Original Research

Effects of Experimental Warming and CO$_2$ Concentration Doubling on ^{13}C CPMAS NMR Spectra of Humin in Coniferous Forest Ecosystems of the Eastern Tibetan Plateau in China

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Abstract

Despite the perceived importance of humin to forest ecosystem C sequestration, few studies have attempted to examine the responses to elevated temperature (ET, +2.5ºC) and elevated atmospheric CO$_2$ concentration (EC, 700 ppm) on the chemical structures of humin. Solid-state ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CPMAS NMR) spectroscopy was applied to evaluate the 6-year effect of ET and EC on the chemical structure of humin under the coniferous forest ecosystems in the eastern Tibetan Plateau of China. Results showed that ET treatment decreased soil organic carbon (SOC), whereas EC and ETC treatment increased SOC. ET treatment decreased aromatic C and carbonyl C of humin while increasing alkyl C, the ratios of alkyl C/O-alkyl C, aliphatic C/aromatic C, and hydrophobic C/hydrophilic C. Compared with ET treatment, the ETC treatment had a similar but reduced impact on the chemical structure of humin, while EC had slightly and undetectable impact on the chemical structure of humin. The 6-year exposure to ET decreased SOC and changed the molecular structure of soil humin to be more alkyline and hydrophobic, and it was a protection mechanism to the sequestration and stability of organic C in soil.

Keywords: ^{13}C CPMAS NMR, humin, elevated temperature, elevated CO$_2$ concentration

Introduction

Soil organic matter (SOM), an important C reservoir, is influenced strongly by global climate change [1]. Temperature and atmospheric CO$_2$ concentration are two of the key factors controlling the C cycling processes in terrestrial ecosystems. IPCC [2] predicted that an increment of global mean air temperature is about 0.6-4.0ºC and that atmospheric CO$_2$ concentration will be about 490-1260 ppm by the end of this century. Much work has been done to probe the ecosystem response to climate change, but debate on the C balance between input and output is still ongoing due to the inhomogeneous ecosystem, such as vegetation types, the

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relative sizes of C pools, and the chemical composition and initial turnover rates of SOM [3-5]. Global environmental change is projected to alter the efflux of CO_2 from soil respiratory C loss through altering the quality and quantity of SOM [6-9]. Moreover, the functions and C sequestration capacity of SOM are closely related to their chemical structure [10-13]. Chemical recalcitrance of specific molecules is regarded as an important mechanism for the stabilization of soil organic matter (SOM), determining an intimate interaction between C cycling and structure stability [12]. Therefore, many efforts have been made to clarify the structural composition of humic substances [10, 14]. During recent years, solid-state ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C-CPMAS-NMR) has already been increasingly applied to identify a detailed molecular characterization and the contents of humified constituents in either bulk soils or isolated fractions [15-19]. Analysis of soil chemical composition with molecular techniques can further our understanding of how SOM functions as a source and sink for atmospheric CO_2.

On the basis of the solubility in aqueous solution of different pH values, humic substances can be divided into three fractions, namely humic acid, fulvic acid, and humin [20-22]. Humin, one of the most importantly stable fractions of soil humic substances, is a highly heterogeneous organic compound formed during biogeochemical degradation of plant animal residues and condensation of the degraded segments. To date, most studies have focused on the response of humic and fulvic to the environmental changes, due to its relative sensitivity, which provided a more pronounced feedback in the early stage [23]. However, humin, the most relative recalcitrant fraction, is regarded as an important fraction of soil organic matter [24, 25], determining the long-term response to climate change. Although it plays an important role in the C sequestration, much uncertainty remains regarding its chemical structure owing to inhomogeneity, structural complexity, and insolubility [26, 27].

The Qinghai-Tibetan Plateau (the 'third pole' of earth) is the world's largest plateau. With a pace of about twice the observed rate of global warming, the temperature has increased nearly by 0.2ºC per decade over the past 30 years [28]. *Abies faxoniana* is one of these keystone species and is widely distributed in the subalpine coniferous ecosystems of this region [29, 30]. Changes of soil C function groups can be used to predict possible future soil carbon storage under global climate change. Hence, in this study we conducted an experiment to examine the response of the chemical structure of humin in the soils of *A. faxoniana* plots under ET and EC. Moreover, we hypothesize that: (1) ET, EC, or ETC would change the functional groups of humin differently and (2) the molecular structure of soil humin would be prone to be more hydrophobic under ET and EC.

Material and Methods

Site Description

The experiment was conducted in the Maoxian Ecological Experimental Station of the Chinese Academy of Sciences in Sichuan Province, southwestern China (31°41′N, 103°63′E, 1820 m a.s.l). In this region, the mean annual temperature is 9.3ºC, mean annual precipitation is 826.2 mm·y^{-1}, and the average amount of annual sunshine is 1373.8 hours. Approximately 70% of annual precipitation falls during the summer. The soil was classified as mountain brown soil series (Chinese taxonomy). The vegetation at this site was dominated by *Picea asperata*, *A. faxoniana*, and *Pinus tabulaeformis*.

Experimental Design and Treatments

The experiments were carried out in the enclosed-top fumigation chambers. In September 2004, the planting boxes were filled with undisturbed bulk soil (about 30 cm) taken from a natural *A. faxoniana* forest, and 8-year-old *A. faxoniana* seedlings of a uniform size from a local nursery were transplanted to 48 planting boxes (50×30×30 cm). There were 6 boxes [2(chambers)×3(boxes)] of each treatment. These seedlings were watered frequently to keep the soil water content at 30%. The planting boxes were subjected to CO_2 concentration and temperature enhancement treatments during the growing season (from April to October).

The chamber consisted of two parts, including a 1.5 m-high cylindrical structure of 11 glass walls and a hemispherical roof, with internal volume of 24.6 m^3 and ground area of 9.4 m^2. The total light received by the seedlings was about 70% of ambient light during the experimental period. Additional parameters of the chambers were uniform with a previous similar study [31]. A computer-controlled heating and cooling system (LT/WSK-PLC, BQHGCT Co. Led., Beijing, China) linked to a set of magneto electric valves to control the pure CO_2 supply was installed on the top of each chamber, which kept the temperature and CO_2 concentration adjusted automatically inside the chambers to ensure the outside ambient concentration (about 350 ppm), or to achieve a two-fold CO_2 concentration of the outside ambient concentration (about 700 ppm) and/or a rise in temperature (+2.5ºC above the ambient temperature). The four treatments in this study were as follows: (1) ET: elevated temperate, (2) EC: elevated CO_2, (3) ETC: a combination of elevated temperate and elevated CO_2, and (4) CK: control.

Soil Sampling and Processing

Soil was sampled in October 2010 with a hand auger from depths of 0-15 cm in three boxes of each treatment. Soil samples at field-moist conditions were further picked by hand to remove visible plant material

and passed through a 2-mm sieve, air dried, and stored for later analyses. Due to the high cost of ^{13}C CPMAS NMR, the soil samples from each treatment were pooled to form one soil sample per sampling. Soil organic carbon (SOC) was determined through an elemental analyzer (Milti N/C 2100S).

The pretreatment method of soil sample for ^{13}C CPMAS NMR was based on the procedures described by Preston et al. [32] and Zhang et al. [26] 10 g of air-dry soil sample and 80 ml of distilled water were added into each plastic centrifuge tube and shaken for 12 hours at 26ºC in a thermostatic water bath oscillator. Each tube was centrifuged at 3500r/min for 15 minutes and the supernatant was discarded to remove the poorly decomposed light fraction floating in the water. The same treatment was performed by replacing distilled water with equal volumes of 0.026 m H_2SO_4 to remove carbonates. And then 80 ml of 0.1 m NaOH + 0.1 m $Na_4P_2O_7$ with 6% (w/v) $Na_2SO_4 \cdot 10H_2O$ was added to the soil residue to remove humic and fulvic acids. This treatment was repeated about eight times until the supernatant was nearly colorless and the remaining alkaline-insoluble solid residual in the tube was HM. Then HM was treated successively with 80 ml 0.6, 2.6, 6, 10, 20, 30, and 40% (v/v) HF-HCl solution for either 7, 7, 11, 6, 4, or 2 times, and once, respectively. The residues should then repeatedly be washed with distilled water and centrifuged to remove the supernatant until it was free of chloride anions as determined by $AgNO_3$. The humin was freeze-dried and then ground to pass a 0.1-mm sieve before analysis of the ^{13}C CPMAS NMR.

Analysis of Humin

The solid-state ^{13}C CPMAS NMR experiments were obtained on a Bruker AVANCE III 600 spectrometer (Switzerland) operating at 150.9 MHz and a spinning rate of 7 KHz. The contact time was 2 ms, recycle time was 4s, and 2048 data points were collected. Chemical shifts were referenced relative to external Hexamethylbenzene (HMB).

The chemical shift regions were expressed as the percentage of the area of the total ^{13}C NMR spectrum and divided into four main chemical shift regions according to the spectral characteristics through a MestReNova software package. The spectral areas were consistent with that of Li et al. [15] and Mathers and Xu [16]. These regions are as follows: 0-50 ppm (alkyl C), 50-110 ppm (O-alkyl C), 110-160 ppm (aromatic C), and 160-200 ppm (carbonyl C). Moreover, O-alkyl C was further divided into a region of methoxyl C (50-60 ppm), carbohydrate C (60-95 ppm), and di-O-alkyl C (95-110 ppm); and aromatic C detailed allocated in a region of aryl C (110-145 ppm) and phenolic C (145-160 ppm). The ratios of alkyl C to O-alkyl C, aliphatic C to aromatic C, and hydrophobic C to hydrophilic C were calculated according to Zhang et al. [26]. Aliphatic C/Aromatic C = (Alkyl C+O-alkyl

Table 1. The content of SOC under different treatments.

Treatment	SOC(g/kg)
ETC	126.46
EC	103.20
ET	60.96
CK	91.39

C)/Aromatic C; Hydrophobic C/Hydrophilic C = (Alkyl C+Aromatic C)/(O-alkyl C+ Carbonyl C).

Results and Discussion

SOC Content

The values of soil organic carbon (SOC) under different treatments are shown in Table 1. The SOC contents, ranging from 60.96 to 126.46 g/kg, varied among different treatments. In comparison with the CK treatment, SOC increased by 12.92% and 38.37% under the EC treatment and the ETC treatment, respectively. The extent of increment under the ETC treatment was more than that under the EC treatment. The increase of SOC is mainly attributed to the greater aboveground and underground products for the improvement of photosynthetic capacity under the exposure to EC and ETC. The extents of photosynthetic enhancement vary greatly among different plant species, duration of elevated CO_2 exposure, and soil nutrient status [33]. Though the photosynthesis rate is initially stimulated by elevated CO_2, the initial stimulation of growth usually cannot last for a long time due to all kinds of reasons, such as soil nutrient limitations [34]. However, the ET treatment decreased the SOC content by 33.29% compared with the CK treatment. Temperature is a key factor that regulates the carbon balance between soil organic matter decomposition and C uptake through photosynthesis. In this paper, warming decreased the SOC content and led to a faster decay when the forest soils store high concentrations of organic carbon in cold regions. The impacts of warming on the decomposition of SOC might be either due to effects of elevated temperature on microbial community structure and activity or soil nutrient availability.

Solid-State ^{13}C NMR Spectroscopy of Humin Samples

The relative proportion of organic C functional groups identified by solid-state ^{13}C CP MAS NMR spectroscopy (Fig. 1) indicated that humin samples from all the treatments displayed similar spectral patterns, with a predominance of the O-alkyl-C (50-110 ppm) region (about 48.6% of total intensity). O-alkyl C was followed by alkyl C (25.5% of total intensity) and aromatic C (15.5%), and the smallest intensity was
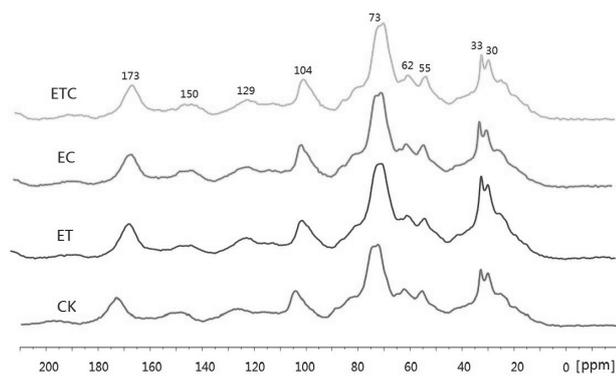
Fig. 1. ^{13}C CPMAS NMR spectra of soil humin in soil under different fertilizer treatments: (i) (ET), (ii) (EC), (iii) (ETC), (iv) (CK).

observed in the carbonyl C region (10.5%). Moreover, carbohydrate C showed the greatest intensity (31.6%) in the O-alkyl C region, and the intensity of aryl C (10.4%) was the highest in the aromatic C region. Humin from the Tibetan region included alkyl C, O-alkyl C, aromatic C, and carboxyl C, and it was shown to follow the order of O-alkyl-C > alkyl-C > carboxyl-C > aromatic-C. The O-alkyl-C structures, with much protonated C, were easily degradable chemical compounds [35]. Meanwhile, the aromatic-C mainly derived from lignin and tannin, and bacterial resynthesized compounds consisting of alkyl-C and carboxyl-C [36]. The relatively high O-alkyl-C content and the relatively low aromatic-C content indicated the poor humification degree of humin in this kind of soil ecosystem. The chemical structure characteristics of humin were similar to those observed in other reports [37]. However, the peaks of the specific compounds detected in our experiment were different from those of Zhang et al. [26], who identified larger intensity and more dominant peaks in the alkyl C region of humin and a lower O-alkyl C region in a typical Hapludoll substrate of northeast China as compared with this study. A reason for the difference might be attributed to a preferential accumulation of O-alkyl-C group in forest soil due to the high quantities of organic carbon in the high-altitude forest soil of the cold region, while the extent of SOM decomposition by microbial oxidation was greater to meet crop demands under cultivation tillage. This kind of difference mainly

depends on the different ecosystem types and largely influenced the quantitative and qualitative functional groups of SOM.

In the alkyl C region, the dominant peak at 30 ppm represented the resonance of the amorphous $-(CH_2)n-$, and the resonance at 33 ppm was attributed to crystalline $-(CH_2)_n-$[38]. In the O-alkyl C region, four peaks (at 55, 62, 73, and 104 ppm) were attributed to methoxyl C, carbinol alcohol-C structure, the -CH(OH)- in carbohydrate, and double oxygen-C in polysaccharide, respectively [37,39]. In the region for aromatic C, the signal of aryl C, appearing at 129 ppm, could be mainly attributed to the aromatic C substituted by -COOH or -COOMe and the unsubstituted aromatic meta carbons bearing an oxygen or nitrogen atom [40]. And the signal of 150 ppm was very weak and indicated the O-substituted aryl carbons [38]. In the carbonyl-C region, the resonance peak of 173 ppm corresponded to carboxyl-C of the amide and polypeptide [37]. The resonance was a declining trend in the order of 73>33>30~31>62>55>104>173>129>150, namely, the -CH(OH)- in carbohydrate > crystalline -(CH$_2$)n- > the amorphous $-(CH_2)n-$ > carbinol alcohol-C structure > methoxyl C > and double oxygen-C in polysaccharide > carboxyl-C of the amide and polypeptide > the aromatic C substituted by -COOH or -COOMe and the unsubstituted aromatic meta carbons bearing an oxygen or nitrogen atom > O-substituted aryl carbons.

Response of Humin to Climate Change

The extents of change for different C functional groups under the ET treatment were generally greatest among the different treatments (Table 2). Compared with the CK treatment samples, ET treatment increased alkyl C by 13%, while O-alkyl C, aromatic C, and carbonyl C decreased by 2%, 10%, and 7%, respectively. This result indicated that ET treatment led to higher decomposition of humin as compared to CK. In general, the increment of alkyl-C during decomposition was mainly attributable to the accumulation of polymethylene during the original plant materials' aerobic decomposition [41].

These different C functional groups of humin had slightly or undetectable responses under the ETC and EC treatment, and the extent of intensity change for these C functional groups were less than 3% relative to CK treatment. The extent of intensity changes for

Table 2. Distribution of different C types from ^{13}C CPMAS NMR spectra of soil humin under different treatments.

Treatment	Alkyl C	O-alkyl C				Aromatic C			Carbonyl C
		Methoxyl C	Carbohydrate C	Di-O-alkyl C	Total	Aryl C	Phenolic C	Total	
ETC	25.30	7.35	31.21	9.69	48.25	10.42	5.24	15.66	10.8
EC	24.37	7.30	31.59	9.94	48.83	10.68	5.42	15.10	10.71
ET	27.79	7.12	31.59	9.51	48.22	9.84	4.41	14.25	9.75
CK	24.50	7.15	32.06	9.90	49.11	10.60	6.26	15.85	10.54

the C functional groups were much less under the EC treatment than under the ET treatment. It was probable that ET treatment can elicit changes in molecular structure via direct effects on microbial or via plant activity. However, EC only elicited little changes in molecular structure. The response to ETC was intermediate and smaller than the response to ET alone in this experiment. The interactions of ET and EC in soil were potentially complex. Dieleman et al. [42] also indicated that interactive effects of elevated CO_2 and warming may not simply result in additive responses, but might elicit synergistic or antagonistic responses.

Compared with the CK treatment samples, the increased extents for the ratios of alkyl C/O-alkyl C, aliphatic C/aromatic C, and hydrophobic C/hydrophilic C of humin under the ET treatment were generally greatest (15.5%, 14.9%, and 7.2%, respectively), followed by the ETC treatment (5.1%, 1.1%, and 2.5%, respectively). The smallest was the EC treatment (nearly undetected change) (Table 3). The proportion of alkyl-C/O-alkyl-C has been used as an important index of the degree of decomposition or humification of soil SOC. In this study the larger value of alkyl C/O-alkyl C ratio under ET was attributed by a relative enrichment of alkyl C and a decrease in the O-alkyl C region, indicating that ET increased the stability of humin and the resistance to further C loss as compared with CK. The majority of the alkyl C can be attributed to carbons in rigid structures with attached hydrogens, such as surface waxes, lipids, cutins, and resins, and be relatively stable against microbial attack [43]. However, O-alkyl C is a relatively easily decomposable fraction. The increase in this region was also consistent with another report in the Qinghai-Tibetan Plateau [11], which indicated that warming enhanced most labile SOC oxidation and enlarged the amount of recalcitrant C. A probable reason for this was that the elevated temperature increased soil microbial activity. Then ET treatment led to the increased utilization of O-alkyl C in humin and a relative enrichment of alkyl C.

The aliphatic C/aromatic C ratio has been used as an index of the aliphatic degree of humic substances. The increase of aliphatic C/aromatic C ratio under ET reflected a less aromatic structure and a smaller extent of condensation in humin, as compared with CK.

The molecular structure of humin tended to be simplified under a 6-year ET treatment. The larger value of aliphatic C/aromatic C ratio under ET was attributed from a relative enrichment of aliphatic C and a decrease in the aromatic C region. The increased decomposition of aromaticity was in accordance with the decrease of SOC under the ET treatment. This change might partly depend on the shifts in the soil microbial community structure and microbial substrate utilization patterns under ET [44]. However, the result in this paper was contradictory to that of Balaria Ankit et al. [45], who indicated that warmer soils under low-elevation sites were richer in aromatic C due to the accumulation of lignin and lignin derivatives during decomposition. Much of the increment in the aromatic C under ET may be attributed to the quantity of litter from broad-leaved forests in the low-elevation region. But litter in this experiment would have contributed little to soil during the 6-year exposure period due to the little new-formed litter from evergreen coniferous seedlings.

Increments of the hydrophobic C/hydrophilic C ratio have also been found in this study, which indicated that the hydrophobic degree and the stability of humin were enhanced under the ET treatment, as compared with CK. The trend in the hydrophobic C/hydrophilic C ratio of humin was identical to that of the alkyl C/O-alkyl C ratio, which further suggested that ET was favorable for degradation of ROC and then the sequestrated stability of soil organic C. The stability of soil was greater under ET than the control because of the accumulated recalcitrant C. The enrichment of hydrophobic structure of humin may increase the stability of SOM and was more resistant to further rapid C loss from forest soils, which corresponded to the mechanisms of C sequestration obtained by Zhou et al. [46], who suggested that differentially stimulating genes for degrading labile but not recalcitrant carbon was one of the primary mechanisms to maintaining long-term soil carbon stability and storage through integrated metagenomic and functional analyses of a long-term warming experiment in a grassland ecosystem.

Conclusions

ET treatment decreased SOC and decreased aromatic C and carbonyl C of humin while increasing alkyl C, the ratios of alkyl C/O-alkyl C, aliphatic C/aromatic C, and hydrophobic C/hydrophilic C. Compared with ET treatment, the ETC treatment had a similar but reduced impact on the chemical structure of humin, while EC had slight and undetectable responses to the chemical structure of humin. The 6-year exposure to ET increased the decomposition of soil organic matter and changed the molecular structure of soil humin to be more alkyl and hydrophobic, and it was beneficial to the sequestration and stability of organic C in soil.

Table 3. Ratios of alkyl C to O-alkyl C, aliphatic C to aromatic C, and hydrophobic C to hydrophilic C from ¹³C CPMAS NMR spectra of soil humin under different treatments.

Treatment	Aliphatic C/Aromatic C	Alkyl C/O-alkyl C	Hydrophobic C/Hydrophilic C
ETC	4.70	0.52	0.69
EC	4.55	0.50	0.68
ET	5.33	0.58	0.73
CK	4.64	0.50	0.68

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Conflict of Interest

The authors declare no conflict of interest.

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