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Highly elevated CO_2 and fertilization with nitrogen stimulates significant *schima superba* growth and mediates soil microbial community composition along an oligotroph-copiotroph spectrum

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Abstract

Purpose Substantial studies report about the extent of elevated CO_2 (eCO_2) and nitrogenous (N) fertilization or deposition above- and belowground. Although CO_2 concentrations are expected to be more than 1000 μmol·mol^{-1} by 2100 (IPCC 2014), there are relatively few studies about the effects of highly concentrated eCO_2 plus N fertilization on woody plants.

Materials and methods *Schima superba* seedlings were exposed to eCO_2 and N fertilization in open-top chambers (OTCs), including ambient air (400 μmol·mol^{-1}), 550, 750, and 1000 μmol mol^{-1} and 5, 10, or 0 g N m^{-2}·year^{-1}, respectively. Plant photosynthesis (Pn), leaf/root carbon (C) and N, and biomass were analyzed; furthermore, soil microbial community structure was examined.

Results and discussion After only one growing season, the combination of eCO_2 and N fertilization increased Pn. N fertilization also increased plant biomass. The combined effect of higher CO_2 concentration with N fertilization further stimulated plant biomass. Soil fungal community structure was altered by eCO_2 via affecting leaf N and C/N. Moreover, N fertilization changed the composition of soil bacterial communities, which in part was driven by soil NO_3^-, as well as root C/N. Although eCO_2 and N fertilization yielded a direct relationship of synergistic effects on Pn and plant biomass, they elicited contrasting effects on soil copiotrophic and oligotrophic groups, which mediate the soil microbial community structure and nutrient cycling.

Conclusions Plant growth and soil microbial communities could be affected within short time scales by global change. Experimental manipulations that focus on the singular effects of either CO_2 or N fertilization alone may underestimate the effects of global change on woody plants.

Keywords Elevated CO_2 · N fertilization · *Schima superba* · Photosynthesis · Plant growth · Soil microbiology

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1 Introduction

Many studies report the beneficial effects of eCO_2 on plant performance (Ainsworth et al. 2005; Hikosaka et al. 2005; Reich et al. 2018; Li et al. 2019), as well as CO_2-induced negative effects (Gleadow et al. 2009) or "no plant" growth stimulation (Van Der Sleen et al. 2015). Previous work has proposed that the capacity for a plant to store additional carbon (C) under eCO_2 would be inhibited by the limitation of nitrogen (N) availability (Luo et al. 2004). A majority of research in this area shows that long-term eCO_2 leads to decreased leaf N availability (review by Peterson et al. 1999; review by Taub and Wang 2008; Sharwood et al. 2017) because the higher growth rate under eCO_2 would dilute the N tissue concentration (Johnson 2006). A reduction in leaf N under eCO_2 conditions would cause downregulation of N

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reallocation in carboxylation and electron transport (Crous et al. 2010), thus resulting in declined leaf photosynthesis with reduced Rubisco activity and amount (Gutiérrez et al. 2009). However, when eCO_2 is combined with increased N deposition, these limiting effects of N availability under eCO_2 may be alleviated. In addition to eCO_2, N deposition is another important environmental issue that impacts global ecosystems. Numerous studies show that eCO_2 and N deposition affect plants (Lipson et al. 2005; Feng et al. 2010; Deng et al. 2016).

Both N deposition and eCO_2 are in direct relationship to the extent of C and N cycling within plants via impacting photosynthesis, allocation of photosynthetic products, soil properties, and soil microbial community structure, inevitably leading to changes in the functioning of terrestrial ecosystems (Aber et al. 2001; Chung et al. 2007; Knops et al. 2007). The growth, composition, and functions of soil microbiology can be indirectly affected by eCO_2, which induces more rhizodeposits and changes the composition of root exudates (Jones et al. 2009; Blagodatskaya et al. 2010; Drigo et al. 2010). Additionally, eCO_2 often increases soil moisture through reducing plant stomatal conductance, and thereby has beneficial effects on soil biota (Field et al. 1995; Adair et al. 2011). Also, enhancement of aboveground productivity of eCO_2 could increase the amount of litter input, which would supply more C to soil microorganisms and increase microbial biomass (Reich et al. 2006; Eisenhauer et al. 2012). Commonly, the more abundant organics from root exudates resulting from eCO_2, would modify the ecological strategy of soil microbiome with a higher proportion of fast-growing r-strategists, which could quickly metabolize available substrates (Blagodatskaya et al. 2010). Conversely, N addition was reported to decrease belowground C allocation and reduce rhizodeposits, resulting in lower microbial biomass (DeForest et al. 2004; Högberg et al. 2010); reduced microbial enzyme activity, modified soil community composition, and reduced microbial biodiversity (Frey et al. 2004; Treseder 2008). It is possible that N fertilization favors some opportunistic microbial taxa (Drigo et al. 2010). Hence, some reports indicated that the effects of eCO_2 on soil microbial communities were counterbalanced by N addition (Chung et al. 2007; Blagodatskaya et al. 2010). Some previous studies also reported the influence of eCO_2 on fungi was greater than bacteria (Lipson et al. 2005; Carney et al. 2007), due to the decrease in N concentration of plant residues (Cotrufo et al. 1998), and N deposition had a greater effect on soil bacterial community (Feng et al. 2010; Fierer et al. 2012; Cederlund et al. 2014).

Anthropogenic greenhouse gas emissions have increased, as concentrations of CO_2, methane, and nitrous oxide (N_2O) are being emitted at unprecedented rates within the past 800,000 years (IPCC 2014). According to the Intergovernmental Panel on Climate Change's (IPCC's) 5th Evaluation Report, which sheds light on the pathways of representative concentrations used for making projections based on the aforementioned factors, these representative concentration pathways (RCPs) describe a range of different projected pathways that will putatively represent greenhouse gas (GHG) emissions for the twenty-first century with respect to heightened air pollutant emissions and emissions generated from land uses (IPCC 2014) as a function of atmospheric concentrations. These RCPs designate one highly strict scenario for mitigation (i.e., RCP2.6; stringent mitigations), as well as two scenarios which could be considered intermediate (i.e., RCP4.5 and RCP6.0; intermediate mitigations); furthermore, these RCPs include a scenario projecting no mitigation efforts that yielded very high GHG emissions (RCP8.5). The CO_2 concentrations are projected to be between 430 and 480 $\mu mol \cdot mol^{-1}$ in RCP2.6, between 580 and 720 $\mu mol \cdot mol^{-1}$ in RCP4.5, and between 720 and 1000 $\mu mol \cdot mol^{-1}$ in RCP6.0; in most scenarios for RCP8.5 without additional mitigation efforts, and highly emissive projections, CO_2 concentration will likely result in greater than 1000 $\mu mol \cdot mol^{-1}$ by the year 2100. Meanwhile, N_2O emissions will increase with increasing CO_2 concentrations and will reach upward of 20 T $g \cdot year^{-1}$ until 2100 for scenario RCP8.5 (IPCC 2014). Data clearly indicates an increase of N_2O emissions over time, and most of this emission is deposited back to land and water bodies (Xu et al. 2015). This trend will probably lead to increased rates of N deposition. Also, on a global scale, current N emissions in most regions will significantly increase along with heightened N deposition. By the year 2030, it is estimated that over 30 kg N $ha^{-1} \cdot year^{-1}$ will be produced within a variety of tropical ecoregions, especially within India and China (Bobbink et al. 2010).

Most previous studies set CO_2 concentrations at approximately double that of ambient air. Within even fewer studies, higher CO_2 concentrations, such as 1000 $\mu mol \cdot mol^{-1}$, were investigated, for determining the effects of high eCO_2 on agricultural crops (Lambreva et al. 2006; Aranjuelo et al. 2013), grass (Yoder et al. 2000; Ryan et al. 2014), or shrubs (Polley et al. 1997). Other studies reported the effects of 1000 $\mu mol \cdot mol^{-1}$ CO_2 on water use efficiency and gas exchange in plant species, such as *Tamarix ramosissima* (Chang et al. 2016), or on stomatal conductance, transpiration, and plant biomass in *Populus deltoides* (McDonald et al. 2002). Although little to no studies have examined the consequences of 1000 $\mu mol \cdot mol^{-1}$ CO_2 combined with N fertilization on plant growth and soil microbial community structure, previous work has investigated the combined effects of N fertilization and eCO_2 above- and belowground (Feng et al. 2010; Fransson and Johansson 2010; Weber et al. 2013), especially within forests in subtropical China.

Schima superba is a tree species representative of subtropical forests in China; *S superba* is also widely used

in urban landscaping. Previous studies showed that eCO_2 (about 700 μmol·mol^{-1}) and N deposition affected photosynthesis, N:P ratio, biomass accumulation and allocation, and soil respiration of *S. superba* (Sheu and Lin 1999; Deng et al. 2010; Liu et al. 2013a, b). Yet, there is a dearth of information about how plant growth of *S. superba* responds to highly eCO_2 (1000 μmol·mol^{-1}) together with the effects of N deposition, as well as how soil microbial community structure associated with *S. superba* may respond to eCO_2 and N deposition.

For this study, we investigated *S. superba* growth responses and changes to the associated soil microbial community composition from exposure to highly concentrated eCO_2, both alone and in combination with N deposition. We conducted CO_2 gradients based on RCPs, including ambient air, 550, 750, and 1000 μmol·mol^{-1} and different levels of N fertilization. As CO_2 concentrations increase, N availability will become a limiting factor; therefore, we hypothesize that (1) compared to ambient CO_2, the stimulation on photosynthesis and biomass would not continuously increase for the scenario of RCP8.5, as it did for RCP 4.5 and RCP 6.0; in this case, limitations may be alleviated by N fertilization. Moreover, we predict that (2) the impacts of eCO_2 on soil microbial community structure will vary by affecting plant photosynthesis and plant C and N allocation belowground. Finally, we hypothesize that (3) soil fungal communities are more affected by eCO_2 while N fertilization will have a greater influence on soil bacteria than eCO_2.

2 Materials and methods

2.1 Study site and experimental design

We executed this study at the Qianyanzhou Ecological Station, associated with the Chinese Academy of Sciences (115°04′13″E, 26°44′48″N), located in the southeast Chinese province of Jiangxi, which experiences a subtropical monsoon climate. The elevation ranges are between 60 and 150 m. For our field experiments, we used 2-m-diameter open-top chambers (OTCs) that were 2.2 m high. Prior to this study, we used these OTCs to study the responses of plants to O_3; details of the OTC design can be found in Yu et al. (2018) and Chen et al. (2019).

CO_2 was provided by a compressed CO_2 cylinder (≥ 99.9%), and the concentration of CO_2 inside the OTCs was monitored with a CO_2 analyzer (FGD2-C-CO_2, Shenzhen Xin Hairui Science and Technology Development Co., Ltd, Shenzhen, China). CO_2 treatments were set according to IPCC and were set as ambient air (about 400 μmol mol^{-1}, hereafter CO2_400), or we based our treatments according to the RCPs, as 1000 μmol·mol^{-1} (hereafter CO2_1000), 750 μmol·mol^{-1} (hereafter CO2_750), and 550 μmol·mol^{-1} (hereafter CO2_550). These four different CO_2 treatments were conducted in separate OTCs, with three OTCs for each CO_2 gradient, for a total of 12 OTCs.

According to a national monitoring network's data, the total N deposition averaged around 4 g N m^{-2}·year^{-1} and the maximum value exceeded 8 g N m^{-2}·year^{-1} (Xu et al. 2015). As one of several regions in the world for highest known levels of N deposition (Yu et al. 2019), China showed an increase of about 60% over the past three decades (Liu et al. 2013a, b). In consideration of this increasing trend of N deposition, in this study, we conducted three N fertilization treatments: no treatment (i.e., 0 g N m^{-2}·year^{-1}; N0), 5 g m^{-2}·year^{-1} (N1), or 10 g m^{-2}·year^{-1} (N2) for each category of CO_2 gradient. In total, there were 12 N fertilization treatments including CO2_400 plus N fertilization (N0_400, N1_400, N2_400), CO2_550 plus N fertilization (N0_550, N1_550, N2_550), CO2_750 plus N fertilization (N0_750, N1_750, N2_750), and CO2_1000 plus N fertilization (N0_1000, N1_1000, N2_1000) with three OTC replicates per CO_2 gradient.

2.2 Plant materials

In April 2018, 1-year-old *S. superba* seedlings were transplanted to pots (20 cm × 30 cm) with local soil under ambient condition. Baseline contents of soil nutrients were measured and found to contain 20.1 mg·kg^{-1} of available potassium, 1.58 mg·kg^{-1} of available phosphorus, and 500 mg·kg^{-1} of total N; soil pH was 4.70, and the organic matter content of these soils was 8.63 g·kg^{-1}. Seedlings with similar growth parameters were transplanted into OTCs at the end of April, with 15 plants in each OTC. After 7 days, the plants were fumigated with ambient or eCO_2 air. In each OTC, five plants were selected to receive N fertilization, and from June 15, 2018, KNO_3 solution was added once a month until November 2018. During the whole growing season, well water was used to irrigate the seedlings when needed.

2.2.1 Photosynthesis analysis

Rates of photosynthesis were calculated by measuring photosynthates from 09:00 to 11:30 a.m. and 02:00 to 03:30 p.m. respectively in the middle of July, August, and September. In each N fertilization treatment, two plants were randomly selected from five independent plant replicates in each OTC. For each plant, two middle leaves were analyzed with a portable photosynthetic measuring device (Li-COR model LI-6400, Li-COR Corp., USA, Lincoln, NE). Flux density of photosynthetic photons was found to be 1000 μmol m^{-2} s^{-1}, with gas flow rates calculated at 500 ml·min^{-1}.

2.2.2 Plant growth analysis

Plants were harvested on October 26, 2018, after the growing season had ended. Two plants were randomly collected in each N fertilization treatment from each OTC, and leaves, stems, and roots were sampled. To determine biomass, we dried roots, stems, and leaves until they reached constant weights at 70 °C; sub-samples of these dried botanical parts were ground into a powder and sifted through a 2-mm sieve for plant C and N analysis. We used potassium dichromate oxidation to determine plant C content; an automated Kjeldahl apparatus (KD310, Opsis, Sweden) was used to calculate plant N.

2.3 Soil collection and analysis

After plants were harvested, soil was mixed homogeneously within each pot. Soil sub-samples of about 500 g was collected from each pot with the same N fertilization treatment. Within each OTC, sub-samples with the same N fertilization were completely mixed into a composite sample. We used air-dried soil combined with distilled water at 1:5 (weight/volume) to measure soil pH with a Mettler-Toledo Delta-series pH-meter (Delta 320, Mettler-Toledo, Shanghai, China). We titrated with potassium dichromate oxidation to measure soil organic matter. We used a flow analyzer (AA3, Seal, Germany) to measure the quantities of soil NH_4-N and NO_3-N. We used chloroform fumigation-extraction to measure both soil microbial biomass N (SMBN) and soil microbial biomass C (SMBC), as per Vance et al. (1987).

DNA of soil microorganisms was extracted from aliquoted soil, as per Chen et al. (2019). For targeted amplicon sequencing, we targeted hypervariable regions for the V3/V3 and the V4/V4 regions of the ribosomal 16S rRNA gene for targeting prokaryotes (i.e., archaea and bacteria), as well as a hypervariable region for the internal transcribed spacer for fungi (i.e., fungal ITS1). For this molecular analysis, we conducted polymerase chain reactions (PCRs) for amplifying these hypervariable regions. Our PCR products were purified using gel extraction (Axyprep DNA Gel Extraction Kit; Axygen, USA); then, we used QuantiFluor™-ST (Promega, USA) to subsequently quantify the resultant PCR products. For both targeted loci, we used purified and quantified PCR products for generating our pooled library, which contained purified PCR product for each individual sample. These samples were pooled by concentration, which was calculated to be equimolar, for downstream Illumina sequencing on the PE300 MiSeq sequencer (Illumina Corp., CA, USA). For more details, please see the supplemental information (SI).

2.4 Statistical approach

When plants were exposed to the same CO_2 concentration, each of the different N fertilization treatments were pooled together; this allowed us to isolate the effects of eCO_2 on plant and soil, resulting in four treatments including: CO2_400 (N0_400, N1_400, N2_400), CO2_550 (N0_550, N1_550, N2_550), CO2_750 (N0_750, N1_750, N2_750), and CO2_1000 (N0_1000, N1_1000, N2_1000). Meanwhile, all of the different CO_2 treatments with the same loads of N fertilization were considered as the same N treatment, which included N0 (N0_400, N0_550, N0_750, N0_1000), N1 (N1_400, N1_550, N1_750, N1_1000) and N2 (N2_400, N2_550, N2_750, N2_1000). Additionally, we analyzed each of the 12 treatments to investigate any combined effects of N fertilization and eCO_2.

SPSS statistical software (SPSS Inc., Chicago, IL, USA) was used to compare treatment means. The independent effects and the combined effects of eCO_2 and N fertilization on soils and plants were determined by a two-way ANOVA. The averages of the relative abundances (RA) of operational taxonomic units (OTUs; RA > 1%) within each treatment were used to compare the differences between the treatments. For the OTU-based analysis, Shannon diversity was calculated using the Shannon index and the Chao index, which allowed us to estimate microbial diversity within each sample. To compare soil microbial diversity between treatments, we used Tukey's honest significant difference (HSD) methods, based on the Studentized range statistic, to conduct pairwise comparisons and evaluate the outcomes of these post hoc tests along with our one-way ANOVAs (Zhao et al. 2014). Soil microbial composition based on OTU composition was compared across all samples; we generated Bray–Curtis (BC) distances to detect correlations with the relative abundances of particular microbial phyla along with associated environmental variables. We used these distance matrices for visualizing community composition and calculated redundancy analysis (RDA) and generated ordinations with principal coordinates analysis (PCoA). To determine the relative importance of edaphic, botanic, eCO_2, and N fertilization for fungal and bacterial communities, a variation partitioning analysis (VPA) was conducted with "varpart" function of the "vega" package. This analysis enabled us to parse the effects of each environmental factor. We used PERMANOVA and analysis of similarities (ANOSIM) with these BC dissimilarity matrices, using the function adonis in the R package vegan (Anderson et al. 2008; Oksanen et al. 2012). RDA was performed on soil microbial community. Before RDAs, environmental factors were screened with variance inflation factor (VIF) analysis, such that $p > 0.05$; factors with VIF > 10 were removed from further analyses. Hence, pH, SOM, NH_4-N, leaf N, leaf C/N, NO_3-N root N,

and root C/N were found to have VIF values greater than 10 and were thereby removed from downstream analyses. To further explore the pathways of N fertilization and CO_2 impacts on the composition of soil microbial communities, the relationship between CO_2 or N fertilization and plant and soil properties was quantified by Spearman's correlation analysis.

3 Results

3.1 Photosynthesis

ECO_2 alone and N fertilization alone significantly increased net Pn. Compared to N0_400, all of the treatments of N1 and N2 increased Pn, with the highest rate observed from N2_1000 treatments in July. In August, the treatments of N1 and N2, with the exception of N1_400, significantly increased Pn, as compared to N0_400; N2_750 revealed the highest levels of Pn. In September, there were no significant differences found among Pn in different N loads under 400 μmol·mol^{-1}. Likewise, when OTCs were not fertilized with N, no effects were detected from eCO_2 treatments. In July and August, CO_2 and N fertilization significantly affected Pn (SI-Table 1). Additionally, we found significant combined effects of N fertilization and CO_2 ($p = 0.022$ and 0.001, respectively for July and August). Although CO_2 had no effect on Pn during the month of September, we found both significant combined effects of CO_2 and N and significant effects of N fertilization on Pn (Fig. 1). In August and September, for the N0 treatment, eCO_2 did not affect Pn; yet, when N fertilization was supplied, eCO_2 significantly increased Pn, as compared to N0_400.

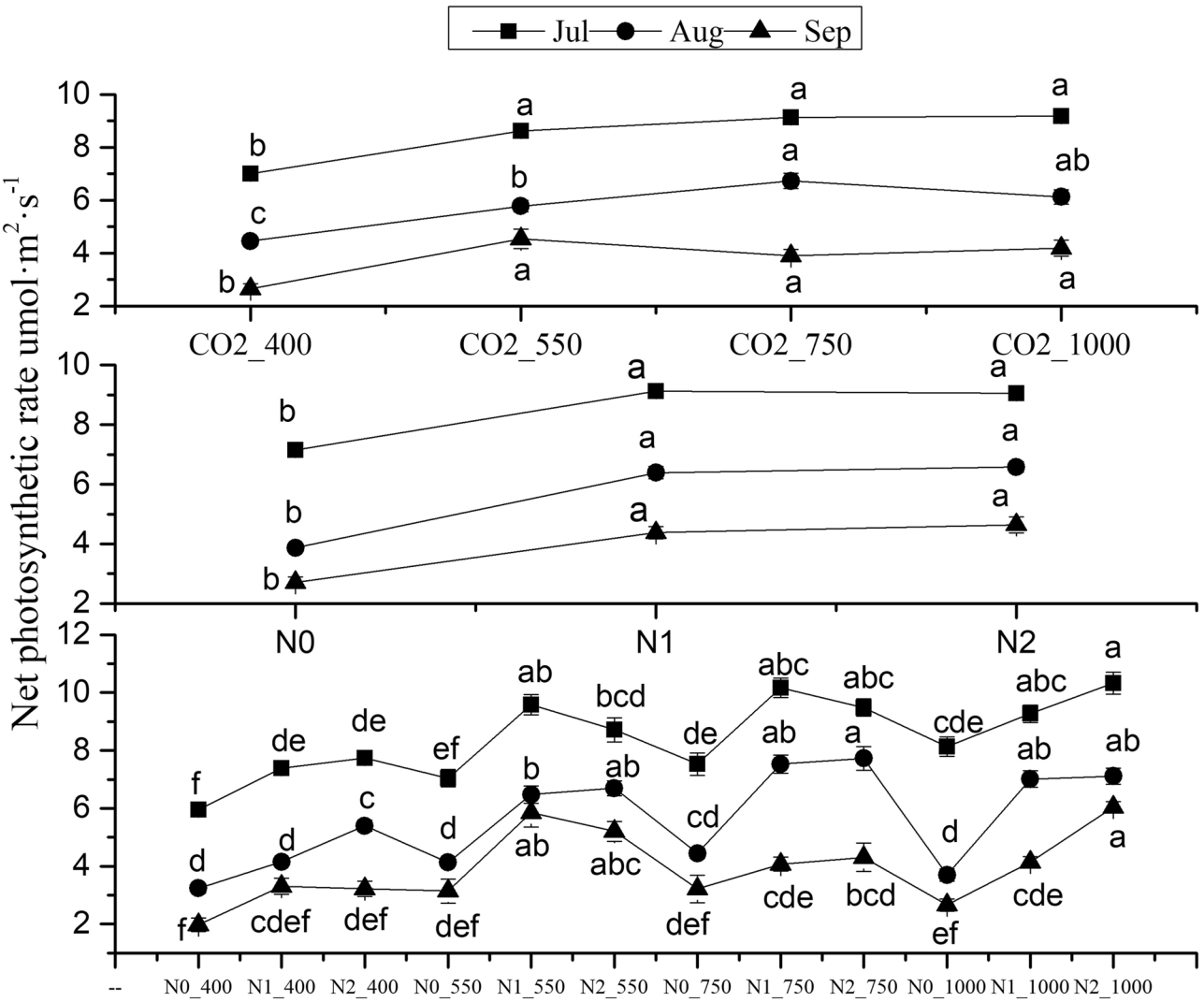

Fig. 1 The impacts of elevated CO_2 and N fertilization on photosynthesis in months July, Aug., and Sept. Different letters indicate significance at the 0.05 level

3.2 Plant biomass and plant C and N

While N fertilization alone significantly increased biomass (Fig. 2A, B), there were no detectable effects of eCO_2 alone on plant biomass. When both eCO_2 and N fertilization were considered in the analyses, our findings show that in combination with N0 treatments, eCO_2 did not affect biomass, as compared to ambient air; however, with N1 and N2 treatments, eCO_2 significantly increased stem, leaf, and total biomass in most treatments, with maximum biomass levels found in N2_1000 and N2_750 (Fig. 2C).

Regardless of CO_2 concentration or N fertilization, there were no detectable differences among treatments in total C content for either leaves or roots. Plant N content increased with increasing N loads; at any CO_2 concentration, N2 significantly increased the content of N, as compared to N0. Only CO2_1000 significantly decreased N content in leaves, as compared to ambient CO_2 (400 μmol·mol^{-1}) with N0 and N1 treatments. N content in roots decreased by CO2_1000, as compared to ambient CO_2 coupled with N1 treatments. The impacts of eCO_2 and N fertilization on plant N content resulted in opposite C/N responses, as N2 decreased C/N and eCO_2 increased C/N. Although no combined effects were detected between CO_2 and N fertilization (Fig. 3), both CO_2 concentration and N fertilization notably affected plant N content and C/N ratio ($p < 0.0001$).

3.3 Soil properties

Among CO_2 or N fertilization treatments, no differences were detected in soil organic matter and NH_4^+. When plants were exposed to ambient air and CO2_750 treatments, N2 significantly increased the content of soil NO_3^- compared to N0. The effects of CO_2 only persisted in plants with N1 fertilization; CO2_550, 750, and 1000 respectively decreased NO_3^- by 69%, 58%, and 66%, as compared to ambient air. When seedlings were exposed to ambient air, 550, and 750 μmol·mol^{-1} CO_2, N2 increased SMBC and SMBN, as compared to N0 and N1; however, these increments were only significant in plants fumigated with ambient air, while the increments in CO2_550 and CO2_750 were not

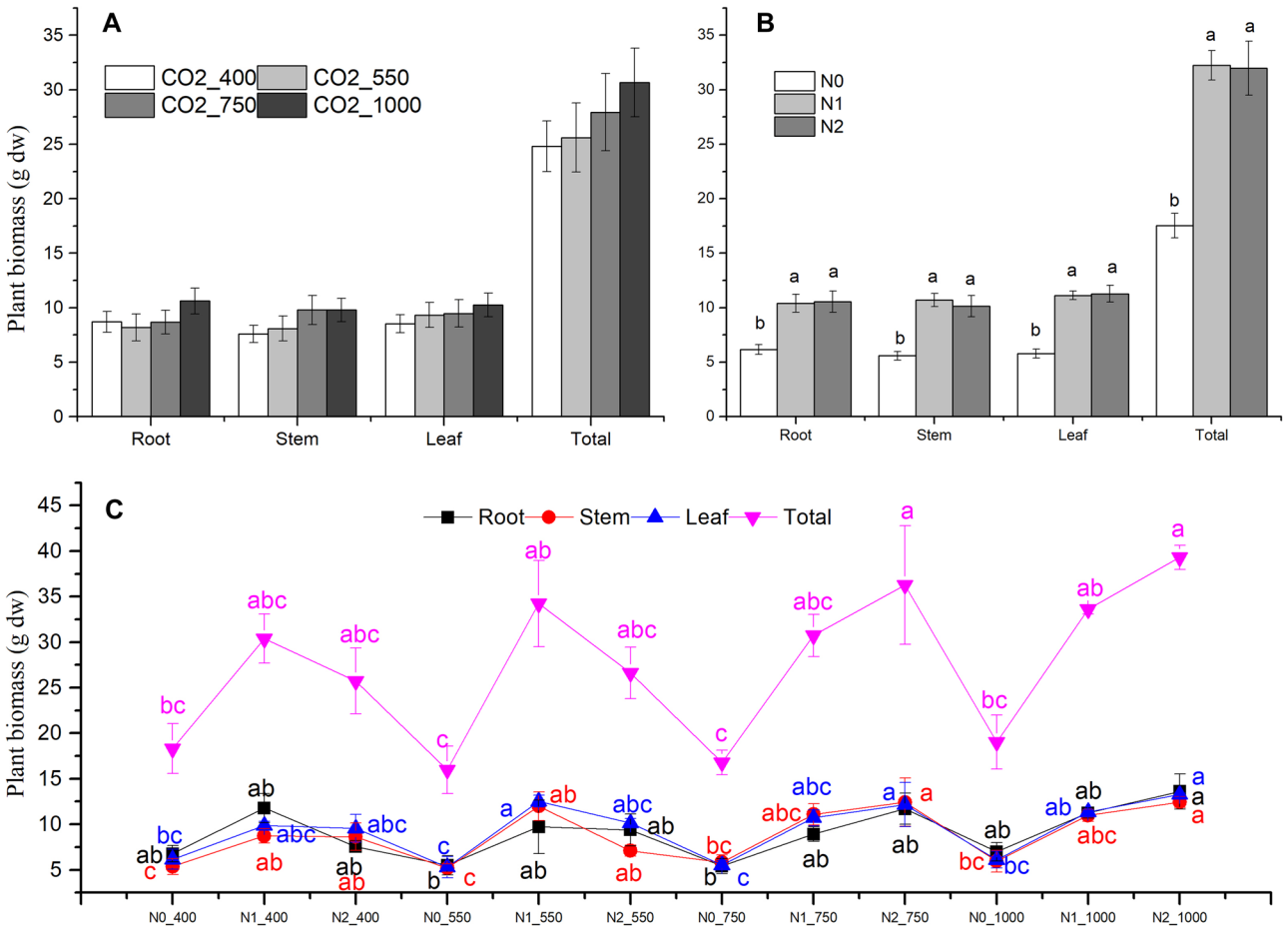

Fig. 2 Plant biomass in different elevated CO_2 and N fertilization treatments. Different letters indicate significance at the 0.05 level

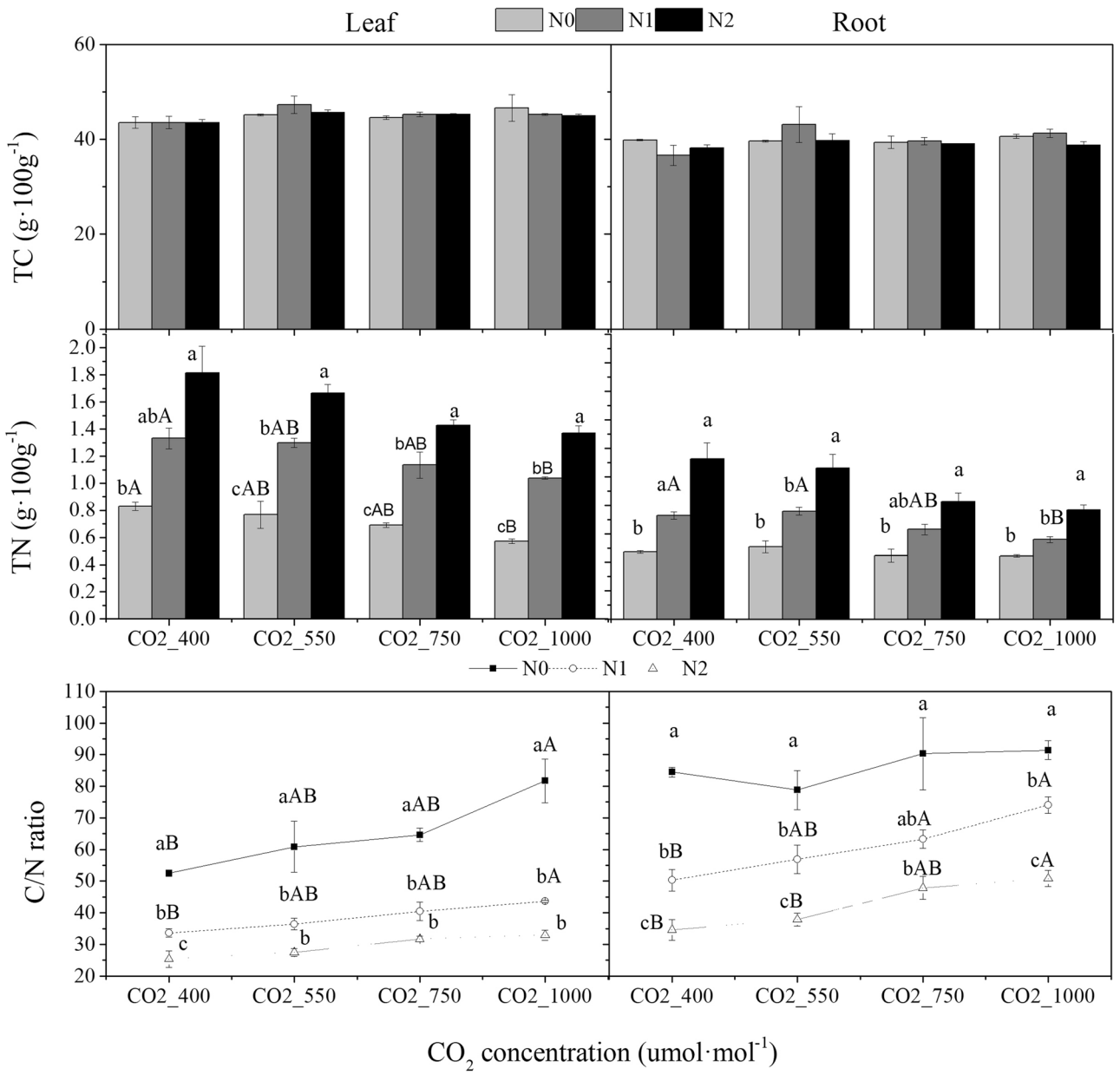

Fig. 3 The responses of C, N content and their C/N ratio in leaf and root to elevated CO_2 and N fertilization. Different capital letters indicate significance at the 0.05 level among different CO_2 concentrations under the same N treatment. Different lowercase letters indicate significance at the 0.05 level among different N fertilizations at the same CO_2 concentration

significant different, ostensibly because of large variance within the replicates (Table 1).

3.4 Soil microbial community structure

3.4.1 Soil microbial diversity

CO_2 and N fertilization did not affect soil bacterial Shannon diversity, while 750 and 1000 μmol·mol^{-1} CO_2 significantly decreased soil fungal Shannon diversity, as compared to ambient air in plants with N1 fertilizer. Two-factor ANOVA analyses indicated that CO_2 and N fertilization were not significant drivers of soil bacterial diversity; however, higher CO_2 (750 and 1000 μmol·mol^{-1}) significantly decreased soil fungal Shannon diversity under N1 and N2 treatments compared to N1_400, along with detectable interactions between CO_2 and N fertilization on ITS1 Chao (Table 2).

Table 1 The effects of elevated CO_2 and nitrogen fertilization on soil properties

	NH_4^+ mg·kg^{-1}			NO_3^- mg·kg^{-1}		
	N0	**N1**	**N2**	**N0**	**N1**	**N2**
CO2_400	0.25 ± 0.06	0.55 ± 0.029	1.12 ± 0.445	0.95 ± 0.035b	8.36 ± 1.27abA	25.87 ± 7.48a
CO2_550	0.57 ± 0.17	0.36 ± 0.035	0.68 ± 0.232	1.34 ± 0.196	2.56 ± 0.32B	19.21 ± 8.58
CO2_750	0.33 ± 0.08	0.44 ± 0.276	0.84 ± 0.144	1.10 ± 0.083b	3.52 ± 1.15bB	19.78 ± 1.63a
CO2_1000	0.33 ± 0.00	0.57 ± 0.285	0.58 ± 0.096	4.13 ± 3.115	2.82 ± 0.20bB	8.12 ± 5.16
CO_2	NS			NS		
N	0.016			< 0.0001		
CO_2 × N	NS			NS		
	SMBC mg·kg^{-1}			**SMBN mg·kg^{-1}**		
	N0	N1	N2	N0	N1	N2
CO2_400	5.36 ± 1.52bB	5.52 ± 1.24b	18.07 ± 4.97a	2.87 ± 0.47b	2.71 ± 0.45b	10.42 ± 2.25a
CO2_550	6.64 ± 0.60B	10.17 ± 2.99	16.48 ± 0.42	2.21 ± 0.88	2.74 ± 0.66	6.99 ± 2.86
CO2_750	5.89 ± 3.06B	6.07 ± 3.31	10.65 ± 4.93	3.21 ± 1.67	1.63 ± 0.78	6.41 ± 2.14
CO2_1000	20.19 ± 2.63A	15.99 ± 6.18	9.22 ± 2.67	1.73 ± 0.97	0.90 ± 0.75	2.19 ± 0.91
CO_2	NS			0.035		
N	NS			< 0.0001		
CO_2 × N	NS			NS		

The values are means ± SE (n = 3). Results followed by different capital letters are statistically significant at the 0.05 level among different CO_2 concentrations under the same N treatment; different lowercase letters indicating significance at the 0.05 level among different N fertilizations at the same CO_2 concentration. For the main effect of either eCO_2 or N fertilization, and the interaction between eCO_2 and N fertilization, p-values indicate significant effects of that factor on these soil property variables; "NS" indicate no significant effect of that factor on these response variables

SMBC soil microbial biomass carbon, *SMBN* soil microbial biomass nitrogen

Table 2 The effects of elevated CO_2 and nitrogen fertilization on soil microbial diversity

	Shannon–Wiener Index		**Chao**	
	16S	**ITS**	**16S**	**ITS**
N0_400	5.92 ± 0.234	4.38 ± 0.128abc	2393.98 ± 184.58ab	1261.11 ± 98.57a
N1_400	5.90 ± 0.048	4.59 ± 0.171a	2276.56 ± 45.37ab	793.17 ± 53.52de
N2_400	5.90 ± 0.052	4.31 ± 0.105abcd	2284.09 ± 57.43ab	1062.27 ± 59.74abcd
N0_550	5.94 ± 0.118	3.96 ± 0.248bcd	2158.87 ± 186.50b	724.90 ± 278.59e
N1_550	5.90 ± 0.082	4.27 ± 0.249abcd	2316.79 ± 51.32ab	1133.95 ± 64.04abc
N2_550	5.97 ± 0.044	4.47 ± 0.139ab	2437.64 ± 96.85a	828.77 ± 42.78cde
N0_750	5.84 ± 0.133	4.28 ± 0.191abcd	2491.04 ± 42.81a	1002.83 ± 23.16abcde
N1_750	5.90 ± 0.067	4.00 ± 0.073bcd	2416.02 ± 58.02ab	1097.93 ± 39.69abcde
N2_750	5.94 ± 0.076	3.83 ± 0.005d	2472.16 ± 51.71a	1101.99 ± 16.48abcd
N0_1000	5.95 ± 0.080	3.93 ± 0.128 cd	2425.60 ± 101.22ab	940.826 ± 103.20bcde
N1_1000	5.90 ± 0.103	4.01 ± 0.131bcd	2333.48 ± 50.59ab	1181.36 ± 89.26ab
N2_1000	5.82 ± 0.057	4.04 ± 0.351bcd	2374.45 ± 32.44ab	1170.39 ± 117.95ab
CO_2	NS	0.03	NS	NS
N	NS	NS	NS	NS
CO_2 × N	NS	NS	NS	0.013

The values are means ± SE (n = 3). Results followed by different capital letters are statistically significant at the 0.05 level among different CO_2 concentrations and N fertilization. For the main effect of either eCO_2 or N fertilization, and the interaction between eCO_2 and N fertilization, p-values indicate significant effects of that factor on these soil property variables; "NS" indicate no significant effect of that factor on these response variables

3.4.2 Soil microbial community structure and composition

PCoA illustrated distinct clustering for both soil prokaryote communities (Fig. 4A) and fungal community composition (Fig. 4B) by our CO_2 or N fertilization treatments. For the 16S, N fertilization appeared to be the major driver, as bacterial communities from N2 (dark color) clustered below the origin of PC1 and at the upper range of PC2 (Fig. 4A), which was distinct from bacterial communities from N0 (ANOSIM, $r = 0.1844$, $p = 0.001$). Although for fungal ITS1, CO_2 was a strong driver of differences among treatments, fungal communities from CO2_750 (triangle) and CO2_1000 (plus sign) were predominantly spread below the origin of PC2; fungal communities from CO2_400 (circle) and CO2_550 (square) were more scattered at the upper range of the origin of PC2 (Fig. 4B).

The dominant bacterial phyla across all treatments were *Chloroflexi*, *Acidobacteria*, and *Proteobacteria* (SI-Fig. 1A). ECO_2 remarkably enhanced the relative abundance of *Acidobacteria* ($p = 0.029$) in plants with N2 treatment and had no effects on other phyla (SI-Table 2). *Acidobacterial* and *Gemmatimonadetes* relative abundances both declined with exposure to our N2 treatments; however, when plants were fumigated with ambient air, more *Actinobacteria* was detected. When plants were exposed to ambient air and CO2_750, the relative abundance of the taxon GAL15 decreased, along with increases in the relative abundance of *Firmicutes* with N2, as compared to N0. In plants fumigated with ambient air and CO2_550, less abundance of *Nitrospirae* was detected with N2 (SI-Table 2). Across all the treatments, the most abundant fungal phylum was *Ascomycota*, followed by *Basidiomycota* and *Zygomycota* (SI-Fig. 1B).

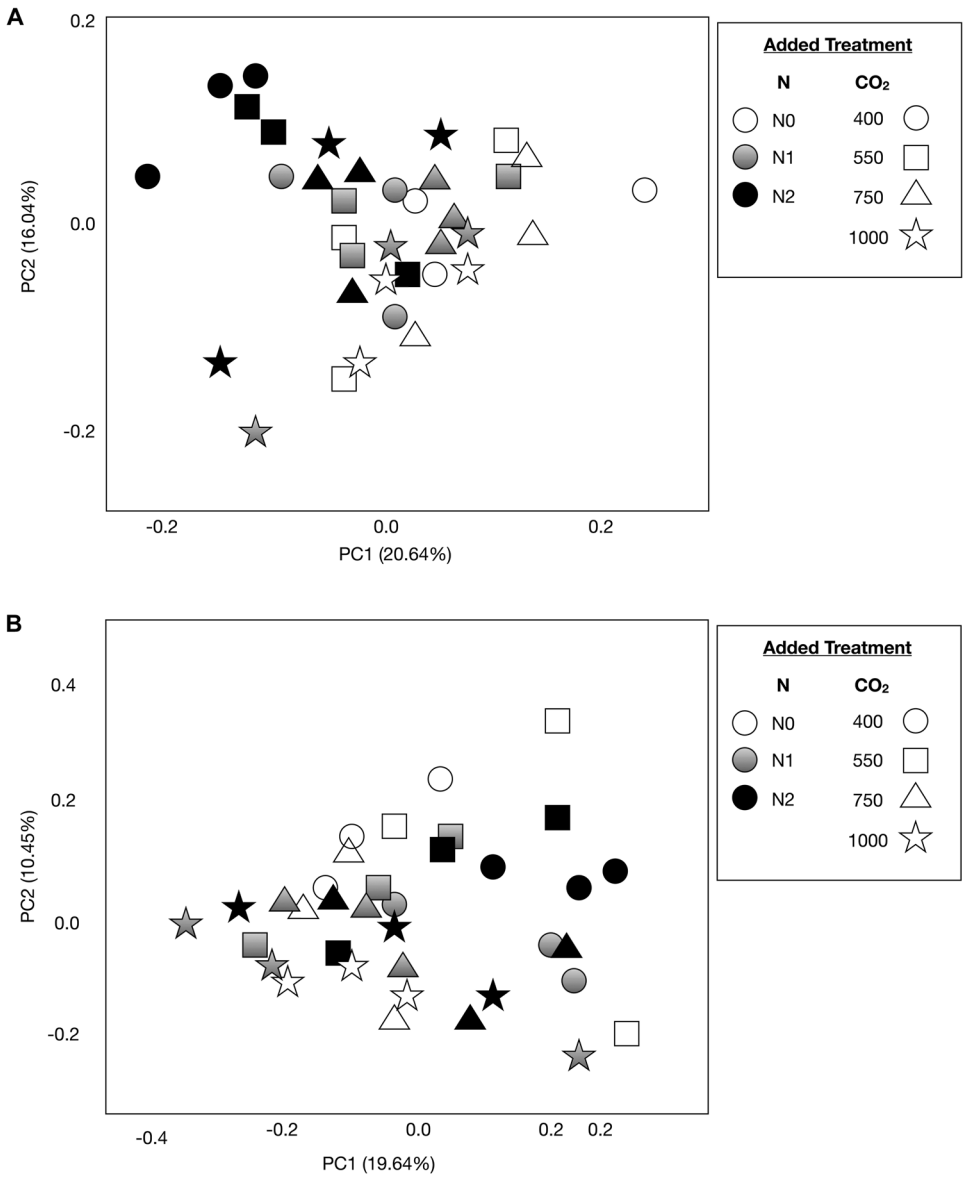

Fig. 4 Principal coordinates analysis of soil bacterial 16S **A** and fungal ITS **B** communities. Analyses of PERMANOVA show remarkable primary effects of N fertilization ($p = 0.001$) on soil bacterial communities, CO_2 ($p = 0.025$) on soil fungal communities, with significant interactive effects between N fertilization and CO_2 ($p = 0.005$ for 16S, $p = 0.044$ for ITS)

Table 3 The correlation among N fertilization, soil available N and plant C and N

	NH_4^+	NO_3^-	Root C	Root N	Root C/N	Leaf C	Leaf N	Leaf C/N
N fertilization	0.521	0.829	0.386	0.904	0.891	0.223	0.909	−0.914
	(0.001)	(<0.0001)	(0.02)	(<0.0001)	(<0.0001)	(0.192)	(<0.0001)	(<0.0001)
NH_4^+			−0.336	0.468	−0.514	−0.066	0.487	−0.503
			(0.045)	(0.004)	(0.001)	(0.703)	(0.003)	(0.002)
NO_3^-			−0.351	0.788	−0.805	0.128	0.829	−0.834
			(0.036)	(<0.0001)	(<0.0001)	(0.456)	(<0.0001)	(<0.0001)
Root C				−0.396	0.523	−0.010	−0.398	0.415
				(0.017)	(0.001)	(0.956)	(0.016)	(0.012)
Root N					−0.976	0.288	0.954	−0.945
					(<0.0001)	(0.089)	(<0.0001)	(<0.0001)
Leaf C								−0.203
								(0.234)
Leaf N								−0.993
								(<0.0001)

p-values (in the brackets) indicate significant correlation

Although eCO_2 had no significant effects on fungal phyla relative abundances, our findings show that both *Ascomycota* and *Chytridiomycota* relative abundances significantly responded to N fertilization (SI-Table 3).

3.4.3 Soil microbial community structure links to environmental properties

The contribution of edaphic and botanic variables, eCO_2, and N addition to both bacterial and fungal communities is illustrated with a modified VPA diagram (Fig. 5). Botanic variables primarily contributed to both 16S and ITS variation (respectively 24.64% and 17.70%) followed by edaphic variables. A slightly greater contribution of N fertilization (2.98%) on soil 16 S was detected compared to eCO_2 (2.17%) (Fig. 5A), while eCO_2 was much more important than N addition for soil 16S assembly, with 15.33% and 9.00% contributions respectively (Fig. 5B). PERMANOVA analyses indicated remarkable effects of N fertilization ($p=0.001$) on soil bacterial communities. Furthermore, CO_2 ($p=0.02$) significantly affected soil fungal communities, with significant interactions detected between N fertilization and CO_2 ($p=0.005$ for 16S, $p=0.044$ for ITS). RDA plots indicated that soil NO_3-N strongly drove patterns of soil bacterial community composition, followed by root C/N (Fig. 6A). However, leaf N and leaf C/N predominantly drove the patterns observed for soil fungal community structure (Fig. 6B). PERMANOVA analyses indicated that root C/N ($p=0.001$), soil NO_3-N ($p=0.001$), leaf N ($p=0.001$), root N ($p=0.001$), SOM ($p=0.003$), soil pH ($p=0.004$), leaf C/N ($p=0.004$), and soil NH_4-N ($p=0.033$) significantly affected soil bacterial community structure. Additionally, leaf C/N ($p=0.004$), root C/N ($p=0.016$), leaf N ($p=0.01$), and root N ($p=0.046$) significantly affected soil fungal community composition.

Nitrogen fertilization was correlated with leaf C/N, soil NO_3-N, root C/N, leaf N, and root N ($p<0.0001$), as per Spearman's correlation analysis; N fertilization was also correlated with root C ($p=0.02$) and soil NH_4-N ($p=0.001$). Furthermore, soil nitrate and ammonium were significantly correlated with both root and leaf nitrogen, as well as with root and leaf C/N (Table 3). When all the treatments were included in Spearman's analyses, CO_2 concentrations were significantly correlated with Pn in both July and August, as

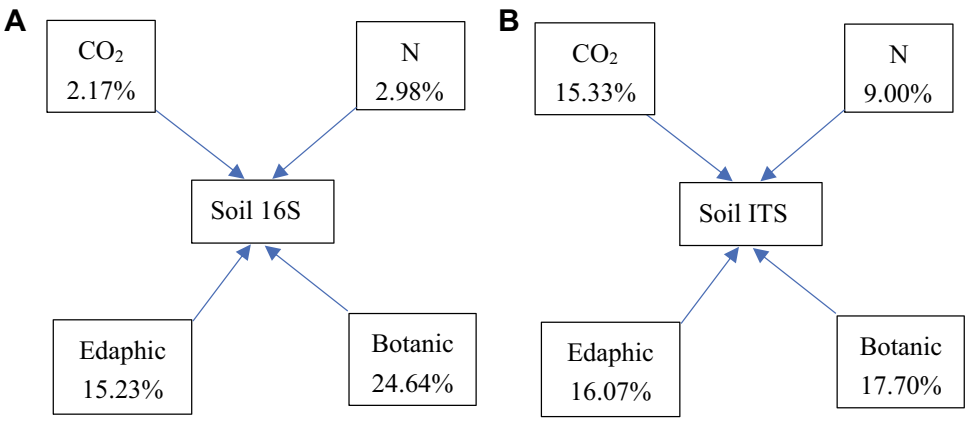

Fig. 5 Variation partitioning analysis (VPA) differentiating effects of soil bacterial **A** and fungal **B** community

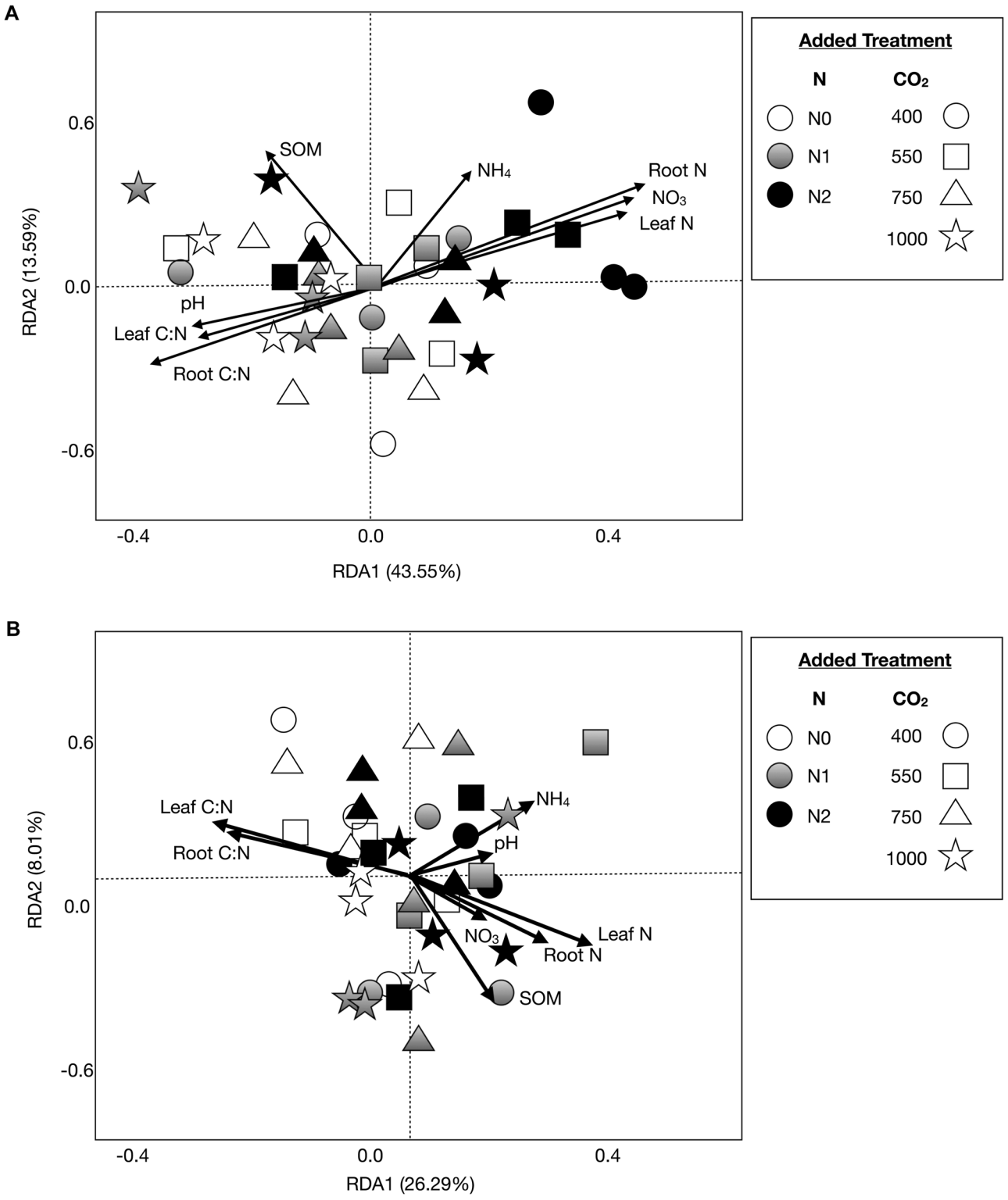

Fig. 6 Redundancy analysis to detect the responses of soil bacterial **A** and fungal **B** community to elevated CO_2 plus N fertilization and correlations with edaphic properties

well as with root C. However, CO_2 concentrations were not correlated with either leaf N or leaf C/N. Yet, we did detect significant correlations between CO_2 and leaf N, as well as between CO_2 and leaf C/N (Table 4).

4 Discussion

Although prior work has examined the effects of N fertilization and eCO_2 on both above- and belowground terrestrial systems (Feng et al. 2010; Fransson and Johansson 2010; Weber et al. 2013), there remains a dearth of information about the extent of higher concentrations of CO_2 (e.g., 1000 μmol·mol^{-1} at RCP8.5) alone on plants and microbial communities in terrestrial ecosystems, or its combined influence with N fertilization on plant growth and soil microbial community structure. In our study, seedlings of *S. superba* were exposed to eCO_2 gradients separately and in combination with N fertilization to investigate the responses of plant growth and soil microorganisms.

Previous studies have shown that eCO_2 positively affects plant gas exchange (Jacotot et al. 2018; Je et al. 2018; Terrer et al. 2018). For instance, the global meta-analysis by Ainsworth and Long (2005) illustrated the effect of eCO_2 on stimulating leaf photosynthesis in CO_2 enrichment experiments (i.e., Free Air CO_2 Enrichment; FACE) over 15 years, corresponding to an overall stimulation of 31%. Likewise, our study showed that eCO_2 stimulated leaf-level Pn within most treatments. Yet, we did detect some context-dependency and temporal variability. When plants were exposed to CO2_1000 without N fertilization, Pn was significantly increased in July, but not in August and September, as compared to CO2_400. This may be attributed to limited foliar N, such that when plants were fertilized with N1 and N2 treatments, eCO_2 significantly increased Pn, as compared to N0_400 for all measured parameters. Irrespective of CO_2 concentration, higher N fertilization may have increased leaf N, resulting in significant stimulation of Pn by N1 and N2, as compared to N0. Similarly, reduced leaf N by eCO_2 was also reported in *Eucalyptus globulus*, ostensibly via photosynthetic adaption of *E. globulus* leaves to eCO_2; this was likely caused by reduced quantities of leaf N and Rubisco (Sharwood et al. 2017). In our study, eCO_2 also reduced leaf N; this may contribute, in part, to the CO_2-induced negative feedback on plant N uptake (Xu et al. 2020). We also detected a strong correlation between leaf N and Pn (Table 4). As reviewed by Peterson (1999), the relationship of photosynthesis and leaf N is usually linear and clearly expressed across a wide range of taxa. Approximately 20% of leaf N is invested into Rubisco and about 75% of the N in leaves is invested into plant photosynthetic organs (Evans and Seemann 1989); therefore, when leaf N decreased in plants exposed to eCO_2, Pn would likely also be inhibited. However, when plants were exposed to higher N fertilization, these resources may have supplied more N transportation to leaves, thereby alleviating leaf N limitation on Pn, especially in conditions where plants were exposed to higher CO_2 concentration.

Although eCO_2 and N fertilization each significantly affected plant photosynthesis, there were no effects of these treatments on either leaf or root C. With the exception of N2 treatments, eCO_2 significantly increased leaf C/N. Root C/N significantly increased, except within N0 treatments. Additionally, N fertilization significantly decreased both leaf and root C/N. This is evidenced by the decrease of eCO_2 and improvement of N fertilization on leaf or root N, as well as the absence of detectable effects of eCO_2 on leaf and root C.

Previous work shows in red maple (*Acer rubrum* L.), systems show that *A. rubrum* seedlings, which were exposed to either 800 or 800 μmol·mol^{-1} CO_2 for one growing season, result in no responses in measured leaf C content; however, in this same system, leaf N concurrently decreased and C/N markedly increased (Li et al. 2019). Across 110 experimental field-based experiments, it was shown that eCO_2 does not significantly affect C concentration (Nie et al. 2013). In this study, Nie et al. (2013) summarized these findings and concluded that eCO_2 consistently had no effect on C concentration across research facilities and ecosystems, even with regard to the duration of experimental treatments. Yet,

Table 4 The correlation among CO_2 concentration, Pn, and plant C and N

	Pn in July	Pn in Aug	Pn in Sep	Leaf C	Leaf N	Leaf C/N	Root C	Root N	Root C/N
CO_2 concentration	0.555	0.472	0.234	0.048	−0.311	0.311	0.340	−0.270	0.328
	(<0.0001)	(0.004)	(0.197)	(0.782)	(0.065)	(0.065)	(0.043)	(0.111)	(0.051)
Pn in July				0.198	0.359	−0.352	0.005	0.368	−0.312
				(0.246)	(0.032)	(0.035)	(0.979)	(0.027)	(0.064)
Pn in Aug				0.141	0.492	−0.501	−0.112	0.487	−0.438
				(0.419)	(0.003)	(0.002)	(0.523)	(0.003)	(0.009)
Pn in Sep				0.239	0.446	−0.464	−0.069	0.406	−0.372
				(0.187)	(0.011)	(0.007)	(0.708)	(0.021)	(0.036)

p-values (in the brackets) indicate significant correlation

for 7.8% of those studies, decreased root N concentration resulted in greater root C/N. In our present study, which was consistent with findings reported by Nie et al. (2013), we showed that CO2_750 and CO2_1000 increased root C/N respectively by 6.9% and 8.2% (SI-Table 4) in plants without N fertilization. On the other hand, when plants received N1 and N2 fertilization, eCO_2 increased root C/N by 9–47%, which may be attributed to an accumulation of root N concentration, as associated with N fertilization (SI-Table 4).

Although eCO_2 alone may differentially affect plant biomass, N2_750 and N2_1000 significantly increased stem, leaf, and total biomass, as compared to N0_400. In the latter, we found positive effects of N fertilization on plant performance, as measured via biomass. Some reports indicate that eCO_2 increased biomass of tree species after only one growing season (Dijkstra et al. 2002; Li et al. 2019). Yet, other research shows that biomass production responses to eCO_2 generally varies between species, growing seasons, and experimental conditions (Ainsworth et al. 2005). Even when the plants of *Betula pendula*, *Alnus glutinosa*, and *Fagus sylvatica* were planted within the same FACE at the same density, after the first year of eCO_2 fumigation, only the aboveground biomass of *A. glutinosa* significantly increased (Smith et al. 2013). With CO_2 as the only environmental factor (i.e., N fertilization was not considered), the relative effects of CO2_1000 (more than 20%) were much greater than that of CO2_550 and CO2_750, as compared to CO2_400 (SI-Table 5), with the exception of stem biomass. As reviewed by Overdieck (2016), after 1 year of approximately 650–700 $\mu mol \cdot mol^{-1}$ CO_2 enrichment, the total biomass of *Prunus serotine* decreased by 0.5% and that of *Sorbus aucuparia* increased by 7.0%; yet, after 2–5 years fumigation, the biomass of some other species increased to a larger extent (from 20.0 to 66.0%). Our results showed that 1000 $\mu mol \cdot mol^{-1}$ gradient with the N2 treatment substantially increased plant biomass, even within one growing season; for RCP8.5 (more than 1000 $\mu mol \cdot mol^{-1}$ CO_2 plus more N deposition), this indicates that the effects of plant biomass may amplify over a greater duration of time, potentially by more than 20.0–66.0%, as was demonstrated in the 650–700 $\mu mol \cdot mol^{-1}$ CO_2 gradient. Therefore, we were forced to reject our first hypothesis that the RCP8.5 treatment would induce slow stimulation of plant biomass.

There are many experiments which indicate that the composition of soil microbial communities is affected solely by changes in eCO_2 (Carney et al. 2007; Zhou et al. 2011; Deng et al. 2016), as well as by N fertilization and eCO_2 (Feng et al. 2010; Weber et al. 2013). In contrast, other studies show that these soil microbial community parameters may not be affected by the eCO_2 factor alone (Klamer et al. 2002; Niklaus et al. 2007). Across other previously published work, similar findings confirmed that eCO_2 primarily affected soil fungal community structure, with little to no effects on soil bacteria (Lipson et al. 2005) or archaea, as was observed for this investigation. In our study, eCO_2 significantly affected soil fungal community structure indicated by VPA and PERMANOVA results. Moreover, both VPA and PERMANOVA confirmed that N fertilization was a main driver of soil bacterial community structure, with significant interactions detected for both soil fungal and bacterial or archaeal community structures. Many reports confirm that eCO_2 affects soil fungal biomass, abundance, diversity, and community structure (Lipson et al. 2005; Carney et al. 2007). This suggests that lower N availability with eCO_2 may constrain fungal growth or processes, as fungi are likely to have greater C/N than bacteria. This higher C/N may ameliorate fungal demand for N (Hu et al. 2001). However, soil C/N was not increased by eCO_2 in this study. RDA and PERMANOVA analysis revealed that leaf N and C/N strongly determined soil fungal community structure, which indicates that the indirect effects of eCO_2 on fungal community structure may have been top-down, as predicted by our second hypothesis. It is generally thought that plant root production and exudation may mediate the influence of eCO_2 on soil microbes (Lipson et al. 2005). In this study, PCoA depicted soil fungal community structures within the CO2_1000 gradient was significantly different than fungal community composition in CO2_400, regardless of different N treatments ($p = 0.013$). Likewise, root biomass increased by 21.97% in CO2_1000, as compared to CO2_400 (SI-Table 5). ECO_2 decreased leaf N, accompanied by increased leaf C/N and alterations to photosynthetic rates, which may affect root exudates and enrich rhizosphere communities with photosynthates. Although measuring the impacts of eCO_2 on root exudation and the relationship between leaf N (leaf C/N) and root exudates were beyond the scope of this study, this avenue of research warrants future investigation.

Although some studies show that bacterial biomass does not respond to N fertilization (Billings and Ziegler 2008), soil bacterial community structures have been known to significantly change, as shown within the Duke Forest FACE investigation (Feng et al. 2010); this was consistent with our results from N fertilization and soil bacteria. Similar to findings from Fierer et al. (2012), our study showed that higher N fertilization stimulated the relative abundance of *Actinobacteria*, while concurrently decreasing that of *Acidobacteria*. In line with Cederlund et al. (2014), higher N fertilization corresponded with decreases in the relative abundance of the *Gemmatimonadetes* microbial group. N fertilization likely directly influences decomposition, by alleviating limiting enzymatic components, therefore increasing potential enzyme activity, as well as indirectly by altering the composition of downstream substrates, resulting from organic matter decomposition. The relative abundance of *Firmicutes* responded with increasing N availability, which is not surprising as many taxa from *Firmicutes* use xylose,

an abundant source of labile C within natural environments (Zhao et al. 2009). Furthermore, gram-positive bacteria from *Actinobacteria* may respond to varying degrees of N availability, as these bacterial taxa are key microbial players in organic matter decomposition (Kramer and Gleixner 2008). In contrast, oligotrophic groups, such as from *Gemmatimonadetes* (Tardy et al. 2015) and *Acidobacteria* (Fierer et al. 2012), are often characterized by lower growth rates along with an increased capacity for metabolic processing of recalcitrant C sources or other nutrient-poor substrates (Fierer et al. 2012). This putative shift from microbial characteristics along an oligotroph-copiotroph spectrum may have resulted directly from the increasing N availability with N fertilization, as growth of copiotrophic taxa tends to require higher N loads than is required by more oligotrophic taxa (Fierer et al. 2012). RDA and PERMANOVA analysis showed that soil NO_3^- and root C/N were the largest drivers for soil 16S community structure. As predicted by our third hypothesis, N fertilization supplies more available N, which directly affects soil bacterial structure, including the composition or abundance of key microbes involved in N cycling. Our work shows that enrichment of available N increases root N content, resulting in lower root C/N, which may affect soil bacterial community structure.

ECO_2 and N fertilization significantly interacted, and the consequences of these interactions likely affect both soil fungal and bacterial communities. For example, the relative abundances of both *Firmicutes* and *Actinobacteria* notably increased by N fertilization when plants were exposed to ambient air; however, this stimulation by N fertilization disappeared when plants were exposed to all eCO_2 treatments. In contrast, we detected decreases in the relative abundances of other microbial groups; most notably, *Gemmatimonadetes* and *Acidobacteria* decreased in treatments fertilized with N in ambient air; yet, when plants were exposed to eCO_2, N fertilization had no effect on these phyla. This indicates that eCO_2 and N fertilization may have yielded contrasting effects on copiotrophic and oligotrophic microbial groups, which mediate soil microbial community structure and nutrient cycling in these forested ecosystems.

5 Conclusion

When seedlings of *Schima superba* were exposed to eCO_2 and N fertilization for only one growing season, with conditions based on RCPs defined in IPCC 2014, the highest CO_2 concentrations, which reached an upward limit of 1000 μmol mol^{-1} significantly increased Pn in July; however, these effects were not evident in either August or September. Yet, when CO_2 gradients were combined with N fertilization treatments, Pn also significantly increased in August and September. Although eCO_2 alone did not affect plant biomass, eCO_2 together with N fertilization significantly increased plant biomass, as compared to those seedlings exposed to ambient air, without any additional N. Soil fungal community structure was driven mainly by eCO_2, which may lead to eCO_2-induced modifications of plant-associated C and N. Moreover, our analyses revealed that fertilization with N primarily drove compositional differences in soil bacterial communities. This highlights the effects of N fertilization belowground, via supplying greater available N, thus indirectly stimulating N content aboveground through increasing root N. In this study, after only one growing season, the significant effects of eCO_2 combined with N fertilization have been found both above- and belowground. These findings, within such a short time frame, suggest that the effects of global change may exacerbate both functional changes and structural impacts to soil microbial communities, as well as plant development, which could in turn affect global C budgets. Given that these complex ecological challenges, as predicted by global change models, elicit effects on both above- and belowground ecosystems, we have greater evidence that these feedback mechanisms will require deft mitigation and motivation for future studies on the putative effects of these climate factors on forested ecosystems.

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Declarations

Conflict of interest The authors declare no competing interests.

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