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Nitrogen-Fixing Plants Enhance Soil Phosphorus Availability by Promoting Transformations Among Phosphorus Fractions in a Subtropical Karst Forest

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Abstract: Nitrogen (N)-fixing plants are commonly employed in the restoration of degraded terrestrial ecosystems due to their ability to increase soil N capital and boost ecosystem productivity. Given the close coupling between N and phosphorus (P) in soil, the effects of N-fixing plants on soil P fractions and availability in karst forests remain largely unexplored. Herein, we compared soil P pools, fractions, and availability in the rhizosphere and non-rhizosphere soils of N-fixing and non-N-fixing plants, and explored associated drivers, such as soil, microbial, and plant properties, in a subtropical karst forest. The results showed that the N-fixing plants increased total P, inorganic P, and available P in both the rhizosphere and non-rhizosphere soils. The nitrogen-fixing plants increased soil labile P (LP) and non-labile P (NLP), but decreased moderately labile P (MLP), particularly in the rhizosphere soils, due to transformations among different soil P fractions. Soil P fractions were primarily influenced by soil inorganic P, root and leaf N, and microbial biomass N in the N-fixing plant treatment, whereas soil inorganic P, dissolved organic carbon (DOC), and dissolved organic N (DON) were the key factors in the non-N-fixing plant treatment. Consequently, soil properties, microbial attributes, plant nutrients, and soil P fractions collectively exerted both direct and indirect effects to increase soil P availability in the N-fixing plant treatment. In contrast, soil P fractions directly and soil properties indirectly influenced soil P availability in the non-N-fixing plant treatment. Our results revealed the unique role of N-fixing plants in driving soil P availability in subtropical karst forests. These findings are essential for developing effective strategies for P nutrient management and guiding the selection of appropriate plant species for vegetation restoration in karst regions.

Keywords: karst forest; nitrogen-fixing plant; rhizosphere soil; soil phosphorus fraction; soil phosphorus availability

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1. Introduction

Phosphorus (P) is an essential nutrient for plants and is closely coupled with carbon (C) and nitrogen (N) in biogeochemical cycles [1], thereby maintaining the stability of

structures, functions, and services in terrestrial ecosystems [2,3]. Although the soil is reported to be the largest P pool in terrestrial ecosystems [4], soil P availability is often a major limiting factor for plant production in many ecosystems [1,5,6]. This limitation is especially pronounced in karst ecosystems [7–9], where P deficiency severely constrains vegetation restoration and ecosystem stability [9,10]. Studies have indicated that soil P dynamics and availability are closely linked to climate, topography, soil biochemical processes, and vegetation [3,4,11,12]. Additionally, soil P variations and availability are significantly influenced by global climate change, including rising atmospheric carbon dioxide (CO_2) levels, increased air temperature, and nitrogen deposition in terrestrial ecosystems [13–16]. Furthermore, soil P fractions, including labile P (LP), moderately labile P (MLP), and non-labile P (NLP), play crucial roles in regulating soil P dynamics and availability [17–19]. Although several studies have examined how these factors, individually or in combination, influence soil P cycling and availability in forest and agricultural soils [3,8,9,20,21], limited research has been conducted in karst ecosystems. Therefore, understanding how soil P fractions and availability change, along with the factors that influence them, is essential for the sustainable management of P and the maintenance of ecosystem stability in karst areas.

Karst landforms are among the dominant geomorphological types globally, covering over one-third of China's land area, predominantly distributed across southwestern regions such as Yunnan, Guangxi, and Guizhou provinces [22,23]. However, the ecological environment of karst ecosystems in this region is extremely fragile [22,24], making it crucial to the ecological security of southwest China and the entire country [23,24]. Although a series of environmental protection measures have been implemented since the mid-1980s, the restoration of vegetation in previously degraded forest ecosystems has been slow due to shallow soil layers, limited nutrient pools, and a weak nutrient supply rate [24]. For example, studies have shown that vegetation restoration in karst areas of Guangxi and Guizhou provinces is significantly influenced by soil N and P availability and their respective cycling processes [9,10]. In addition, soil erosion occurs frequently due to the high rainfall in this region, causing soil P to be easily lost through surface runoff and leaching [25]. Moreover, the increase in N deposition, atmospheric CO_2 concentration, and global warming has significantly promoted plant growth, thereby intensifying plant demand for P, which exacerbates soil P deficiency [13,15,16]. Therefore, exploring the status of soil P in karst forest ecosystems and implementing appropriate measures to improve soil P availability may accelerate the process of vegetation restoration in the southwestern karst regions.

Nitrogen-fixing plants, such as *Albizia odoratissima*, *Dalbergia hupeana*, *Acrocarpus fraxinifolius*, *Cajanus cajan*, *Bauhinia brachycarpa*, *Sophora davidii*, *Lespedeza juncea*, *Trifolium repens*, *Campylotropis yunnanensis*, and *Indigofera mengtzeana*, are widely distributed in tropical and subtropical karst forests [9,26–29]. Studies have shown that N-fixing plants can enhance N capital and alleviate N limitation [30,31], thereby promoting the growth of non-N-fixing plants and enhancing ecosystem productivity [32–34]. Consequently, these plants are expected to be widely utilized for vegetation restoration in degraded karst ecosystems. For example, studies have reported that planting N_2-fixing plant species, such as *Dalbergia odorifera*, *Acrocarpus fraxinifolius*, *Acacia mangium,* and *Acacia auriculiformis*, can enhance nutrient accumulation and benefit vegetation restoration in a degraded karst region of subtropical China [9,35]. Given the close coupling relationship between N and P nutrients, the increase in soil N levels induced by N-fixing plants can significantly influence the acquisition of soil P by plants and various soil P cycling processes [26,36]. A few studies have demonstrated that N-fixing trees can significantly enhance soil P availability in karst regions [9,26]. Research in tropical forests has shown that with the increase in the

N-fixation rate of N-fixing plants, soil P concentrations have significantly increased [36,37]. It is suspected that the improvement in soil P availability by N-fixing plants may be due to their stimulation of soil microorganisms and enzyme activities [9,37]. Specifically, N-fixing plants improve soil N availability, which can increase soil microbial biomass, including bacteria, fungi, and actinomycetes, and regulate soil microbial communities and functions, thus enhancing phosphatase activity and the secretion of organic acids and facilitating soil P cycling [9,30,33,34]. Meanwhile, plants may release chemicals into the soil through root exudates, shaping the soil microenvironment and thereby building the rhizosphere microbial community structure, which may play a crucial role in soil nutrient cycling [38,39]. However, the specific underlying mechanisms of enhanced soil P availability involved in the transformation of different soil P fractions driven by N-fixing plants, remain unclear. Consequently, a comprehensive investigation into the effects of N-fixing plants on soil P transformation and availability will provide novel insights for understanding soil nutrient cycling processes and constructing effective vegetation restoration models in karst ecosystems.

The subtropical karst forest in Yunnan province, located in southwest China, represents a typical karst fault basin landform. Due to the significant elevation difference between the plateau and the basin, the vertical development of the karst landform is pronounced, resulting in high vulnerability to severe soil erosion and poor site conditions [40]. The natural vegetation restoration in these rocky desertification areas progresses very slowly owing to the constraints imposed by geological and climatic factors [41,42]. Following years of artificial vegetation restoration involving mixed afforestation with *Dodonaea viscosa* Jacquem., *Pinus yunnanensis* Franch., and other species [43], the vegetation community now exhibits varied structure and growth status, with N-fixing plants being widely distributed [27,44]. This provides an ideal platform to understand the differential response of soil P dynamics to N-fixing versus non-N-fixing plants. In this study, we investigated soil P pools, fractions, and availability in both rhizosphere and non-rhizosphere soils of N-fixing and non-N-fixing plants, and explored the associated drivers (e.g., soil properties, microbial attributes, soil enzyme activity, plant nutrients) to elucidate the regulatory mechanisms by which N-fixing plants influence soil P dynamics in karst forests. Thus, we hypothesized that: (1) N-fixing and non-N-fixing plants would differently affect soil P fractions, including LP, MLP, and NLP fractions, in subtropical karst forests; and (2) N-fixing plants would enhance soil P availability through the transformation of soil P fractions driven by increased soil N and an increase in microbial and enzyme activities.

2. Materials and Methods

2.1. Study Site

This study was conducted at the Jianshui Research Station in Jianshui County of Honghe Hani and Yi Autonomous Prefecture, Yunnan Province, southwest China (23°36′–23°43′ N, 102°54′–102°56′ E, 1350–1700 m above sea level, Figure S1). The region experiences a typical subtropical monsoon climate, with an average annual temperature of 18.5 °C, average annual precipitation of 805 mm, and an average annual evaporation rate of 2230 mm [43]. There are distinct wet and dry seasons, with the wet season occurring from May to October, during which over 80% of the annual precipitation occurs, and the dry season from November to April, characterized by lower precipitation. The soil is classified as Calcareous Cambisols based on the FAO soil classification system [45]. The karst landscape in Jianshui County covers approximately 2578 km^2, making up 68% of the county's total area, while the area affected by rocky desertification is about 417 km^2. Since the 1980s, afforestation projects with different tree species have been extensively implemented in this area to restore rocky desertification lands. After nearly forty years of

afforestation, the vegetation community exhibits varied successional stages, ranging from grass-shrub to woody vegetation, depending on topographical and edaphic conditions.

2.2. Experimental Design and Sampling Methods

In August 2023, three representative mixed forest sites were selected in the study area (Figure S1), with consideration for both terrain and species composition. A 20 m × 20 m sample plot was randomly established in each site based on the common practices in forest ecology research. The plant community composition in each plot was investigated using the typical plot survey method, identifying the dominant N-fixing and non-N-fixing plants. Species were selected based on the following criteria: they needed to be present at all three sites and have at least five individuals in each plot. Finally, eight leguminous N-fixing plants were chosen, including *Bauhinia brachycarpa*, *Sophora davidii*, *Dalbergia hupeana*, *Lespedeza juncea*, *Indigofera pseudotinctoria*, *Indigofera mengtzeana*, *Phanera calciphila*, and *Campylotropis yunnanensis*, and five non-N-fixing plants were chosen, including *Fraxinus malacophylla*, *Dodonaea viscosa*, *Pistacia chinensis*, *Pinus massoniana*, and *Rhus chinensis* (Figure S2). Rhizosphere and non-rhizosphere soils were collected from three comparable individuals of each species in each plot. Specifically, the roots of each plant were carefully excavated, and the bulk soil loosely adhering to the root surface was shaken off and collected as non-rhizosphere soil samples. The soil still adhering to the root surface was brushed off and collected as rhizosphere soil samples. The collected soil samples from each plot were composited and placed in plastic ziplock bags and kept cool (<4 °C) in a portable ice box during transport to the laboratory. Simultaneously, the leaves and roots of selected plants were collected from each plot and composited for the same plant species. Upon arrival at the laboratory, visible plant litter or detritus was removed from all soil samples and the samples were then passed through a 2 mm sieve and divided into two portions. One portion was stored in a refrigerator at 4 °C for the determination of soil-dissolved organic C (DOC), dissolved N (DN), NH_4^+-N, NO_3^--N, and microbial properties, while the other was air-dried at room temperature for the determination of other soil physicochemical properties and P fractions. The leaf and root samples were washed with distilled water, oven-dried at 105 °C for 30 min, and then dried at 65 °C until a constant weight was reached. The samples were then ground and passed through a 0.15 mm sieve for total C, N, and P analyses.

2.3. Analyses of Soil and Plant Samples

Soil pH was determined by measuring air-dried soil samples with a soil-to-water ratio of 1:2.5 (w/v) using a pH meter (PHS-3C, Leici Instruments Ltd., Shanghai, China). Soil organic carbon (SOC) and total nitrogen (TN) concentrations were analyzed with an elemental analyzer (vario EL cube, Elementar Analysensysteme GmbH, Hamburg, Germany) after air-dried soil samples were ground with a ball mill to pass through a 0.15 mm sieve, followed by the removal of soil inorganic carbon using 1 mol L^{-1} HCl. Soil dissolved organic carbon (DOC) and dissolved nitrogen (DN) concentrations were measured using a TOC analyzer (Multi N/C 3100, Analytik Jena GmbH, Jena, Germany) after fresh soil samples were extracted with 0.5 mol L^{-1} K_2SO_4 and filtered through a 0.45 μm mesh. Soil NH_4^+-N and NO_3^--N concentrations were determined using a continuous flow analyzer (AA3, SEAL Analytical Ltd., Watford, UK) after fresh soil samples were extracted with 2 mol L^{-1} KCl at a soil-to-solution ratio of 1:5 (w/v). The concentrations of total Ca, iron oxide (Fe_2O_3), and alumina (Al_2O_3) were determined by inductively coupled plasma optical emission spectroscopy (Optima 7000 DV, PerkinElmer, Waltham, MA, USA). Soil total P (TP) and available P (AP) concentrations were determined by the colorimetric method using a UV–Vis spectrophotometer (Cary 300, Agilent Technologies Inc., Santa Clara, CA, USA) after air-dried soil samples were digested with H_2SO_4-$HClO_4$ for TP

or extracted with 0.5 mol·L^{-1} $NaHCO_3$ for AP. Soil organic P (OP) concentration was measured as the difference in extractable phosphorus between burnt and unburnt soils at 550 °C in a Muffle furnace. Soil inorganic P (IP) concentration was calculated as the difference between TP and OP. All soil properties were presented on an oven-dry basis.

The concentrations of soil microbial biomass C (MBC), microbial biomass N (MBN), and microbial biomass P (MBP) were determined using the chloroform fumigation-extraction method [46–48]. The MBC and MBN in the extracts were analyzed with a TOC analyzer (Multi N/C 3100, Analytik Jena GmbH), while the MBP was measured with a UV–Vis spectrophotometer (Cary 300, Agilent Technologies Inc.). The activity of soil alkaline phosphatase (ALP), an extracellular enzyme responsible for P acquisition, was measured by the fluorescent microplate method [49]. Enzyme activity was expressed as nmol of fluorescence per gram of dry soil per hour.

The TC and TN contents in leaves and roots were analyzed with an elemental analyzer (vario EL cube, Elementar Analysensysteme GmbH). The TP content in leaves and roots was determined by the colorimetric method using a UV–Vis spectrophotometer (Cary 300, Agilent Technologies Inc.) after the sample was digested with H_2SO_4-H_2O_2.

2.4. Soil P Fraction Measurement

Soil P fractions were analyzed using a modified sequential extraction procedure, as outlined by Tiessen and Moir [50], which is based on the technique developed by Hedley et al. [51]. Briefly, different chemical extraction agents, including deionized water (containing anion exchange resin membrane strips), 0.5 mol·L^{-1} $NaHCO_3$ (pH = 8.5), 0.1 mol·L^{-1} NaOH, 1 mol·L^{-1} HCl, and concentrated HCl, were sequentially used to separate various soil inorganic phosphorus (Pi) fractions. The persulfate oxidation method was then employed to determine the total phosphorus (Pt) fraction in each extract, with the difference between Pt and Pi representing the corresponding organic phosphorus (Po) fraction. The Pi and Pt fractions in each extract were measured by the colorimetric method using a UV–Vis spectrophotometer (Cary 300, Agilent Technologies Inc., USA). Throughout the extraction procedure, a total of nine P fractions were obtained, including Resin-P, $NaHCO_3$-Pi, $NaHCO_3$-Po, NaOH-Pi, NaOH-Po, D-HCl-Pi, C-HCl-Pi, C-HCl-Po, and Residual-P. According to previous studies [3,17,51,52], Resin-P and $NaHCO_3$-P are LP, NaOH-P, and D-HCl-P are MLP, and C-HCl-P and Residual-P are NLP.

2.5. Statistical Analyses

A one-way analysis of variance (ANOVA) was employed to evaluate significant differences in soil pH, SOC, TN, DOC, DN, NH_4^+-N, NO_3^--N, Ca, Fe_2O_3, and Al_2O_3 between rhizosphere and non-rhizosphere soils of N-fixing and non-N-fixing plants. Tukey's multiple comparison test was used as the post hoc test to detect treatment effects at an α value of 0.05. A two-way ANOVA was performed to examine the effects of rhizosphere soil, N-fixing plants, and their interaction on soil P pools, LP, MLP, NLP fractions, microbial biomass, and ALP activity. A *t*-test was utilized to determine significant differences in each soil P fraction of rhizosphere and non-rhizosphere soils, as well as C, N, and P contents in plant leaves and roots between N-fixing and non-N-fixing plants. Variation partitioning analysis (VPA) was conducted to evaluate the contribution of soil properties, microbial parameters, and plant nutrients to soil P fractions using the "vegan" package in R. To identify the primary factors among soil properties, microbial parameters, and plant nutrients affecting soil P fractions, a redundancy analysis (RDA) was conducted by the "ade4" package in R using soil P fraction contents as response variables. Multicollinearity among explanatory variables was assessed using the variance inflation factor (VIF) in a regression model. Variables with strong correlations (VIF > 10) were removed, and further RDA analysis was conducted with

the remaining variables [53]. Based on known and potential relationships (e.g., the primary factors identified in the RDA), structural equation models (SEMs) were constructed to elucidate the direct and indirect effects of plant, microbial, and soil variables, and soil P fractions on P availability under the influence of N-fixing and non-N-fixing plants, using Amos 26.0 (IBM Corp., Armonk, NY, USA). All statistical analyses were performed using R software v4.2.1 [54].

3. Results

3.1. Soil Properties

The N-fixing and non-N-fixing plants differently affected the soil properties of both the rhizosphere and non-rhizosphere soils (Table 1). Concentrations of SOC, TN, and NO_3^--N were significantly higher in the rhizosphere soils, while soil Ca concentrations were significantly lower in the N-fixing plants compared to the non-N-fixing plants ($p < 0.05$). No significant differences were observed in soil pH, or the concentrations of soil DOC, DN, NH_4^+-N, Fe_2O_3, and Al_2O_3 between the N-fixing and non-N-fixing plants. In the non-rhizosphere soils, in addition to higher concentrations of SOC, TN, and NO_3^--N, soil pH was also significantly higher in the N-fixing plants than in the non-N-fixing plants ($p < 0.05$). Conversely, soil DOC and Ca concentrations were significantly lower in the N-fixing plants than in the non-N-fixing plants ($p < 0.05$). Concentrations of soil DN, NH_4^+-N, Fe_2O_3, and Al_2O_3 did not differ between the N-fixing and non-N-fixing plants. In the N-fixing plants, soil pH and Ca concentrations were lower, and soil DOC and DN concentrations were higher in the rhizosphere soils than those in the non-rhizosphere soils ($p < 0.05$). In the non-N-fixing plants, except for significantly higher soil DOC and DN concentrations and significantly lower soil Ca concentration in the rhizosphere soils than in the non-rhizosphere soils ($p < 0.05$), there were no significant differences in other soil properties between the rhizosphere and non-rhizosphere soils.

Table 1. Effects of vegetation restoration duration on soil properties in the 0–10 cm soil layer in karst regions of southwest China.

Parameter	Rhizosphere Soil		Non-Rhizosphere Soil	
	N-Fixing Plant	Non-N-Fixing Plant	N-Fixing Plant	Non-N-Fixing Plant
pH	6.6 ± 0.1 b	6.5 ± 0.1 b	6.8 ± 0.1 a	6.5 ± 0.0 b
SOC (g kg^{-1})	48.3 ± 2.6 a	39.7 ± 2.0 b	45.5 ± 3.3 a	33.6 ± 1.3 b
TN (g kg^{-1})	3.3 ± 0.3 a	2.5 ± 0.3 bc	3.1 ± 0.2 ab	2.1 ± 0.2 c
DOC (mg kg^{-1})	131.4 ± 7.3 ab	137.5 ± 7.8 a	95.9 ± 4.7 c	124.3 ± 8.6 b
DN (mg kg^{-1})	39.3 ± 3.8 a	34.9 ± 4.9 ab	30.6 ± 2.8 bc	28.7 ± 0.7 c
NH_4^+-N (mg kg^{-1})	7.9 ± 1.9 a	9.8 ± 3.3 a	6.5 ± 1.3 a	9.3 ± 1.9 a
NO_3^--N (mg kg^{-1})	13.0 ± 3.3 a	4.4 ± 1.6 b	9.9 ± 2.6 a	4.4 ± 1.9 b
Ca (g kg^{-1})	7.0 ± 0.5 c	8.8 ± 0.7 b	10.4 ± 0.8 b	12.2 ± 0.4 a
Fe_2O_3 (g kg^{-1})	166.0 ± 2.0 a	171.2 ± 2.8 a	168.9 ± 2.0 a	172.5 ± 2.5 a
Al_2O_3 (g kg^{-1})	277.7 ± 3.3 a	281.3 ± 5.5 a	274.8 ± 2.9 a	285.1 ± 4.4 a

Notes: Data in the table are mean ± standard error of the means. Different lowercase letters in the same row indicate significant differences among treatments for each soil parameter ($p < 0.05$). SOC, soil organic carbon; TN, total nitrogen; DOC, dissolved organic carbon; DN, dissolved nitrogen.

3.2. Soil P Pools and Fractions

The effects of N-fixing and non-N-fixing plants on soil P pools differed for both rhizosphere and non-rhizosphere soils (Figure 1). The N-fixing plant had significantly higher TP, IP, and AP concentrations in both rhizosphere and non-rhizosphere soils compared to the non-N-fixing plant, while it did not affect soil OP concentrations ($p < 0.05$). For the N-fixing plant, soil OP and AP concentrations were significantly higher in the rhizosphere

soil compared to the non-rhizosphere soil ($p < 0.05$). In contrast, for the non-N-fixing plant, soil IP and AP concentrations were significantly higher in the rhizosphere soil compared to the non-rhizosphere soil ($p < 0.05$).

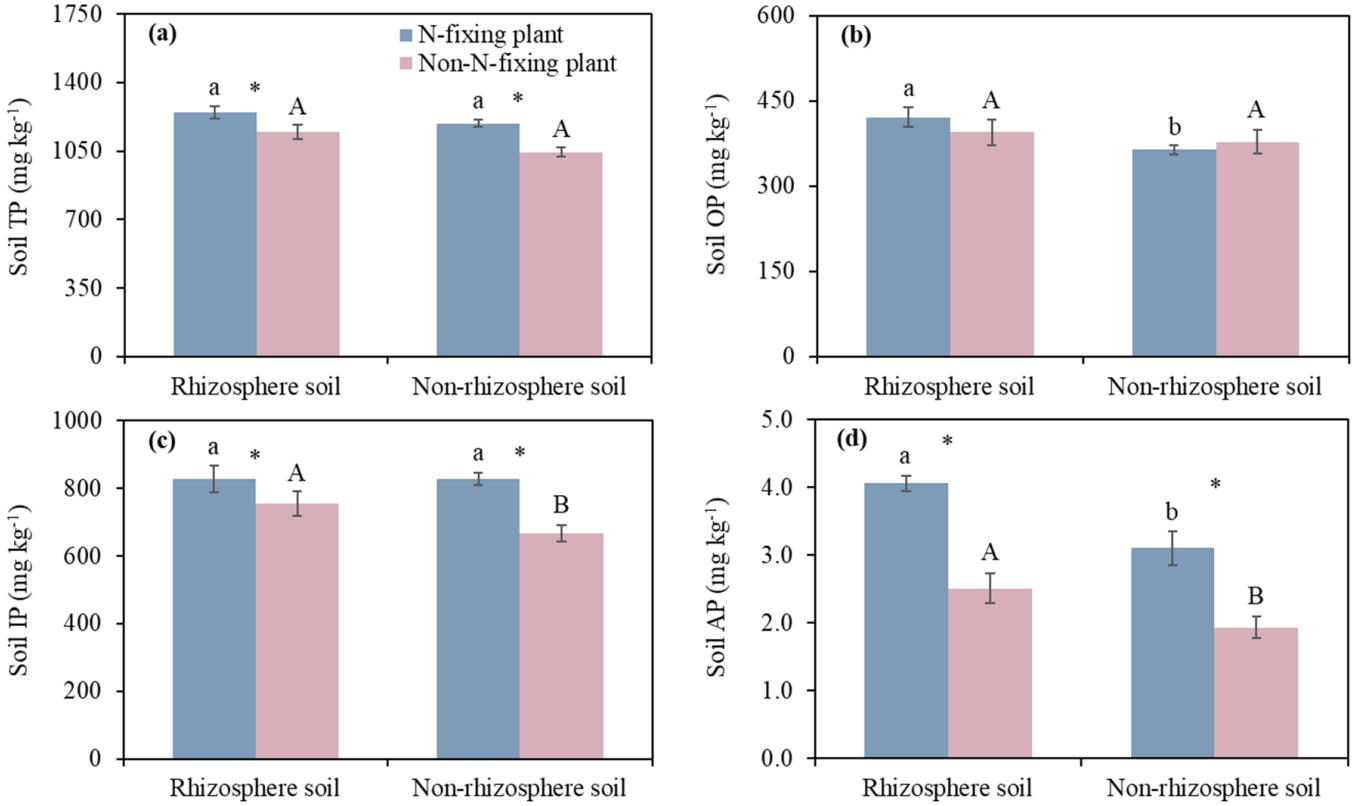


Figure 1. Soil total P (**a**), organic P (**b**), inorganic P (**c**), and available P (**d**) contents in rhizosphere and non-rhizosphere soils of N-fixing plants (n = 8) and non-N-fixing plants (n = 5) in a subtropical karst forest in China. Different lowercase and uppercase letters indicate significant differences between rhizosphere and non-rhizosphere soils for N-fixing and non-N-fixing plants, respectively ($p < 0.05$), and * indicates significant differences between N-fixing and non-N-fixing plants for rhizosphere soil or non-rhizosphere soil at $p < 0.05$.

Soil P fractions ranged from 4.16 mg kg^{-1} of Resin-P to 297.7 mg kg^{-1} of C-HCl-Pi for the N-fixing plant, and from 3.65 mg kg^{-1} of Resin-P to 278.0 mg kg^{-1} of C-HCl-Pi for the non-N-fixing plant, reflecting a large variation in soil fractions across both soil zones (Figure 2). Concentrations of Resin-P and $NaHCO_3$-Pi in the rhizosphere soil of the N-fixing plants, as well as $NaHCO_3$-Pi in the non-rhizosphere soil, were significantly higher than those of the non-N-fixing plants ($p < 0.05$, Figure 2), consequently leading to higher soil LP concentrations in both soil zones ($p < 0.05$, Figure 3a). Although soil D-HCl-Pi concentrations were significantly higher in the N-fixing plants than in the non-N-fixing plants, soil NaOH-Pi and NaOH-Po concentrations were significantly lower than in the non-N-fixing plants in the rhizosphere soil (Figure 2), thereby reducing the soil MLP concentration in the rhizosphere soil ($p < 0.05$, Figure 3b), as NaOH-Po constituted the largest portion of soil MLP (Figure 2). There were no significant differences in soil NaOH-Pi and NaOH-Po concentrations between the N-fixing and non-N-fixing plants in the non-rhizosphere soil. However, soil D-HCl-Pi concentration was significantly higher in the N-fixing plant compared to the non-N-fixing plant (Figure 2), resulting in no variation in soil MLP concentration in the rhizosphere soil (Figure 3b). Additionally, concentrations of soil D-HCl-Po in the rhizosphere soil of the N-fixing plants, and soil C-HCl-Pi, and Residual-P in the non-rhizosphere soil, were significantly higher than those of the non-N-

fixing plants ($p < 0.05$, Figure 2), thereby contributing to higher NLP concentrations in both soil zones ($p < 0.05$, Figure 3c).

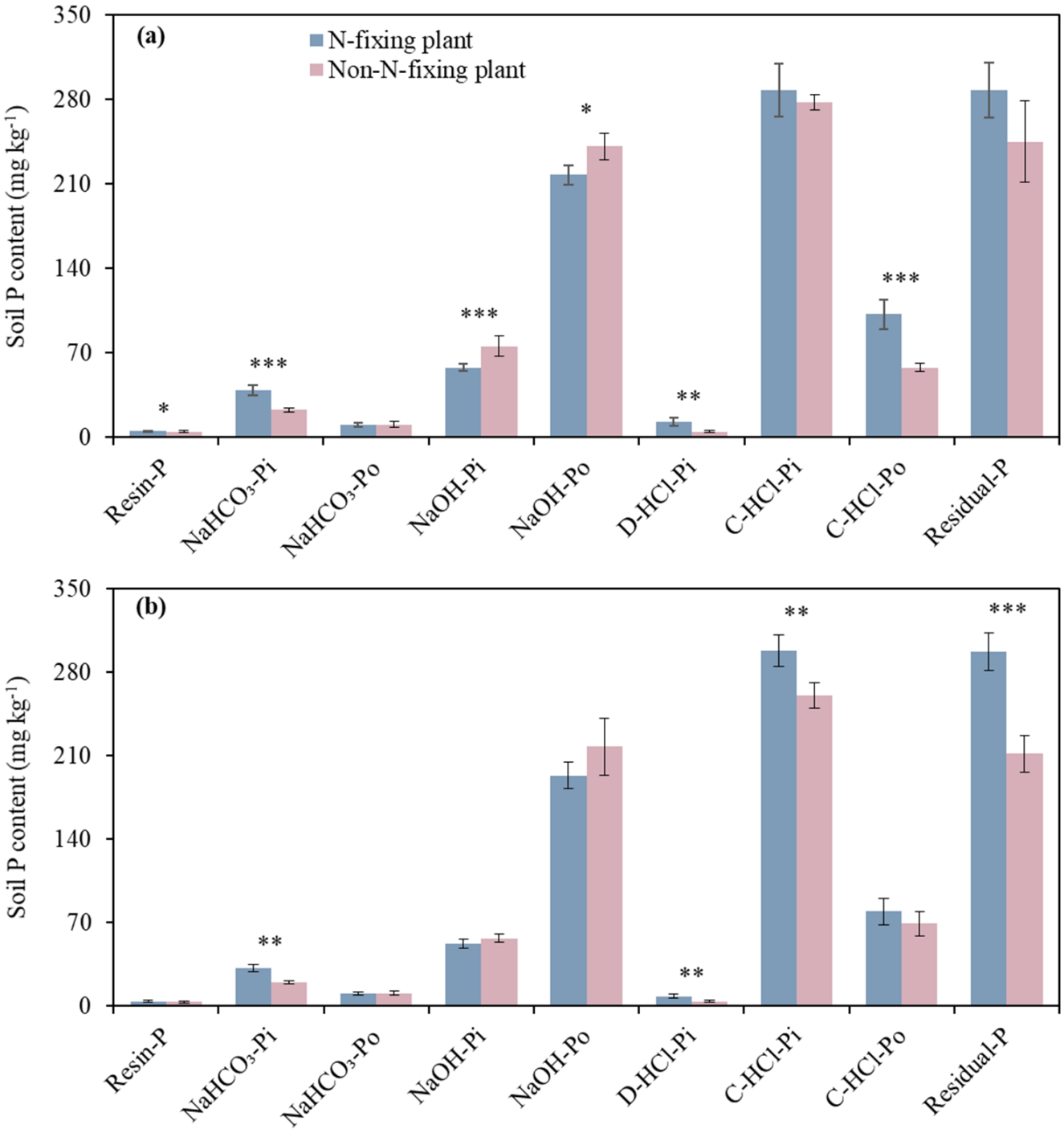


Figure 2. Soil P fractions in rhizosphere (**a**) and non-rhizosphere (**b**) soils of N-fixing plants (n = 8) and non-N-fixing plants (n = 5) in a subtropical karst forest in China. Asterisks indicate significant differences between N-fixing and non-N-fixing plants for each soil P fraction. *, **, and *** indicate significance levels at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

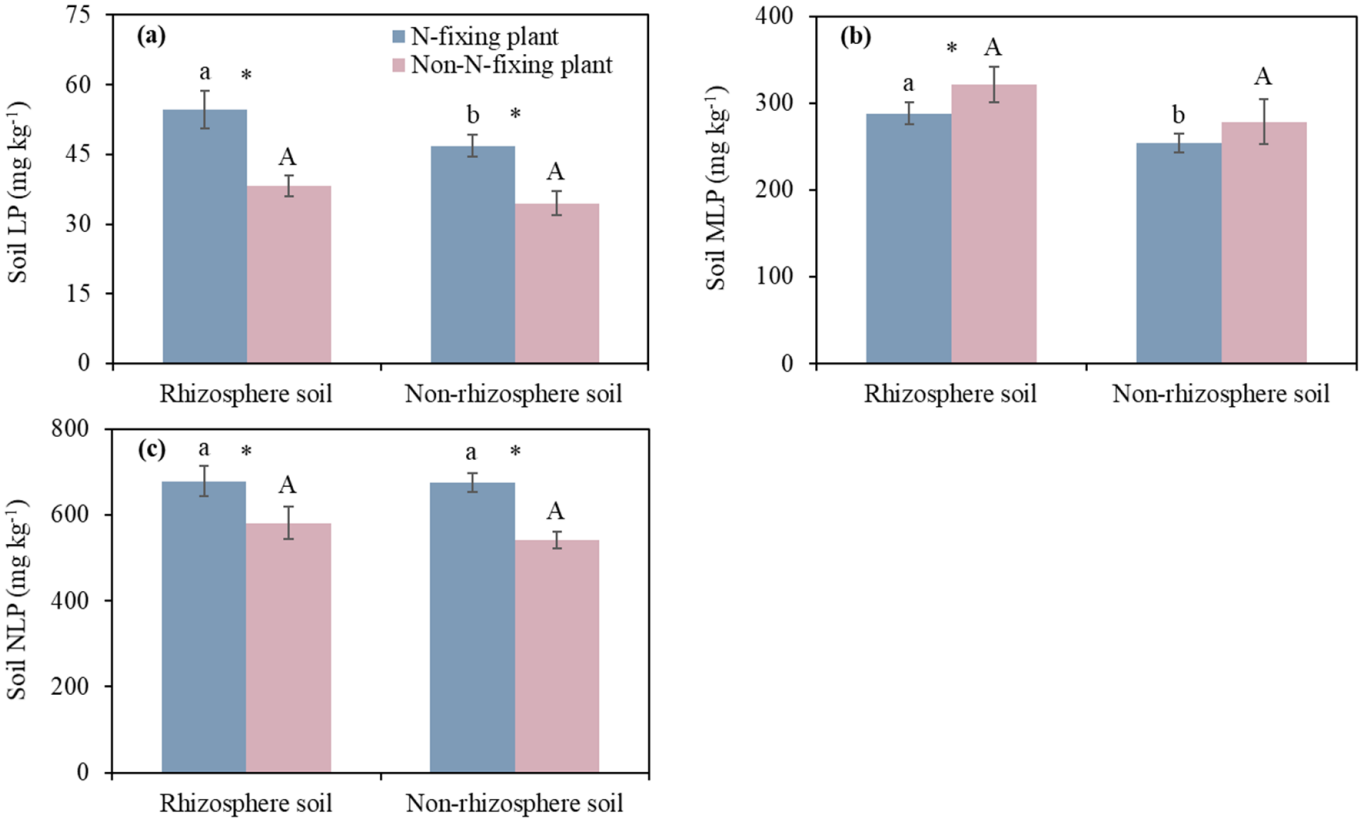


Figure 3. Soil labile P (**a**), moderately labile P (**b**), and non-labile P (**c**) contents in rhizosphere and non-rhizosphere soils of N-fixing plants (n = 8) and non-N-fixing plants (n = 5) in a subtropical karst forest in China. Different lowercase and uppercase letters indicate significant differences between rhizosphere and non-rhizosphere soils for N-fixing and non-N-fixing plants, respectively ($p < 0.05$), and * indicates significant differences between N-fixing and non-N-fixing plants for rhizosphere soil or non-rhizosphere soil at $p < 0.05$.

3.3. Soil Microbial Biomass, Enzyme Activities and Plant Nutrients

Concentrations of soil MBC, MBN, and MBP were significantly higher in both the rhizosphere and non-rhizosphere soils of the N-fixing plants compared to the non-N-fixing plants ($p < 0.05$, Figure 4). Additionally, they were also significantly higher in the rhizosphere soil compared to the non-rhizosphere soil ($p < 0.05$), except for soil MBN concentration in the non-N-fixing plants. Soil ALP activity of the N-fixing plant was 277.1 and 195.7 nmol g^{-1} h^{-1} in the rhizosphere and non-rhizosphere soils, respectively, both of which were significantly higher than those in the corresponding soils of the non-N-fixing plant ($p < 0.05$, Figure 4d). Moreover, soil ALP activities were significantly higher in the rhizosphere soils compared to the non-rhizosphere soils for both plant types ($p < 0.05$). There were no significant differences in leaf and root C concentrations between the N-fixing and non-N-fixing plants (Figure 5a), whereas both leaf and root N and P concentrations were significantly higher in the N-fixing plants than those in the non-N-fixing plants ($p < 0.05$, Figure 5b,c).

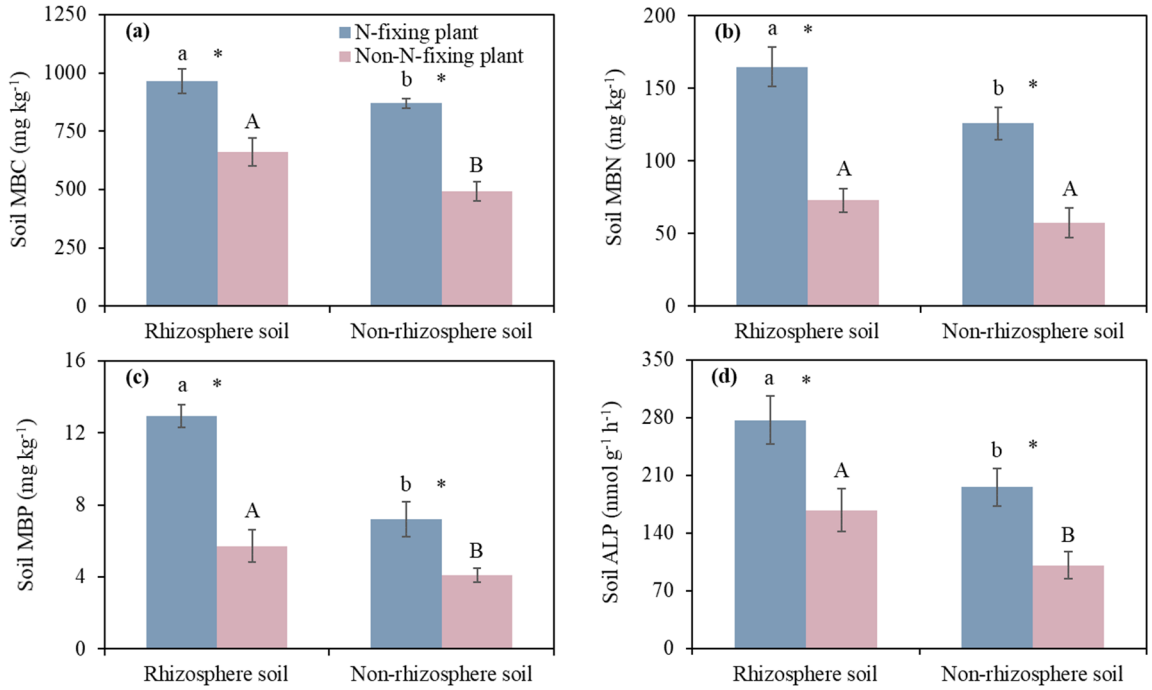


Figure 4. Soil microbial biomass carbon (**a**), microbial biomass nitrogen (**b**), and microbial biomass phosphorus (**c**) contents and alkaline phosphatase (**d**) activity in rhizosphere and non-rhizosphere soils of N-fixing plants (n = 8) and non-N-fixing plants (n = 5) in a subtropical karst forest in China. Different lowercase and uppercase letters indicate significant differences between rhizosphere and non-rhizosphere soils for N-fixing and non-N-fixing plants, respectively ($p < 0.05$), and * indicates significant differences between N-fixing and non-N-fixing plants for rhizosphere soil or non-rhizosphere soil at $p < 0.05$.

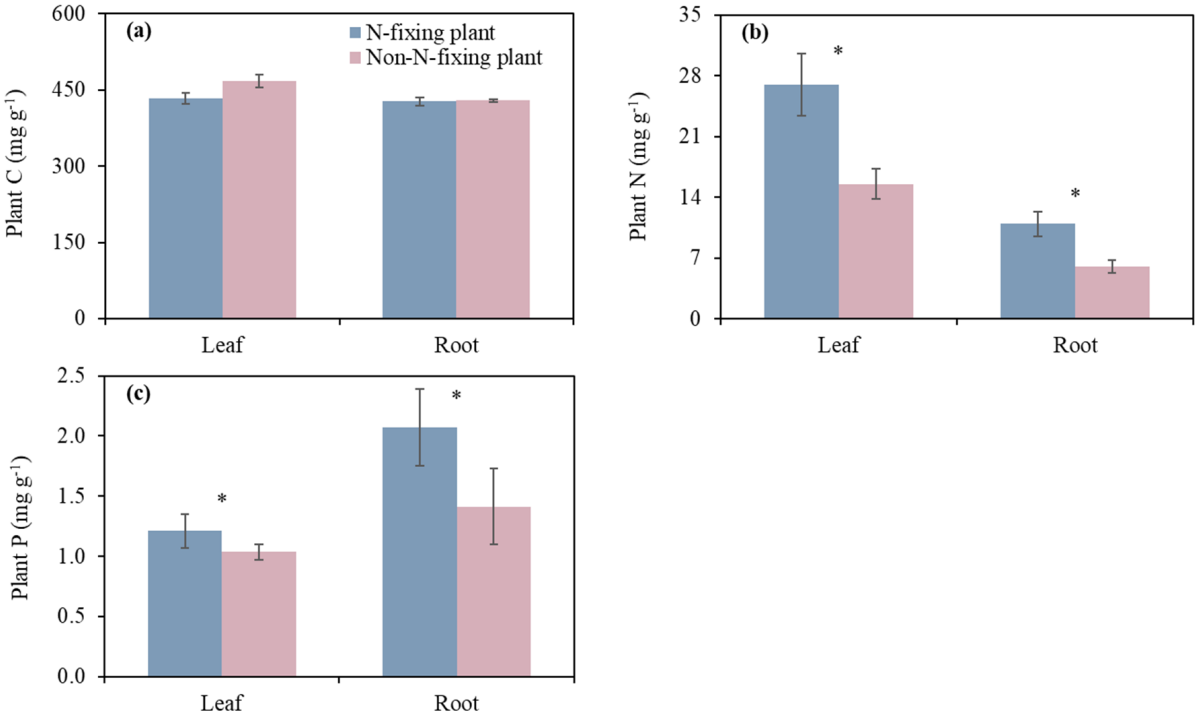


Figure 5. Carbon (**a**), nitrogen (**b**), and phosphorus (c) contents in the leaves and roots of N-fixing plants (n = 8) and non-N-fixing plants (n = 5) in a subtropical karst forest in China. * indicates significant differences between N-fixing and non-N-fixing plants for each nutrient at $p < 0.05$.

3.4. Controlling Factors on Soil P Fractions and Availability

The VPA results showed that soil properties, microbial properties, and plant nutrients accounted for 12.8%, 6.7%, and 3.4% of the variation in soil P fractions in the N-fixing plants, respectively, while these factors explained 12.4%, 0.1%, and 4.8% of the variations in the non-N-fixing plants, respectively (Figure 6a,b). The RDA results revealed that the primary factors contributing to changes in soil P fractions were IP (61.0%), MBN (6.7%), leaf N (4.2%), DN (3.7%), root N (1.0%), and root P (0.8%) in the N-fixing plants (Figure 6c). In contrast, the main factors were IP (53.8%), DOC (14.3%), DN (5.7%), leaf N (4.7%), and MBN (0.8%) in the non-N-fixing plants (Figure 6d). The SEM results showed that soil properties, plant nutrients, and soil P fractions directly affected soil P availability, while also indirectly influencing it through changes in soil P fractions in the N-fixing plants (Figure 7a). Soil properties and microbial attributes exhibited positive total effects on soil P availability, whereas plant nutrients and soil P fractions had negative total effects (Figure 7c). Conversely, in the non-N-fixing plants, only soil P fractions directly affected soil P availability, with soil properties indirectly influencing soil P availability via their effects on soil P fractions (Figure 7b). Both soil properties and soil P fractions exhibited negative total effects on soil P availability (Figure 7d).

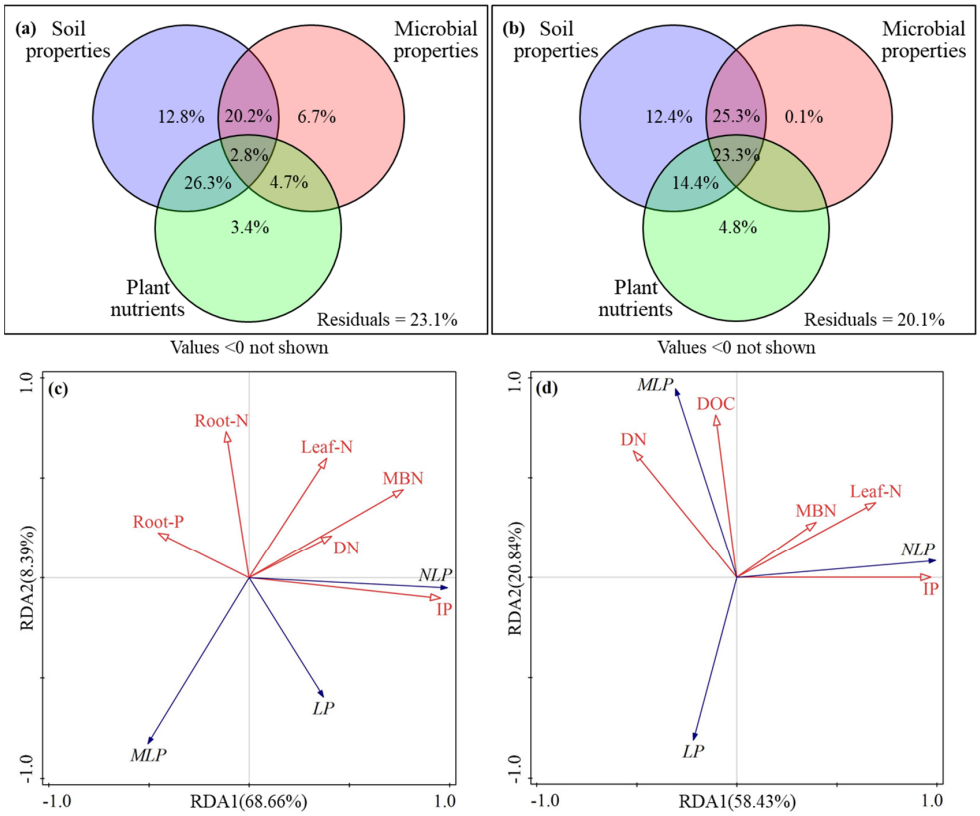


Figure 6. Variation partitioning analysis (VPA) illustrating the unique contributions of soil properties, microbial properties, and plant nutrients to soil P fractions in N-fixing plants (**a**) and non-N-fixing plants (**b**) in a subtropical karst forest in China, and redundancy analysis (RDA) of soil P fractions in N-fixing plants (**c**) and non-N-fixing plants (**d**). The percentages shown in the VPA represent the proportion of variance in soil P fractions explained by each factor (or combination of factors), with the remaining variance attributed to the residual (unexplained) component. The red and blue lines in the RDA represent explanatory and response variables, respectively. LP, labile P; MLP, moderately labile P; NLP, non-labile P; IP, inorganic P; DOC, dissolved organic carbon; DN, dissolved nitrogen; MBN, microbial biomass nitrogen.

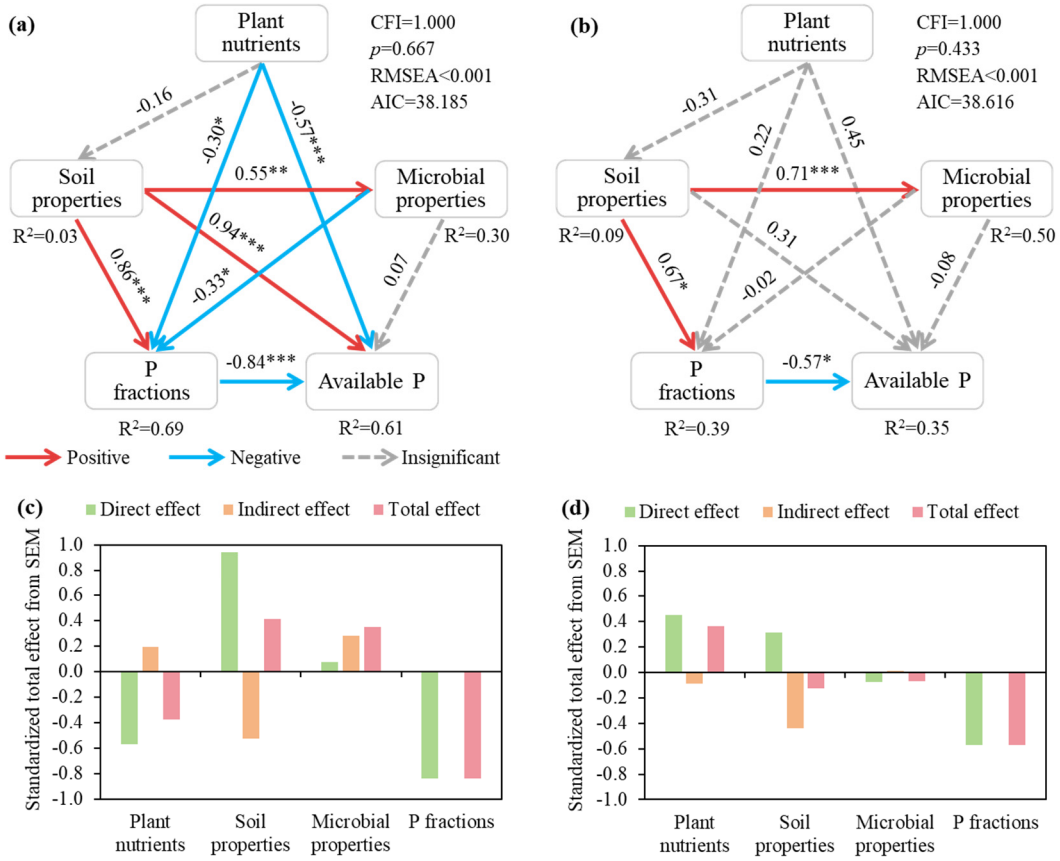


Figure 7. Structural equation models (SEMs) revealing the multivariate effects of soil properties, microbial properties, plant nutrients, and soil P fractions on soil available P in N-fixing plants (**a**) and non-N-fixing plants (**b**) in a subtropical karst forest in China. Standardized total effects of variables from the SEMs are depicted for N-fixing plants (**c**) and non-N-fixing plants (**d**). The solid red and blue arrows indicate positive and negative pathways, respectively, while the dashed grey arrows represent pathways with no significant effect. The numbers next to the arrows are the standardized path coefficients. Asterisks (*, **, ***) denote significance levels at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The R^2 values displayed below the response variables show the proportion of variation explained by the relationships with other variables.

4. Discussion

4.1. Effect of N-Fixing Plants on Soil P Pools

Soil P pools, including OP, IP, and AP pools, are primarily derived from soil weathering and litter decomposition [52,55,56], and they can transform into one another to maintain soil P balance [4,25,57]. In this study, the N-fixing plants significantly increased soil IP and AP concentrations in both the rhizosphere and non-rhizosphere soils (Figure 1), which can be attributed to the following factors. Karst forest soils are typically calcareous, developed from carbonate rocks with low P and high calcium content [58]. Consequently, soil P readily combines with calcium to form calcium-bound IP, significantly reducing the soil AP pool [59,60]. However, the N-fixing plants significantly decreased the calcium concentration in both the rhizosphere and non-rhizosphere soils (Table 1), potentially reducing calcium-bound IP and enhancing the soil AP pool. Meanwhile, compared to non-karst forest vegetation in the same area, karst forest vegetation is generally shorter, sparser, and has lower biomass, significantly reducing litter P input [61]. The higher P content in the leaves and roots of the N-fixing plants likely increases the amount of P returned to the soil through litter decomposition, thereby enhancing the soil TP pool to some extent (Figure 5c). Moreover, the increase in soil organic matter (higher SOC and TN contents)

due to N-fixing plants reduced the stability of OP at soil binding sites (Table 1), making soil OP more accessible to soil microorganisms. This, in turn, significantly enhanced soil OP mineralization and increased the soil IP pool [62]. Although some studies have reported that variations in soil P pools may be attributed to changes in soil pH, and concentrations of iron and aluminum oxides or minerals [14,63,64], no evidence was found in this study to suggest that these factors influenced soil P pools (Table 1).

4.2. Effect of N-Fixing Plants on Soil P Fractions

In this study, the N-fixing and non-N-fixing plants differently affected soil Resin-P, $NaHCO_3$-P, NaOH-P, D-HCl-Pi, C-HCl-P, and Residual-P fractions, thus leading to higher soil LP and NLP in both the rhizosphere and non-rhizosphere soils, and a lower soil MLP in the rhizosphere soil under the N-fixing plant treatment (Figure 3), which agreed with our first hypothesis. Previous studies reported that increased soil N availability could enhance P absorption by plants, and thereafter significantly reducing soil LP fractions in forest soils, particularly soil Resin-P and $NaHCO_3$-Pi fractions [14,65]. In this study, the N-fixing plants significantly increased soil N availability, as indicated by higher TN and NO_3^--N concentrations, and improved plant P absorption, as evidenced by higher leaf and root P contents (Table 1 and Figure 5c). Soil Resin-P and $NaHCO_3$-Pi fractions remained higher in the N-fixing plant treatment compared to the non-N-fixing plant treatment, suggesting the effectiveness of N-fixing plants in improving soil LP. Additionally, the soil NaOH-Po fraction within MLP accounted for the largest proportion of soil OP, which is crucial for maintaining the effective P levels required by organisms [66]. The increased N availability may enhance phosphatase production and accelerate soil OP mineralization to meet the P demand of microbes and plants in P-limited soils, as phosphatase is an N-containing enzyme, with N comprising approximately 15% of its elemental composition [67]. The increased soil ALP activity and decreased NaOH-Po concentration observed in this study indicate that N-fixing plants facilitate the transformation of soil OP, thereby reducing soil MLP and consequently supplementing soil LP [7]. This finding aligns with studies conducted in a *Castanopsis carlesii* natural forest and a *Larix gmelinii* forest plantation, both of which demonstrated a reduction in soil NaOH-Po fraction concentration with increased N input [65,68]. Although N-fixing plants significantly increased soil NLP (C-HCl-Po in rhizosphere soils, and C-HCl-Pi and Residual-P in non-rhizosphere soils), soil NLP is extremely difficult for plants or microbes to utilize under normal conditions [69]. Therefore, we suggest that N-fixing plants play a crucial role in maintaining high soil P availability to meet the P demands of organisms by regulating the transformation of key soil P fractions, such as Resin-P, $NaHCO_3$-Pi, and NaOH-Po in karst forests.

4.3. Factors Driving Soil P Availability by N-Fixing Plants

Soil P availability depends on the presence of specific soil P pools and the transformation of various P fractions. Notably, soluble P normally represents a very small fraction of TP in soils, and only a small portion of soluble P in soil solution (<1%) can be directly absorbed and utilized by organisms [25]. Richter et al. [70] have shown that soil LP has the highest bioavailability compared to soil MLP and NLP fractions and is regarded as the primary P source available to plants and microorganisms [59]. In this study, N-fixing plants significantly increased both soil LP and AP concentrations. For example, soil AP concentration increased from 1.93 to 3.10 mg kg^{-1} in non-rhizosphere soils and from 2.51 to 4.06 mg kg^{-1} in rhizosphere soils (Figure 1d). This finding supports our second hypothesis, suggesting that N-fixing plants were effective in improving soil P availability in karst forests. The improvement in soil P availability can be attributed to the enhanced transformation of soil P fractions, driven by soil properties, plant nutrients, and microbial attributes (Figure 7a).

Li et al. [9] also reported that N-fixing plants increased soil AP concentration in a forest plantation in the karst region and argued that OP transformation, primarily driven by ALP, MBN, and arbuscular mycorrhizal, was responsible. The increase in microbial and enzyme activities could accelerate the mineralization of soil organic matter, directly replenishing soil AP [71,72], especially in highly weathered soils where soil P availability is mainly affected by the decomposition and mineralization of litter and soil organic matter [55,56], and a significant portion of soil $NaHCO_3$-Po and NaOH-Po fractions originates from litter decomposition [73]. Consistent with this, the increased soil microbial biomass, ALP activity, and SOC concentration likely contributed to the higher AP concentration observed in the N-fixing plant treatment (Figure 4). This is further supported by the fact that microbial attributes and soil properties exhibited positive total effects on soil P availability (Figure 7c). Previous studies have indicated that when the N:P ratio of leaves is below 14, the plant growth is mainly limited by N. When the N:P ratio exceeds 16, P becomes the primary limiting factor for growth. However, when the N:P ratio falls between 14 and 16, plant growth may be co-limited by both nutrients or not limited by either [74]. It should be noted that the N:P ratio of leaves ranged from 18.2 to 24.9 in this study, indicating that N-fixing plants were experiencing severe soil P limitation, which would act to significantly increase P absorption, as evidenced by the enhanced P content in leaves and roots (Figure 5c). Thus, plant nutrients displayed a negative total effect on soil P availability for the N-fixing plants. In contrast, the N:P ratio of leaves of the non-N-fixing plants ranged from 11.4 to 17.7, suggesting that N was more likely limiting, but at the upper end, both could be co-limiting growth in these plants. The difference in the N:P ratio of leaves can be explained by the fact that N-fixing plants remarkably invest that acquired N into accessing P, thereby widening the gap between leaf N and P contents.

4.4. Implication for Vegetation Restoration in Karst Regions and the Limitation of the Study

The present study provides evidence that N-fixing plants can regulate soil P fractions and improve soil P availability through enhanced microbial and enzyme activities in subtropical karst regions. Therefore, our results have important implications for formulating measures to accelerate vegetation restoration in areas affected by rocky desertification. In karst regions, N-fixing plants can be introduced to promote soil OP mineralization and increase concentrations of AP and unstable P, thereby alleviating P limitation for plant growth, particularly in P-limited soils [9,27,29]. However, the effects of different N-fixing plants on soil P fractions and availability varied to some extent (Figures S3 and S4). Therefore, the selection of candidate N-fixing plants for vegetation restoration strategies should consider both plant adaptability and their impact on improving soil P availability. Our study offers new insights into P biogeochemical cycling in karst soils by integrating the effects of soil properties, microbial attributes, and plant nutrients associated with N-fixing plants. This contributes to a deeper understanding of the factors driving P dynamics in P-limited karst soils and informs the management of soil nutrients in these regions. It should be noted that under future climate change scenarios—such as rising air temperatures, altered precipitation patterns, and increased N deposition—the complexity of plant and soil P cycling is expected to intensify, further complicating nutrient dynamics in karst regions.

Root exudates, serving as a key link between soil and microorganisms [75–77], play an integral role in P cycling [78,79]. However, the effects of root exudates on soil P fractions dynamics were not explored in this study due to difficulties in field sample collection and the high variability in root exudate data. Future research, conducted under controlled conditions, could explore the underlying mechanisms of root exudates from N-fixing plants on soil microbes and subsequent P transformation, which is essential for a deeper under-

standing of the link between soil P dynamics and N-fixing plants [9,36,77]. Additionally, the effect of N-fixing plants on P availability in this study was inferred solely from changes and relationships among different P fractions. Future research would be well served to systemically quantify specific P cycling processes, including adsorption and desorption, precipitation and dissolution, and microbial immobilization and mineralization [4,25,59], as this could be crucial for thoroughly elucidating the regulatory mechanisms that sustain soil P balance and availability.

5. Conclusions

We investigated the effects of N-fixing plants on soil P fractions and availability in a subtropical karst forest in China. Our results revealed that N-fixing plants significantly increased soil labile and non-labile P while decreasing moderately labile P, thereby enhancing soil P availability. This effect may be primarily attributed to the transformation of soil organic P fractions (e.g., NaOH-Po) to inorganic P fractions (e.g., $NaHCO_3$-Pi, Resin-P) in rhizosphere soils, driven by increased soil microbial and ALP activities, as well as the likely diffusion of labile P from rhizosphere to non-rhizosphere soils. Soil inorganic P, root and leaf N contents, and microbial biomass N were identified as key factors enhancing soil P availability in N-fixing plants. In contrast, only soil properties indirectly influenced soil P availability in non-N-fixing plants. Overall, our findings uncover some of the underlying mechanisms by which N-fixing plants regulate soil P fractions and pools in karst forests, highlighting their critical role in enhancing soil P availability, which will be of particular value in developing effective strategies for P nutrient management and guiding the plantation of appropriate plant species for vegetation restoration in karst regions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f16020360/s1, Figure S1: Location of the study area and selected study sites in a subtropical karst forest, southwest China; Figure S2: Images of the dominant N-fixing and non-N-fixing plants included in this study; Figure S3: Soil total P, organic P, inorganic P, and available P contents in rhizosphere and non-rhizosphere soils of different N-fixing plants and non-N-fixing plants (n = 3) in a subtropical karst forest in China. Different lowercase letters indicate significant differences among various N-fixing and non-N-fixing plants for rhizosphere soil or non-rhizosphere soil ($p < 0.05$). BB, *Bauhinia brachycarpa*; SD, *Sophora davidii*; DH, *Dalbergia hupeana*; LJ, *Lespedeza juncea*; IP, *Indigofera pseudotinctoria*; IM, *Indigofera mengtzeana*; PC, *Phanera calciphila*; CY, *Campylotropis yunnanensis*; FM, *Fraxinus malacophylla*; DV, *Dodonaea viscosa*; PC, *Pistacia chinensis*; PM, *Pinus massoniana*; RC, *Rhus chinensis*; Figure S4: Soil labile P, moderately labile P, and non-labile P contents in rhizosphere and non-rhizosphere soils of different N-fixing plants and non-N-fixing plants (n = 3) in a subtropical karst forest in China. Different lowercase letters indicate significant differences among various N-fixing and non-N-fixing plants for rhizosphere soil or non-rhizosphere soil ($p < 0.05$). BB, *Bauhinia brachycarpa*; SD, *Sophora davidii*; DH, *Dalbergia hupeana*; LJ, *Lespedeza juncea*; IP, *Indigofera pseudotinctoria*; IM, *Indigofera mengtzeana*; PC, *Phanera calciphila*; CY, *Campylotropis yunnanensis*; FM, *Fraxinus malacophylla*; DV, *Dodonaea viscosa*; PC, *Pistacia chinensis*; PM, *Pinus massoniana*; RC, *Rhus chinensis*.

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Data Availability Statement: The data supporting the findings of this study can be obtained from the corresponding author upon request.

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