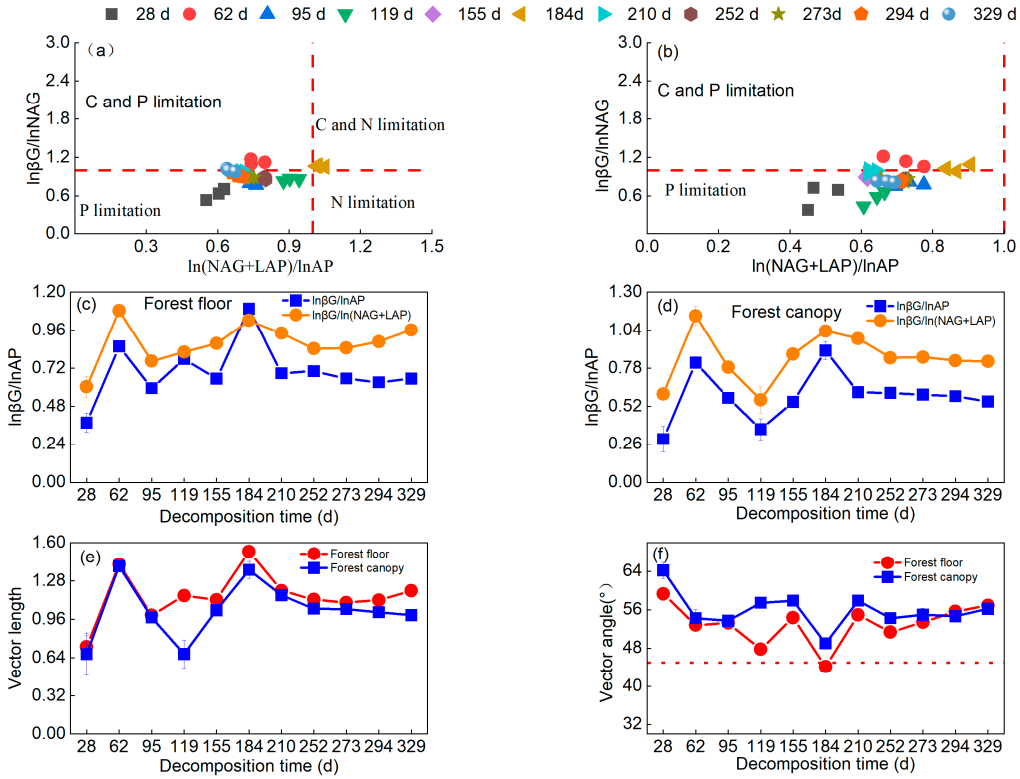

Figure 4. Carbon and nutrient limitation characterized by enzyme stoichiometry of litter leaves in the forest canopy and floor. (**a**,**b**): by using ln(LAP + NAG): lnAP as the x-axis and lnβG:ln(LAP + NAG) as the y-axis four different groups of microbial resource limitations (N limitation, P limitation, C and P limitation and N and P limitation) were categorized;(**c**,**d**): when lnβG:ln(LAP + NAG) is higher than lnβG /lnAP, it denotes P limitation, when smaller it denotes N limitation of microbes; (**e**,**f**):changes of vector length and vector angle were calculated acording to the ratios of the log transformed βG, AP, NAG and LAP. The relatively long vector length represents a stronger carbon limitation; the angles <45° and >45° represent the relative degrees of N and P limitation, respectively.

According to the indicator vector angle concept, microbes were limited by phosphorus in two habitats during litter decomposition; microbes in the forest canopy were more limited by phosphorus than those on the forest floor. Based on the indicators derived from the vector length, microbes were limited by carbon availability in the forest canopy, and the limitation was lower than that on the forest floor.

3.3. Driving Factors for Enzyme Activities during the Decomposition of Leaf Litter

Air temperature strongly affects enzyme activities, enzyme stoichiometry, and enzyme vector features in the forest canopy. Forest floor temperature and litter mass-loss rate in each period significantly affect enzyme activities, enzyme stoichiometry, and enzyme vector features on the forest floor (Figure 5). In the forest canopy, the enzyme activities of LAP were significantly negatively correlated with air temperature, litter mass-loss rate in each period, and litter nitrogen content; AP, URE, LAP, and PHO enzyme activities were significantly negatively correlated with air temperature and litter nitrogen content. We found a significantly negative relationship between the activities of AP and NAG on the forest floor and air temperature, forest floor temperature, and litter mass-loss rate in each period. βG enzyme activities were negatively related to litter carbon, nitrogen, and phosphorus content (Figure 6).

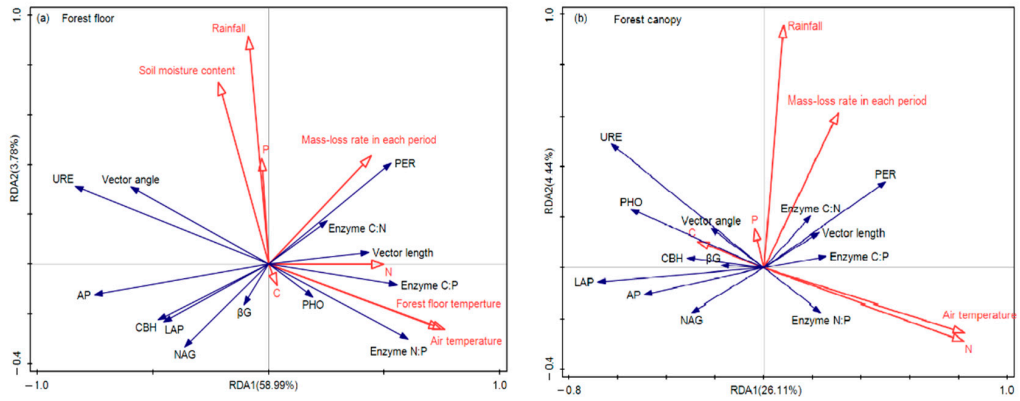

Figure 5. The redundancy analysis (RDA) for enzyme activity, enzyme stoichiometry, enzyme vector features, environmental factors, and litter mass-loss rate in each period in the forest canopy and forest floor. (**a**) Forest floor, (**b**) Forest canopy. βG: β-1,4-glucosidase, CBH: β-D-cellobiosidase, PER: peroxidase, PHO: polyphenol oxidase, LAP: L-leucine aminopeptidase, NAG: β-1,4-N-acetylglucosaminidase, URE: urease, AP: acid phosphatase; Enzyme C:N, lnβG:ln(NAG + LAP); Enzyme C:P, lnβG:ln AP; Enzyme N:P, ln(NAG + LAP):ln AP; C: litter carbon content, N: litter nitrogen content; P: litter phosphorus content.

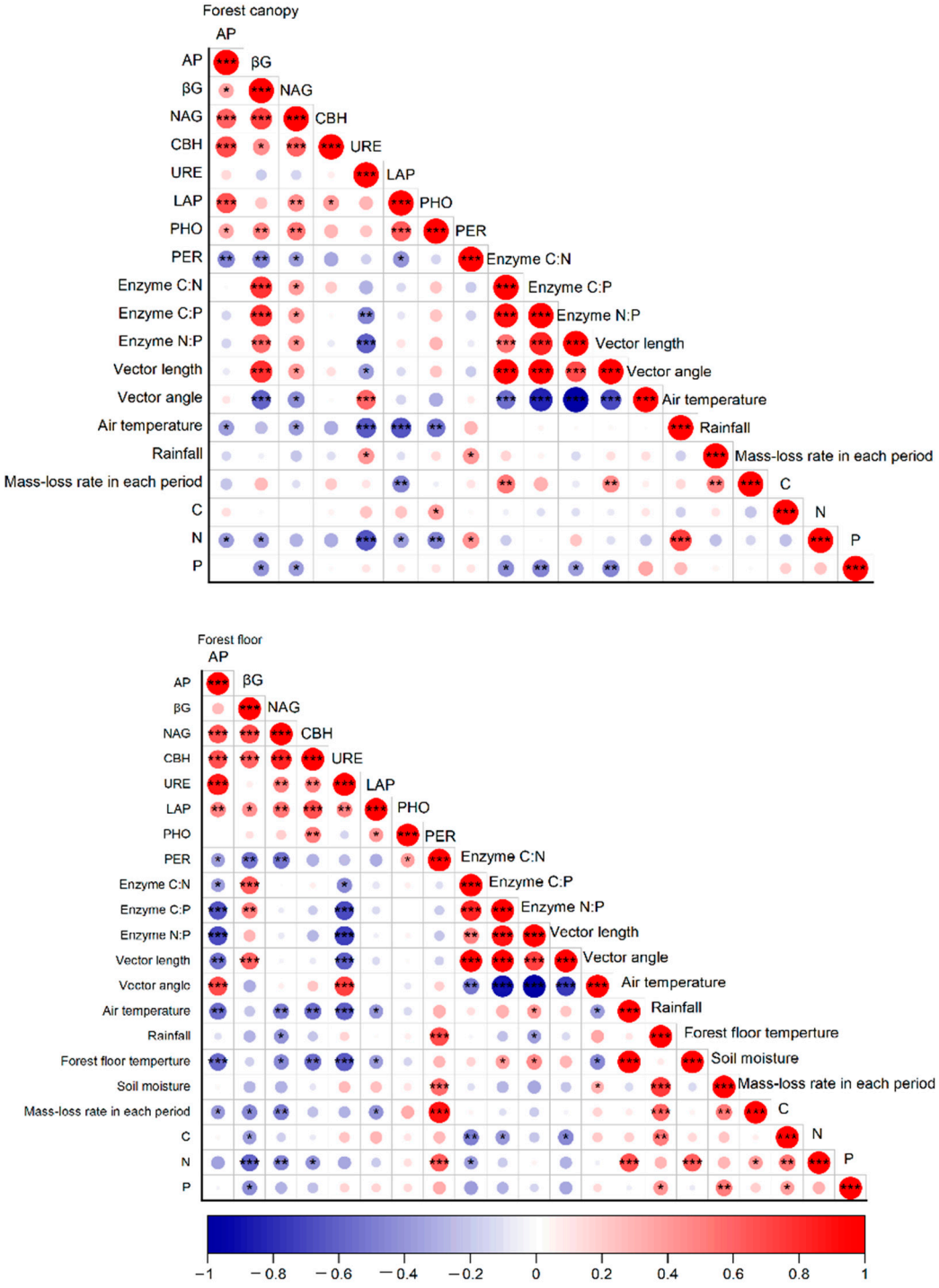

Figure 6. Correlation coefficients among enzyme activity, enzyme stoichiometry, enzyme vector features, environmental factors, and mass-loss rate during leaf litter decomposition in each period in the forest canopy and forest floor. ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

4. Discussion

Microbes in the forest canopy have a higher phosphorus limit compared with the forest floor as our hypothesis. However, microbes on the forest floor are more limited by carbon than forest canopy, which is contrary to our hypothesis. Enzyme activities in different habitats showed different responses to environmental factors (Figure 6), which may lead to further energy and nutrient limits of microbes in different habitats.

Not all enzyme activities in the forest canopy during leaf litter decomposition are lower than the forest floor, and exceptions exist. For example, AP, CBH, URE, and LAP in the forest canopy were higher than those on the forest floor at 119 d incubation. The possible reason may be less rainfall and relatively higher temperature occurred at 119 d incubation. Additionally, the climate condition in the forest canopy is more extreme, and is more vulnerable to wind, sun, and more frequent wet/dry cycles [2]. These harsh conditions may cause microbial death and enzyme release. Meanwhile, AP, LAP, and PHO in the forest canopy were higher than the forest floor at 329 d incubation. Compared with the forest floor, poor nutrient conditions, low nutrient conversion rate, and low microbial activity are the main characteristics of the forest canopy [2,15,17]. At the same time, the study showed that the activity of the tested enzymes was significantly negatively correlated with the litter leaf carbon and nutrient content (Figure 6). With the litter leaf decomposition process, the nutrients and energy in the litter gradually decrease. When the content of nutrients and carbon is low, microorganisms should prioritize spending resources to synthesize related enzymes. Therefore, the enzyme activities of AP, LAP, and PHO in the forest canopy at 329 d incubation were higher than the forest floor.

Scatter plots of enzyme stoichiometry indicated that microbes on the forest floor and forest canopy were limited mainly by phosphorus or carbon, but rarely by nitrogen (Figure 3). In this study, the higher vector length on the forest floor indicates that microbes on the forest floor are subject to greater carbon limitation. When microbes are limited by carbon or the carbon utilization rate is low, it is essential to produce more enzymes to maintain a stoichiometric balance [47]. As a result, the activities of βG, CBH, PER, and PHO on the forest floor are higher than on the forest floor. Compared to the forest floor, phosphorus limitation on microbes is more significant in the forest canopy. This could be attributed to lower phosphorus concentrations in the forest canopy. Compared with the forest floor, the source of phosphorus in the forest canopy is relatively small, mainly from the decomposition of litter leaves and throughfall, while a large part of the phosphorus source on the forest floor comes from rock. A previous study proved that rock contributes 45% of the new phosphorus [48], which is inaccessible in the forest canopy. Additionally, phosphorus usually exists in the organic form in the subtropical regions, and is easily fixed by iron and aluminum substances into complexes, which are difficult to use.

The influencing factors of enzyme stoichiometry in various habitats are different. Previous studies have shown that enzyme stoichiometry is greatly affected by the environment and litter leaf nutrient status [49–51]. This study revealed that soil moisture content strongly affected microbial phosphorus limitation on the forest floor. The result shows that high soil water content can promote microbial metabolism, thereby increasing the microbes' demand for phosphorus sources. In addition, soil moisture changes will affect the transportation and availability of nutrients [52], which will intensify or mitigate the competition for nutrients between plants and microbes in the microenvironment. Litter leaf nitrogen and carbon contents might largely impact enzyme C:N. Our results were consistent with the stoichiometry study of enzyme activity in northern China [53]. Data showed that the microbial carbon limitation of the forest canopy was altered with the change in litter phosphorus content and litter mass-loss rate in each period (Figure 6). This indicated that litter phosphorus content and mass-loss rate in each period are the main factors for microbial nutrient and carbon limitation. Similar results have been found in the enzymatic chemometrics of vegetation restoration in China [54]. The nutrient release of litter in the forest canopy is significantly slower than that in the forest floor [2], which may be one of the reasons why nutrients and energy limit canopy microbes. In addition, water availability in the forest canopy may be another critical factor for microbial carbon and nutrient limitation. The canopy litter is more directly exposed to the sun, and the frequent drying events lower the forest canopy water availability [10]. Water availability would affect the microbial activities in litter leaf, thus affecting the acquisition of microbial carbon and nutrients [55]. Our results stated that the microbial phosphorus limitation was negatively correlated with the microbial carbon limitation, which indicates microbial

communities can maintain stable internal nutrient balance during carbon and nutrient metabolism and is consistent with previous studies [56].

Enzymes are the basic driving force for decomposition [57]. The present study found that enzyme activities and litter mass-loss rate in each period were not well correlated in the forest canopy. However, this result is not entirely surprising. Soluble compounds in litter by leaching can increase mass-loss rate rather than enzyme activity [58]. Many factors, such as litter soluble compounds and polyphenols, can change enzyme activities after the enzymes are produced [59,60]. For example, polyphenols in litter can combine with protein and reduce enzyme activity [61]. Another consideration is that the chemical and physical properties of the litter may have a more significant impact on leaf litter mass loss than enzyme activity [57].

5. Conclusions

In conclusion, the enzyme activity in the decomposition process of the subtropical forest canopy is relatively low compared with the forest floor. According to enzyme stoichiometry, carbon and phosphorus both limited microbial nutrients in two habitats. Furthermore, phosphorus limitation was more robust in the forest canopy, while carbon limitation was lower. Air temperature dominated the changes in enzyme activities. The observations here will provide a new in-depth understanding of litter decomposition and enzyme activities in subtropical forest ecosystems.

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