EFFECT OF NITROGEN ADDITION ON SOIL METABOLISM OF DIFFERENT VEGETATION SUCCESSION STAGES OF KARST ECOSYSTEM IN SOUTHERN CHINA

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Abstract. Nitrogen (N) is a limiting component in the function and stability of karst ecosystems. Different amounts of N additions have been shown to influence the metabolic processes of microbial communities and vegetation. Thus, this study aimed to evaluate how soil metabolism responds to N addition amounts in grassland (GL), shrubland (SL), secondary forest (SF) and primary forest (PF) in karst areas of southern China. Three N addition treatments were applied: no N addition (CN, no N fertilizer), low N addition (LN, 50 kg N ha^{-1} $year^{-1}$), and high N addition (HN, 100 kg N ha^{-1} $year^{-1}$). The results revealed that N addition has a substantial impact on soil metabolite composition and abundance in GL, SL, SF, and PF. Additionally, N addition had distinct impacts on soil metabolism in different vegetation types. N addition significantly reduced several fatty acids in GL, including 9-oxooctadecanoic acid and cis-9,10-epoxystearic acid. N addition to SL resulted in an increase of organoheterocyclic compounds, including clothianidin and gabapentin related compound A. In SF, low N considerably reduced metabolite abundance, while high N dramatically increased metabolite abundance. N addition in PF mainly resulted in the upregulation of soil metabolites. These results provide valuable information for understanding the biochemical impact of N addition in various karst vegetation types.
Keywords: *nitrogen deposition, karst region, soil metabolite composition, metabolomics, vegetation types*

Introduction

Soil nitrogen (N) is one of the most important nutrient elements in ecosystems, influencing ecosystem structure and function. The metabolism of the soil microbiome can be impacted by the direct or indirect interactions of nitrogen with soil (Wang et al., 2018; Zhang et al., 2018). The addition of N significantly increased the concentration of available N in the soil and indirectly altered soil environmental parameters (e.g., soil temperature and humidity), which in turn had a possible impact on soil metabolism (Compton et al., 2004; Weng et al., 2022). Soil metabolites are compounds produced by soil microorganisms, plants and animals as part of their metabolic activities in the soil. These compounds include organic acids, lipids, carbohydrates, fatty acids, and other small molecular compounds (Bhattacharjya et al., 2024). A better overall understanding of the molecular mechanisms associated with environmental cues can be achieved through soil metabolomic profiling (Withers et al., 2020). Previous studies have demonstrated that N addition can influence the metabolic activities of microbes, which in turn influence the production and release of soil metabolites (Weng et al., 2022; Brown et al., 2022). Soil metabolomics has been successful in characterizing close relationships between soil microbiome and metabolome, implying that monitoring soil metabolites can provide an in-depth understanding of metabolic mechanisms for soil microbial community changes (Zhang et al., 2020). However, most studies on the effects of N

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addition have generally focused on changes in the soil microbial community, and few have examined soil metabolites.

Karst landscapes are widely distributed in southern China. Karst is a fragile eco-environment, and its particular geological conditions have influenced the process of vegetation succession (Jiang et al., 2014). N limitation is also one of the important factors in the process of vegetation succession in karst areas. N is one of the key nutrient elements necessary for plant growth, and soil N transformations are likely to change with the vegetation succession (Chu and Grogan, 2010). The primary stage of grasslands in the karst region is N-limited, and N addition has an impact on karst grassland productivity (Zhang et al., 2015; Liu et al., 2018). Previous studies have revealed that N intake influences the soil microorganisms in karst ecosystems (Xiao et al., 2020a, 2020b), but much less attention has been paid to the metabolomic profiling of soils in karst ecosystems. It is unclear how N addition affects the composition and cycling of soil metabolites in the karst region, in particular at the level of individual metabolites. Therefore, studying the effects of exogenous N inputs on soil metabolism in naturally successional karst regions could probe the perturbation of soil metabolism in karst by anthropogenic fertilization. In addition, systematic determination of changes in soil metabolism in response to N addition in various vegetation types will aid in the natural restoration and management of karst degraded ecosystems.

In this study, metabolomic profiling was performed to detect changes in metabolites caused by N addition in karst ecosystems. CH_4N_2O concentrations of 0 kg N ha^{-1} $year^{-1}$, 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$ were applied to natural karst grassland (GL), shrubland (SL), secondary forest (SF) and primary forest (PF), respectively. Soil metabolite profiles were assessed by soil metabolomics studies to determine changes in soil metabolite profiles under four different karst vegetation types following N application. Our hypotheses are that 1) N addition alters the metabolic profile of karst soils, and 2) the magnitude of the impact of N addition on GL, SL, SF and PF will vary among the two N concentrations, with the largest effect expected in GL.

Materials and methods

Site description

This study was conducted in karst ecosystems of GL, SL, SF and PF (*Figure 1*) located in Longzhou County, Chongzuo City, Guangxi, China (22°31′ N, 106°48′ E). The climate is subtropical monsoon, with an average annual temperature of about 20°C-28°C. The soil is calcareous soil.

The soil N addition experiment included three treatments: the control (no N fertilizer, CN), low N addition (50 kg N ha^{-1} $year^{-1}$, LN) and high N addition (100 kg N ha^{-1} $year^{-1}$, HN). There were 9 plots of 2 × 2 m (GL), 5 × 5 m (SL), 10 × 10 m (SF), 10 × 10 m (PF), with 3 replicates of each treatment. Urea (CH_4N_2O) solution was sprayed on the soil of GL, SL, SF and PF in April and July 2023 (i.e. the growing season). To ensure that each vegetation type had the same concentration of N treatment, urea was dose-distributed in each treatment at the following ratios: 4:25:100:100 for GL, SL, SF and PF based on the sample plot area. It was then separately dissolved in 5 L of water and sprayed using a backpack sprayer.

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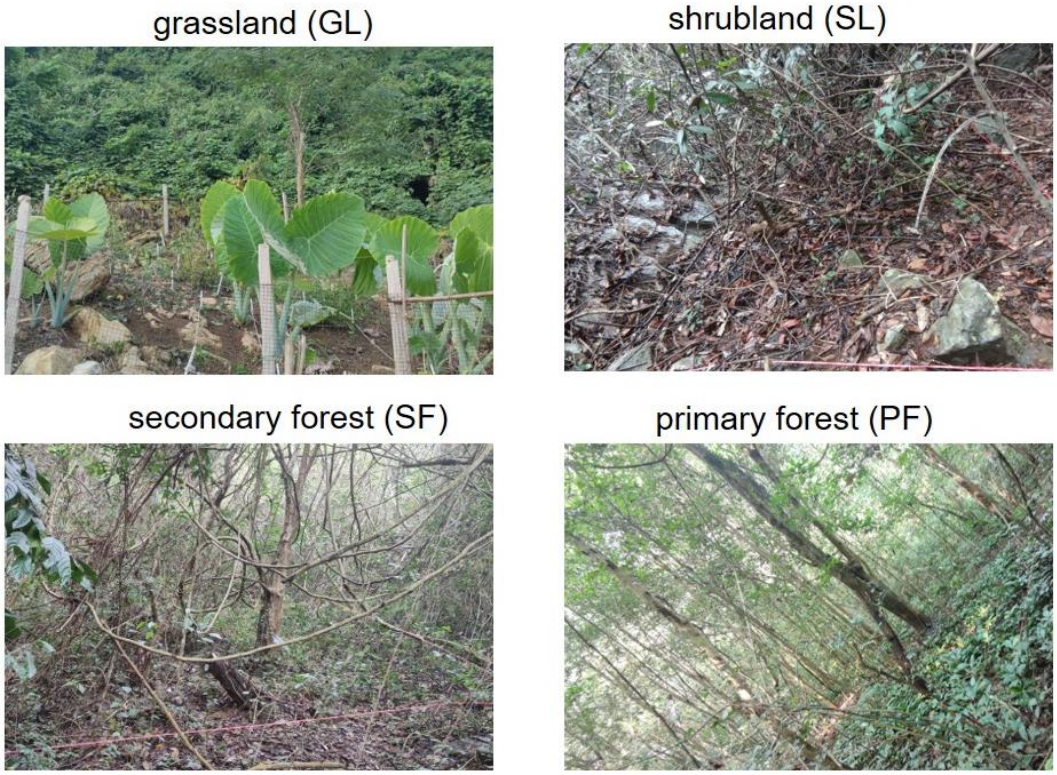

Figure 1. *Experimental habitats of GL, SL, SF and PF located in Longzhou County, Chongzuo City, Guangxi, China*

Soil sampling

In October 2023, soil samples in each plot were collected from the top 0-10 cm of the soil profile. Each soil sample was collected by diagonal sampling method, after removal of apoplastic material, roots and stones, each soil sample was transferred to a 50 ml sterile centrifuge tube and placed in a dry ice bath. The samples were then transferred to the laboratory and stored at -80 °C.

Metabolites extraction

100 mg of sample was transferred to an EP tube. After adding 1000 μL of extract solution (methanol: acetonitrile: H_2O= 2: 2: 1 (v/v), containing isotopically-labelled internal standard mixture), the samples were vortexed for 30 s. This was followed by three repetitions of homogenization in a homogeniser at 35 Hz for 4 min and sonicated in an ice-water bath for 5 min. The samples were then incubated at -40 ℃ for 1 h to precipitate proteins. The sample was then centrifuged at 12000 rpm (RCF=13800(×g), R=8.6 cm) for 15 min at 4 ℃. The resulting supernatant was transferred to a fresh glass vial for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

LC-MS/MS analysis

LC-MS/MS analyses were performed using an UHPLC system (Vanquish, Thermo Fisher Scientific) with a Phenomenex Kinetex C18 (2.1 mm × 50 mm, 2.6 μm) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo). The mobile phase A

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contained 0.01% acetic acid in water, while mobile phase B contained isopropanol and acetonitrile (1:1,v/v). The auto-sampler temperature was 4 ℃, and the injection volume was 2 μL. The Orbitrap Exploris 120 mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate as 50 Arb, Aux gas flow rate as 15 Arb, capillary temperature 320 ℃, full MS resolution as 60000, MS/MS resolution as 15000, collision energy: SNCE 20/30/40, spray voltage as 3.8 kV (positive) or -3.4 kV (negative), respectively.

Data preprocessing and annotation

The raw data was converted to the mzXML format using ProteoWizard and then processed with an in-house program. It was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. The R package and the DB were applied in metabolite identification.

Data analysis

The PLS-DA and OPLS-DA were carried out with SIMCA 18 software. VIP was the weighted sum of OPLS-DA squares, and VIP > 1 and $p < 0.05$ (student's *t*-test) represented significant differences in metabolites.

Results

Soil metabolite profile

In total, 892 individual metabolite compounds were identified across all treatments. These included 181 (20.29%) lipids and lipid-like molecules, 117 (13.12%) organoheterocyclic compounds, 69 (7.74%) fatty acids, 69 (7.74%) benzenoids, 66 (7.4%) shikimates and phenylpropanoids, 63 (7.06%) organic acids and derivatives, 41 (4.6%) phenylpropanoids and polyketides, 36 (4.04%) terpenoids, 35 (3.92%) organic oxygen compounds, and 215 (24.1%) other compounds, including organic nitrogen compounds, alkaloids, carbohydrates, polyketides, amino acids and peptides, etc. (*Figure 2a*).

The multivariate PLS-DA model was applied to obtain general grouping information between the control and N-treated soils. The PLS-DA results for GL, SL, SF and PF showed clear separation between CN, LN, and HN (*Figure 3*). This suggested that the N addition caused significant changes in the metabolite profiles of the karst soils and that these changes may be dependent on the N concentration. As soil metabolites are mainly of microbial origin, this suggested that N addition may alter soil microbial metabolic processes.

Response of soil metabolites to nitrogen addition in four karst vegetation types

In order to more fully extract information on differences in soil metabolites across N addition concentrations, pair-wise comparison analyses were carried out to search for metabolites that differed between CN, LN, and HN. In GL, SL, SF, and PF soils, 94, 48, 56 and 35 metabolites with significant variations were identified, respectively (*Figure 4*).

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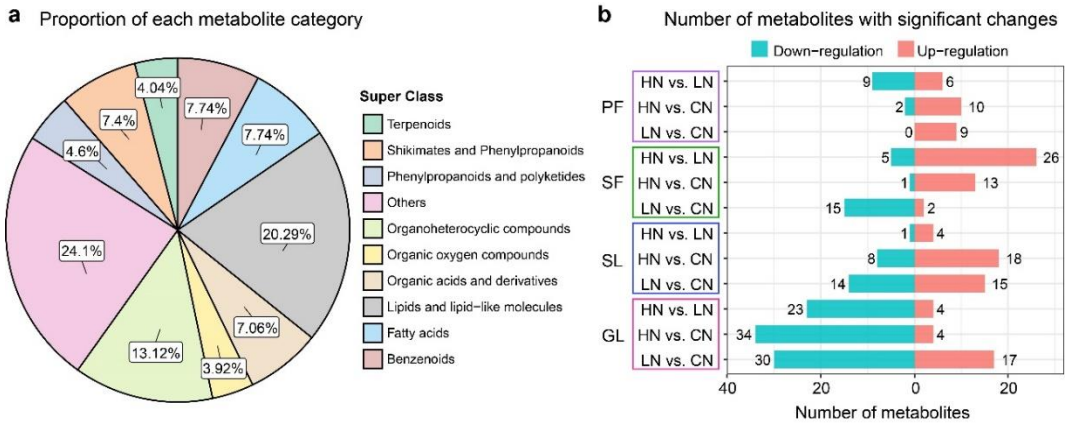

Figure 2. *The soil metabolite profile. (a) Pie plot of identified metabolites in all samples. (b) Summary of the number of compounds significantly different between treatments in GL, SL, SF and PF*

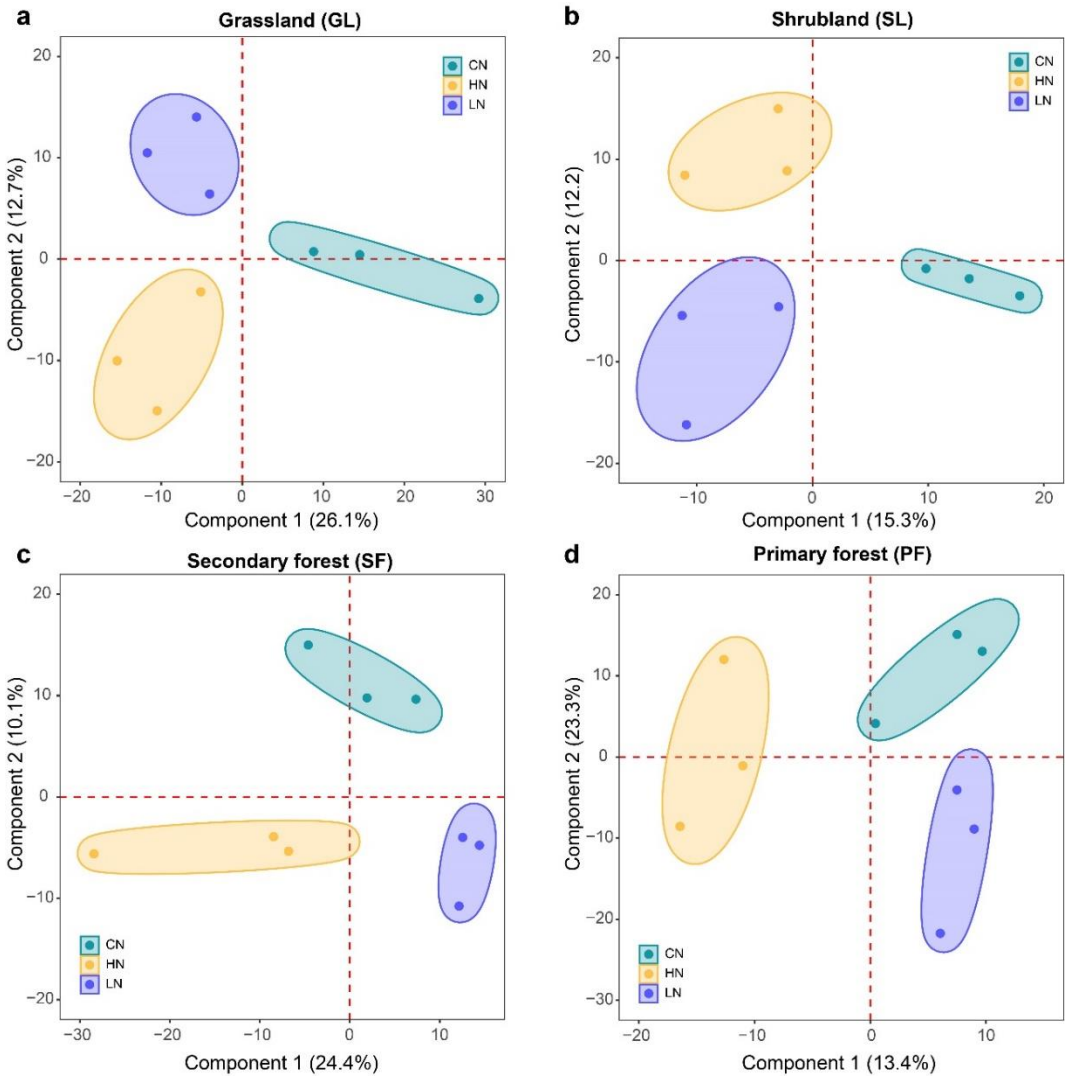

Figure 3. *Score plot (PC1 vs. PC2) of partial least squares-discriminant analysis (PLS-DA) of metabolites in GL (a), SL (b), SF (c) and PF (d)*

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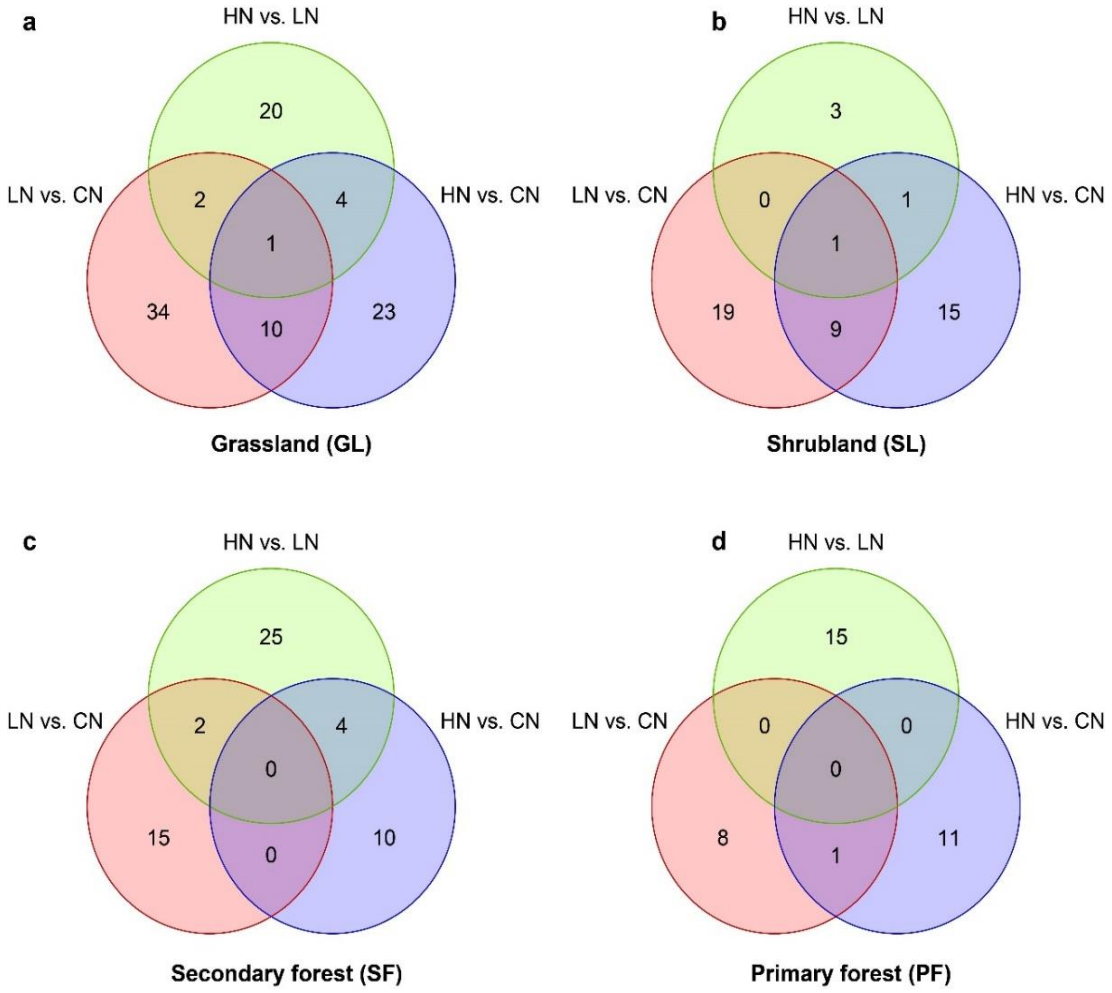

Figure 4. *Venn diagrams showing the overlapping and interconnection between metabolites in GL (a), SL (b), SF (c) and PF (d) at different N additions (CN, LN, HN). Numbers represent the number of metabolites*

Significant differences in composition and abundance of soil microbial metabolites across N concentrations. In GL soils, most of the responsible metabolites decreased with N addition, with the exception of 17 and 4 metabolites that were up-regulated for LN and HN, respectively (*Figure 2b*). In particular, 23 metabolites were down-regulated in HN compared to LN, whereas only 4 were up-regulated in GL (*Figure 2b*). This indicates that N addition had negative impact on the microbe metabolism of GL soil. In addition, these down-regulated metabolites mainly included benzenoids, fatty acids, organoheterocyclic compounds, lipids and lipid-like molecules, organic acids and derivatives, and terpenoids (*Figure 5a*). However, in SL and PF, low and high N additions resulted in an up-regulation of most of the responsible metabolites in comparison CN, especially in PF (*Figure 2b*). In SF, most of the responsible metabolites were down-regulated in the LN and up-regulated in the HN in comparison with the CN (*Figure 2b*).

The magnitude of the effect of N additions on GL, SL, SF and PF varied between the three N concentrations. Most of the responsible metabolites were unique, with only a few changings at both high and low N additions (*Figure 4*). In GL, 11 metabolites were altered by the addition of both low and high N compared to CN, such as allose, preglabridin, and

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allixin (*Figure 4a*, *5a*). Dihydroartemisinic acid, a terpenoids, decreased with the addition of N concentration (*Figure 6a*). It is noteworthy that a number of fatty acids were significantly decreased with N addition in GL soil, of which 9-oxooctadecanoic acid and cis-9,10-epoxystearic acid decreased in both LN and HN (*Figure 6b*, *6c*). In SL, 10 metabolites were changed in both LN and HN compared to CN, such as ganoderiol_G, clothianidin, gabapentin related compound A (*Figure 4b*, *5b*). In PF, only 4-(phenylthio)benzoic acid was altered in both LN and HN compared to CN. In SF, the types of metabolites that changed with the addition of high and low N were completely different.

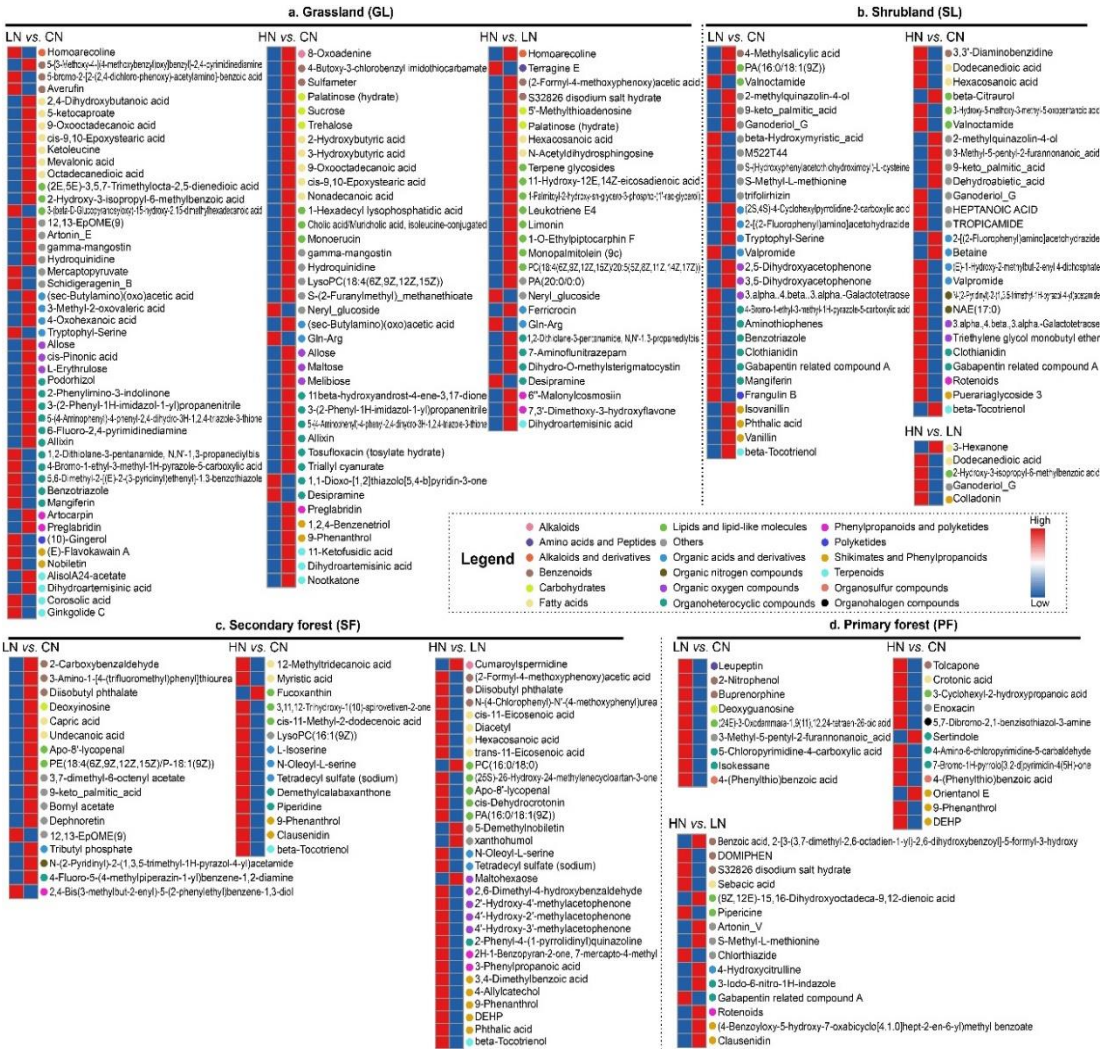

Figure 5. *Summary of metabolite changes between N addition treatments in GL (a), SL (b), SF (c) and PF (d). Red and blue filled cells indicate a significant increase in metabolite content and a significant decrease in metabolite content, respectively*

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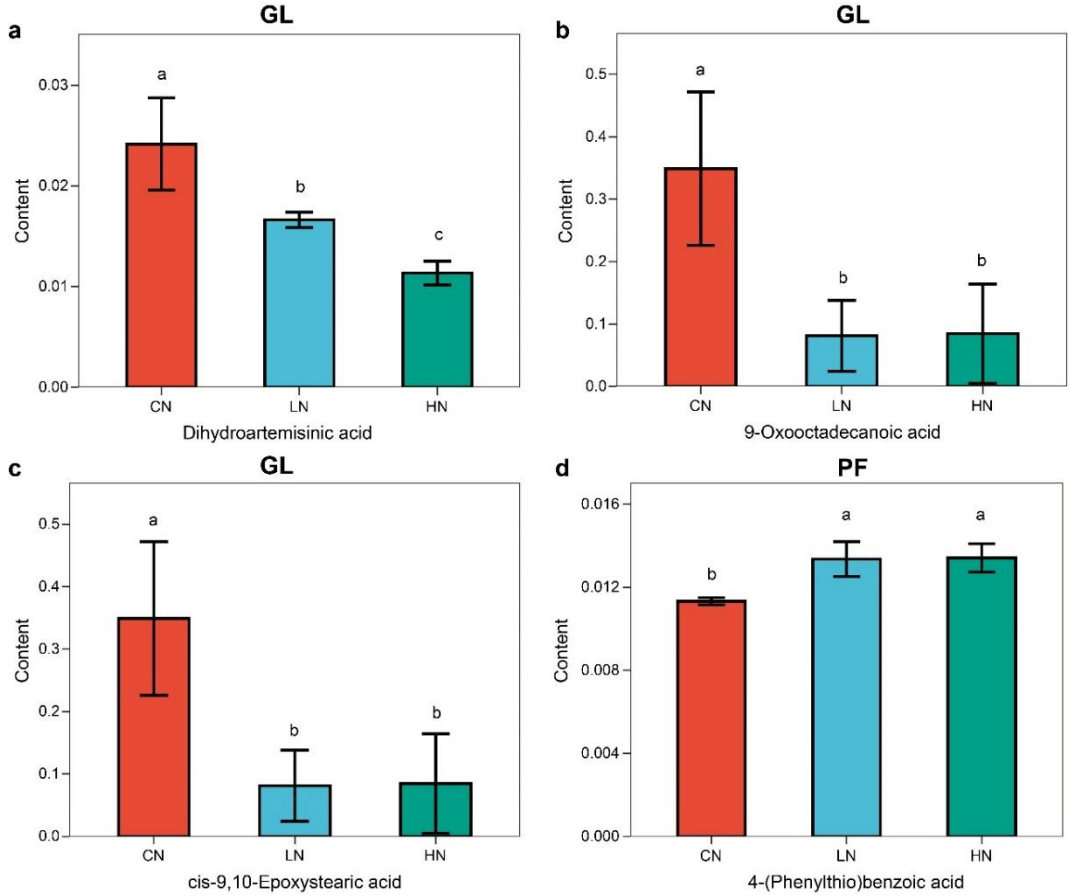

Figure 6. *Response of selected metabolite compounds (dihydroartemisinic acid (a), 9-oxooctadecanoic acid (b), cis-9,10-Epoxystearic acid (c), and 4-(phenylthio) benzoic acid (d)) within the soil in response to N addition. Bars represent mean ± standard error and 3 replicates per treatment. Different letters indicate significant differences between treatment groups (t-test $p < 0.05$)*

Discussion

To date, our knowledge about the effects of N addition on the soil metabolism of karst remains scarce. The N addition experiment in a natural successional karst area can better simulate the effect of anthropogenic fertilization on soil metabolism of karst ecosystems. Exogenous N inputs increase the availability of N in the soil, which affects the metabolic activities of the soil microbial community. The major types of metabolites found in karst soils were lipids and lipid-like molecules, organoheterocyclic compounds, fatty acids, benzenoids, etc. The current study showed that the N addition ultimately has an effect on the composition and content of soil metabolites in karst ecosystems ranging from grasslands to forests. Following N addition, the responsible metabolites of different vegetation types demonstrated the following order compared to CN: GL>SL>SF>PF (*Figure 2b*). Soil metabolic responses to N addition differed by vegetation type, with GL having the most responsible soil metabolites. Different N amounts exhibited varying effects on the soil metabolite profiles. Fang et al. (2014) reported that high N concentrations greatly affected microorganisms' metabolic activity, which may have led

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to soil metabolites. So, alterations in soil metabolism under various N addition conditions may be linked to ecosystem types.

In GL, soil metabolites responded to N addition in the highest numbers. Previous investigations in the karst region have found that N limitation is prevalent in karst grassland soils (Zhang et al., 2015; Liu et al., 2018). Thus, N addition alleviates N constraint and increases soil accessible N content, thereby promoting the functional activity of soil microbial metabolism (Liu et al., 2019). The low N addition reduced the abundance of some fatty acids, organoheterocyclic compounds, organic oxygen compounds, phenylpropanoids and polyketides in grasslands (*Figure 5*). High N levels mostly lowered the content of some carbohydrates, fatty acids, organoheterocyclic compounds, lipids and lipid-like molecules. Previous studies have demonstrated that greater N contents support a higher microbial biomass and may indirectly facilitate an increase of the soil bacteria (Li et al., 2016, 2023). Fatty acids are important for microorganisms to maintain the integrity of cell membranes and cellular functions, and altered fatty acids profile may be a response of microorganisms to direct environmental conditions (Quideau et al., 2016; De Carvalho and Caramujo, 2018). In our study, N addition significantly reduced the content of some fatty acids, such as 2,4-dihydroxybutanoic acid, 9-oxooctadecanoic acid, and cis-9,10-epoxystearic acid (*Figure 6*), which is consistent with previous studies where N deprivation induced storage lipid accumulation (Weng et al., 2014). However, the mechanism of the effect of N addition on microbial metabolic processes in GL soils remains to be further investigated.

In SL, significantly differently abundant metabolites were also screened out. N application significantly increased the levels of organoheterocyclic compounds such as clothianidin and gabapentin related compound A (*Figure 5*). Fifty metabolites were clearly increased even at lower concentrations of N, including aminothiophenes, benzotriazole, and some special materials, such as valnoctamide, valpromide (Radatz et al., 1998). Previous studies have found that N addition led to an increase in organic nitrogen compounds contents (Mooshammer et al., 2014), The results of our study in the addition of high nitrogen showed similar changes, which indicated that there is less requirement for high N use efficiency.

In SF, the addition of low concentrations of N was found to significantly decrease the metabolites content when compared to CN (*Figure 2b*). However, the addition of high concentrations of nitrogen resulted in an increasing trend in metabolite content. The differences in the results of these studies could be attributed to variations in the concentration of N addition. As a result, our findings are comparable with those of Yu et al. (2013), who found that treatment with a high concentration of N improved the metabolic function of soil bacteria in forests whereas low N concentration hindered its activity.

In PF, the response of differential metabolites to N addition tended to be up-regulated in content compared to the CN. N addition significantly increased the 4-(phenylthio) benzoic acid content (*Figure 6*). One possible explanation for the increased the 4-(phenylthio) benzoic acid could be the stimulation of bacterial growth and the decrease of their N demand after the mitigation of soil N limitation by N addition.

In summary, the N addition treatments had a significant effect on the soil metabolism of GL, SL, SF and PF in karst areas, suggesting that increasing N addition rates will always modify the soil properties of the karst environment. Previous studies also found that N addition has a significant impact on soil microbial community structure, accounting for the majority of the variance (Guan et al., 2023; Wang et al., 2023).

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Nevertheless, the metabolomic analysis method can only reflect changes in metabolite type and content, and so cannot fully represent soil microbial functional diversity. Therefore, this approach must be combined with high-throughput sequencing technology to better characterize the variations of microbial functional diversity. In addition, N addition treatments modified the nutrient storage of the soil. Therefore, there is a need for further study of the changes in soil properties and their profound effects on microbial communities and soil metabolism in karst ecosystems.

Conclusions

We conducted an N addition experiment in a karst area with GL, SL, SF and PF located on the southern China. We measured the soil metabolite contents and assessed metabolic response of soil to N addition. The N additions had different effects on soil metabolites profiles, depending on the vegetation type. From GL to PF, there was significant difference in the soil metabolism content at different N concentration. Specifically, GL was more sensitive to N addition. Overall, the detected changes in soil metabolite profiles in this study furnished a new understanding for metabolic activities of karst ecosystems with N addition and provided a theoretical basis for future protection and construction of karst areas.

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