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Elevated Atmospheric CO₂ and Drought Affect Soil Microbial Community and Functional Diversity Associated with *Glycine max*

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Abstract: Under the background of climate change, the increase of atmospheric CO₂ and drought frequency have been considered as significant influencers on the soil microbial communities and the yield and quality of crop. In this study, impacts of increased ambient CO₂ and drought on soil microbial structure and functional diversity of a Stagnic Anthrosol were investigated in phytotron growth chambers, by testing two representative CO₂ levels, three soil moisture levels, and two soil cover types (with or without *Glycine max*). The 16S rDNA and 18S rDNA fragments were amplified to analyze the functional diversity of fungi and bacteria. Results showed that rhizosphere microbial biomass and community structure were significantly affected by drought, but effects differed between fungi and bacteria. Drought adaptation of fungi was found to be easier than that of bacteria. The diversity of fungi was less affected by drought than that of bacteria, evidenced by their higher diversity. Severe drought reduced soil microbial functional diversity and restrained the metabolic activity. Elevated CO₂ alone, in the absence of crops (bare soil), did not enhance the metabolic activity of soil microorganisms. Generally, due to the co-functioning of plant and soil microorganisms in water and nutrient use, plants have major impacts on the soil microbial community, leading to atmospheric CO₂ enrichment, but cannot significantly reduce the impacts of drought on soil microorganisms.

Keywords: enzyme activities, microbial functional diversity, fungi and bacteria, relative abundance.

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INTRODUCTION

Low soil water contents decrease the microbial abundance (Bloem et al., 1992), an effect known to be induced by changes in soil nutrient and water use efficiency (Djekoun and Planchon, 1991; Yi et al., 2007). On the other hand, the atmospheric concentration of CO₂ has been increasing due to emissions from human activities during the last century (IPCC, 2007). It was supposed that increased ambient CO₂ could raise the C/N ratio, altering the soil nutrient components (Hu et al., 1999; Kassem et al., 2008; Wang et al., 2008), consequently affecting the abundance and diversity of the microbial community and influencing soil microbial functions.

However, there is a controversy about the impacts of elevated CO₂ concentration on the soil microbial system (Bazzaz, 1990). The high heterogeneity of soil physical and chemical properties, high variability of soil microbial community, and the highly complex plant-microbial interaction are the reason for different results of studies assessing the influence of elevated CO₂ (Rees et al., 2005). A two-year open-air experiment of carbon dioxide enrichment (FACE) indicated that elevated CO₂ concentrations had insignificant effects on the increase of soil microbial biomass carbon and microbial diversity (Schortemeyer et al., 1996; Rønn et al., 2002; Dam et al., 2017). However, in a previous 8-year study, highly elevated CO₂ significantly changed the soil microbial community structure (Williams et al., 2000). It was concluded that elevated CO₂ induces loop modifications in the plant-microbe system, although the significance of these effects can have different levels, according to the CO₂ enhancement level and exposure time (Wong, 1990).

Although the individual effects of elevated CO₂ or drought on the soil microbe system have been documented in many articles, few studies have addressed the combined effects of elevated CO₂ and drought on soil microbial community structure and functional diversity in cultivated soil (Yonemura et al., 1998). In particular, on the background of climate change, the occurrence frequency of drought tends to increase, significantly affecting the microbial functions of agroecosystem. Thus, a further discussion is necessary to determine their influences and mutual effects.

We hypothesized that: 1) elevated CO₂ concentration insignificantly enhance the metabolic activity in the cultivated soil; 2) severe drought decreases microbial functional diversity and restrain the metabolic activity of soil microbes. In this sense, a greenhouse experiment was conducted to understand the short-term interactive effects of elevated CO₂ and drought on the changes of soil microbial community structure and functional diversity, in soil under *Glycine max* (soybean).

MATERIALS AND METHODS

Experimental design

Soil samples of a Stagnic Anthrosol were collected in March 2013 from the surface layer (0.00-0.15 m) in the Yangtze River Delta, Shanghai. After removing organic debris and rocks, soil samples were sieved (<10 mm), homogenized, and blended into a single composite sample. The samples were filled in pots (0.25 m height × 0.25 m diameter) and divided into 12 treatments, with three replications. A factorial design was used (Table 1), with two representative CO₂ levels, three soil moisture levels, and two soil cover types [with or without *Glycine max* (Yu 19)]. Twelve beans per pot were inserted at 0.05 m depth in the soil. The pots were incubated in a chamber (PRX-2000C-CO₂, China) in random positions. Before germination, the chamber temperature was maintained at 25 °C, light intensity at 600 μmol m⁻² s⁻¹, and relative air humidity at 60 %. Daily watering was performed at 8:00 a.m. No fertilizer was applied during incubation, and CO₂ was monitored with a build-in infrared CO₂ meter (TES 1370, Taiwan).

Table 1. Design of treatments: 3 × 2 × 2 factorial arrangement of soil moisture, elevated CO₂ level, and soil cover (with or without *Glycine max* plant)

Soil moisture	Ambient CO ₂ 350 ± 50 mL m ⁻³		Elevated CO ₂ 700 ± 50 mL m ⁻³	
	bare	soybean	bare	soybean
Wet (80 % WHC)	UAW	PAW	UCW	PCW
Mild drought (60 % WHC)	UAM	PAM	UCM	PCM
Severe drought (40 % WHC)	UAS	PAS	UCS	PCS

WHC: water holding capacity; UAW: unplanted treatment with ambient CO₂ and well-watered condition; UAM: unplanted treatment with ambient CO₂ and mild drought condition; UAS: unplanted treatment with ambient CO₂ and severe drought condition; PAW: planted treatment with ambient CO₂ and well-watered condition; PAM: planted treatment with ambient CO₂ and mild drought condition; PAS: planted treatment with ambient CO₂ and severe drought condition; UCW: unplanted treatment with elevated CO₂ and well-watered condition; UCM: unplanted treatment with elevated CO₂ and mild drought condition; UCS: unplanted treatment with elevated CO₂ and severe drought condition; PCW: planted treatment with elevated CO₂ and well-watered condition; PCM: planted treatment with elevated CO₂ and mild drought condition; PCS: planted treatment with elevated CO₂ and severe drought condition.

Functional and genetic diversity of the soil microbial community

After 60-day incubation, genomic DNA of the soil samples was extracted with the DNA isolation kit (PowerSoil, USA). The 16S rDNA and 18S rDNA fragments were amplified to analyze the functional diversity of the soil microbial community. The F338-GC and R518 were used as bacterium-specific primers (Muyzer et al., 1993). For fungi, 18S rDNA fragments were amplified with the primer pair of FR1-GC and FF390, as described by Vainio and Hantula (2000). The reaction mixture contained 4 µL dNTP, 3 µL MgCl₂, 1 µL of each primer, 0.25 µL Taq polymerase, and 2 µL DNA template.

A touchdown PCR was programmed, according to the methodology described by Zhang et al. (2012). Firstly, 25 µL PCR products were loaded on an 8 % polyacrylamide gel. The denaturing gradient ranged from 40 to 80 %. Electrophoresis was carried out with the Dcode system (Bio-Rad, USA), performed for 12 h at 80 V and 60 °C. Thereafter, the gel was stained with ethylene dibromide 0.5 µg mL⁻¹ for 30 min and visualized under UV with BIO-RAD (BIO-Rad XR, USA).

A bacterial identification system (BIOLLOG MicroPlate, USA) assessed the functional diversity of microorganisms based on the patterns of 31 single carbon sources. At the end of incubation, 10 g of soil sample was added to a 50 mL sterilized Erlenmeyer flask with 0.5 mol L⁻¹ phosphate buffer (pH = 7.0). The mixture was sealed and shaken for 10 min in the dark. A microplate was preheated to 25 °C. Then, 150 µL of diluted supernatant (1:1,000) was added to each well of the plate and incubated at 25 °C. The UV absorption (590 nm) of each well was determined after 12, 24, 48, 72, 129, and 144 h of incubation. The data were corrected based on the initial readings of the control well containing only water.

Statistics

The data are expressed as the mean values of three replicates per treatment. Two-way ANOVA measurement was used to identify significant differences among the treatments at α = 0.05. Statistical procedures were carried out with Software IBM SPSS Statistics (version 11.0). Shannon-Wiener (H') and Simpson (D) indices were calculated to estimate the microbial diversity. Pielou (E) and McIntosh (D_{MC}) indices were used to determine the microbial evenness. Cluster analysis (CA) was conducted to detect similarities of PCR-DGGE banding patterns among treatments with Quantity One Software (Bio-Rad). Microbial carbon source utilization was analyzed with Principal Component Analysis (PCA), to identify latent relationships among the different treatments.

RESULTS AND DISCUSSION

Response of microbial relative abundance and diversity

Denaturing gradient gel electrophoresis patterns showed significant differences among treatments ($p < 0.05$) in the number of bands and density of soil bacteria (Figure 1a). In general, drought significantly affected relative abundance and diversity of soil bacteria. In the crop treatments (PAW, PAM, PAS, PCW, PCM, and PCS), the number of bands and signal density were higher than in the uncultivated treatments, indicating higher bacterial diversity and relative abundance. For instance, bands (1 to 4) in treatment 7 and 12 were much denser than in the other treatments. The main reason for the increase in bacterial diversity in the crop treatments is that dissolved organic carbon (DOC) released by soybean can be used as carbon source for the growth and metabolism of these microorganisms (Wang et al., 2017). It has been reported that the below-ground biomass of *Canna indica* was 4.30 ± 0.83 g per plant, for roots with a diameter of less than 1 mm (Lai et al., 2011). Moreover, the oxygen release and transfer rates of plants can also contribute to the growth of aerobic microbes around the roots of macrophytes. In the literature, similar findings also indicated that the biodiversity was increased by rhizosphere bacteria over that of uncultivated areas (Wang et al., 2017). In crop treatments, bacterial abundance and diversity were increased at elevated CO₂ ($p > 0.05$). However, in uncultivated treatments, drought inhibited soil bacteria significantly ($p < 0.05$). Although the soil bacteria community structure did not change with drought, the relative abundance declined. The effect of elevated CO₂ concentration on relative abundance or density was not significant in any treatment ($p > 0.05$).

The fungi DGGE pattern was presented in figure 1b. The fungal community structure differed significantly among treatments ($p < 0.05$). The number and density of fungi bands were lower than those of bacteria in the same treatment ($p < 0.05$). This indicated a lower fungal than bacterial abundance and diversity, although drought had limited impacts on fungal abundance and diversity. The highest similarity was found in drought treatments. The relative abundance and diversity of fungi in bare soil were higher than in the crop treatments. This difference might be caused by root exudates that inhibited fungal development. In the crop treatments, denser bands (b and c) showed that an elevated CO₂ concentration increased the fungal abundance and diversity ($p > 0.05$).

Diversity and evenness indices of soil bacteria and fungi calculated from DGGE results indicated that drought reduced bacterial abundance (Table 2). Relative abundance and diversity of bacteria decreased with soil moisture, indicating that drought significantly inhibited bacterial growth. However, fungal abundance and diversity increased when soil moisture dropped. The potential of autochthonous *Arbuscular Mycorrhizal* fungal species and particularly their mixture with *Bacillus thuringiensis* of protecting plants against drought and helping plants to thrive in semiarid ecosystems was described by Armada et al. (2016). Thus, we concluded that the increase in fungal species under drought might be beneficial for the growth of soybean plants. Apart from extreme drought conditions, elevated CO₂ concentration would increase fungal abundance and diversity during water stress. Drought was also observed to stimulate fungal development in bare soil. It is known that the adaption of fungi to drought and low matric potential is easier than that of bacteria (Kohler et al., 2010). A reason could be that partial microbial death provided nutrients for fungi during drought adaption. However, fungal abundance did not increase in response to the different CO₂ concentrations, due to the nitrogen restriction (Guenet et al., 2012).

According to the dendrogram of microbial clustering analysis (Figure 2), apart from soil moisture, soil cover was a main factor affecting the microbial community structure. Previous studies indicated that higher CO₂ concentrations (20 %) could benefit the development of fungal abundance when soil water moisture met the demand for plant growth (Xue et al., 2017). Soil microbes were indirectly stimulated by the accumulation

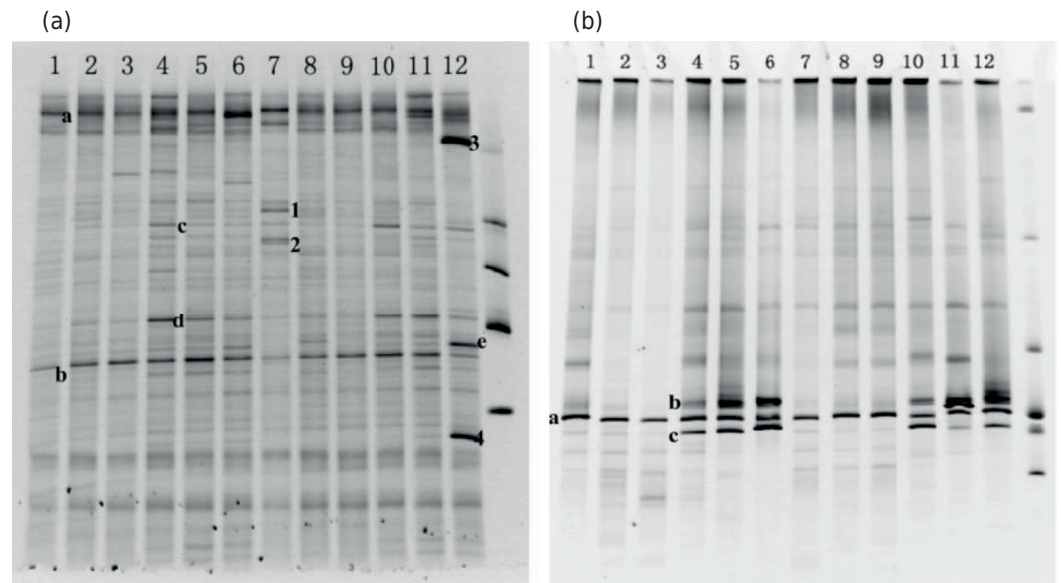


Figure 1. Denaturing gradient gel electrophoresis (DGGE) analyses of 16S rDNA fragments for bacteria (a) and 18S rDNA fragments for fungi (b) in different treatments. 1: unplanted treatment with ambient CO₂ and well-watered condition; 2: unplanted treatment with ambient CO₂ and mild drought condition; 3: unplanted treatment with ambient CO₂ and severe drought condition; 4: planted treatment with ambient CO₂ and well-watered condition; 5: planted treatment with ambient CO₂ and mild drought condition; 6: planted treatment with ambient CO₂ and severe drought condition; 7: unplanted treatment with elevated CO₂ and well-watered condition; 8: unplanted treatment with elevated CO₂ and mild drought condition; 9: unplanted treatment with elevated CO₂ and severe drought condition; 10: planted treatment with elevated CO₂ and well-watered condition; 11: planted treatment with elevated CO₂ and mild drought condition; and 12: planted treatment with elevated CO₂ and severe drought condition.

Table 2. Relative abundance and diversity indices calculated from the DGGE pattern of soil bacterial and fungal community under different treatment of soil moisture, elevated CO₂ level, and soil cover (with or without *Glycine max* plant)

CO ₂	Cover	WHC	H' _{bact}	D _{bact}	E _{bact}	D _{Mc bact}	H' _{fungi}	D _{fungi}	E _{fungi}	D _{Mc fungi}
700 ppm	bare	80 %	3.112	0.916	0.859	0.801	2.374	0.886	0.925	0.682
		60 %	2.844	0.915	0.854	0.753	2.678	0.910	0.926	0.719
		40 %	2.751	0.912	0.877	0.750	2.650	0.911	0.935	0.723
	soybean	80 %	3.207	0.944	0.888	0.811	2.667	0.918	0.941	0.731
		60 %	3.130	0.940	0.903	0.795	2.658	0.919	0.938	0.731
		40 %	2.884	0.919	0.865	0.758	2.151	0.859	0.897	0.643
350 ppm	bare	80 %	2.894	0.906	0.848	0.769	2.335	0.875	0.910	0.667
		60 %	2.931	0.928	0.880	0.770	2.609	0.911	0.941	0.720
		40 %	2.325	0.838	0.742	0.624	2.631	0.910	0.949	0.726
	soybean	80 %	2.919	0.927	0.867	0.776	2.631	0.917	0.949	0.729
		60 %	3.120	0.941	0.908	0.804	2.467	0.902	0.935	0.704
		40 %	2.858	0.922	0.867	0.771	2.427	0.902	0.946	0.703

Shannon-Wiener (H') and Simpson (D) indices were used to estimate the microbial diversity; Pielou (E) and McIntosh (DMc) indices were used to estimate the microbial diversity. WHC: water holding capacity.

of root exudation and potential allelochemicals at elevated CO₂ concentration (Lin et al., 1999). In addition, plant growth can contribute to the decrease of soil nitrogen by root uptake. A reduction in soil nitrogen use may benefit the growth and metabolism of microorganisms (Rakshit et al., 2012). This stimulation was not significant when the soil CO₂ concentration was much higher than in the atmosphere. In bare soil, elevated CO₂ concentration affected soil microbes by changing the oxygen and nutrient supply by enlarging the particle size of soil aggregates (Niklaus et al., 2003), but such influence

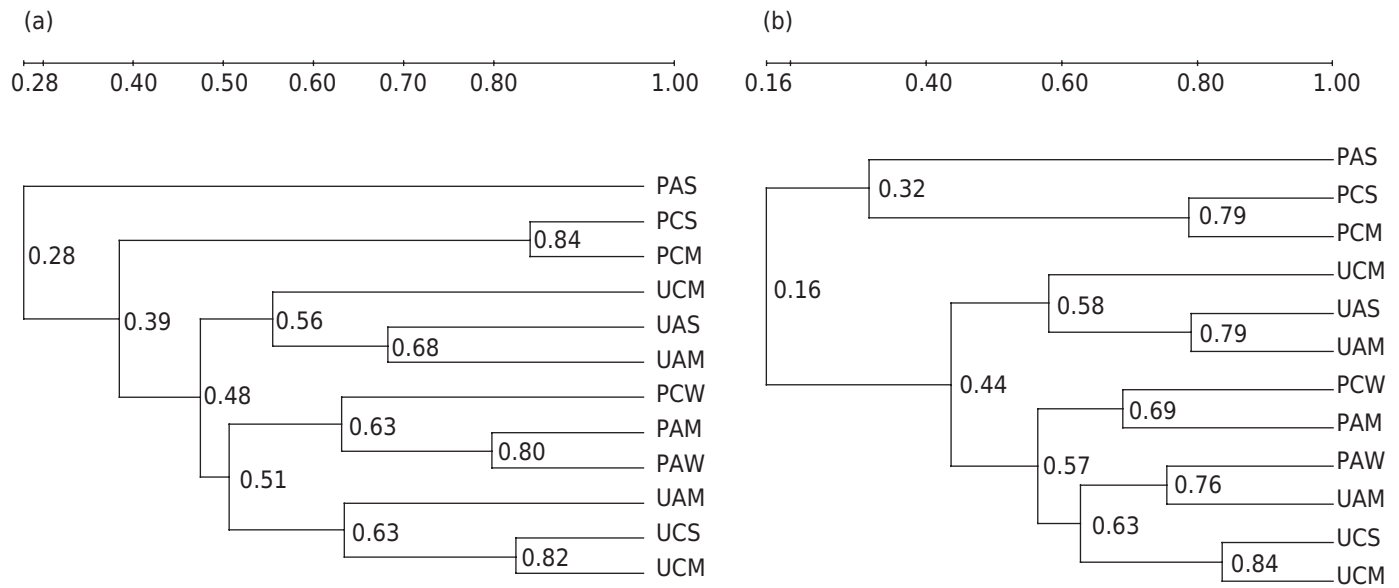


Figure 2. Dendrogram constructed with the complete similarity linkage between bacteria-specific (a) and fungi-specific (b) PCR-DGGE patterns for different treatments. UAW: unplanted treatment with ambient CO₂ and well-watered condition; UAM: unplanted treatment with ambient CO₂ and mild drought condition; UAS: unplanted treatment with ambient CO₂ and severe drought condition; PAW: planted treatment with ambient CO₂ and well-watered condition; PAM: planted treatment with ambient CO₂ and mild drought condition; PAS: planted treatment with ambient CO₂ and severe drought condition; UCW: unplanted treatment with elevated CO₂ and well-watered condition; UCM: unplanted treatment with elevated CO₂ and mild drought condition; UCS: unplanted treatment with elevated CO₂ and severe drought condition; PCW: planted treatment with elevated CO₂ and well-watered condition; PCM: planted treatment with elevated CO₂ and mild drought condition; PCS: planted treatment with elevated CO₂ and severe drought condition.

depended on the sufficiency of nitrogen restriction or soil type (Bruce et al., 2000). Thus, we assumed that plants minimized the bacteria-inhibiting drought impacts.

Response of microbial functional diversity

Based on the utilization patterns of 31 single carbon sources, microbial functional diversity was assessed by testing the capability of the microbial community to adapt their metabolism to various abiotic conditions. The variation in average well color development (AWCD) over time (Figure 3) followed an asymptotic sigmoidal curve, but differed significantly among treatments. Soil microbial carbon source utilization was insignificant in 24 h. However, the increase was rapidly intensified in the following 12 h. After 108 h, the upward trend was slowed down. Compared to those in bare soil, microorganisms showed higher metabolic activity in cultivated soil and developed quickly for environmental adaption ($p < 0.05$). Drought affected the microbial metabolism greatly ($p < 0.01$), regardless of the plant cover. Under drought, the final AWCD ranged from 0.4 to 2.3. Elevated CO₂ concentration affected the metabolic activity positively. Similar reports also demonstrated that the microbial communities collected from the rhizosphere of *Danthonia richardsonii* grown for four years at twice-ambient CO₂ had a significantly higher carbon source utilization than the communities collected from plants grown at ambient CO₂ (Grayston et al., 1998).

To investigate the characteristics of carbon source utilization, 31 single carbon sources were categorized in six classes: saccharides, amino acids (AA), esters, phenols, amines, and carboxylic acids (CA). The final carbon source utilization (after 96 h) differed clearly among treatments (Figure 4). Major available carbon sources included sacchrides, AA, amines, and esters. The results corresponded to the utilization of saccharides, esters, and amines in crop and elevated CO₂ treatments. In bare soil, bioavailable carbon sources were changed from AA and amines to esters. The CO₂ and drought had interactive effects on the higher saccharide and phenol utilization in bare soil ($p < 0.05$). However, the interactive effects were insignificant in cultivated soil treatments ($p > 0.05$).

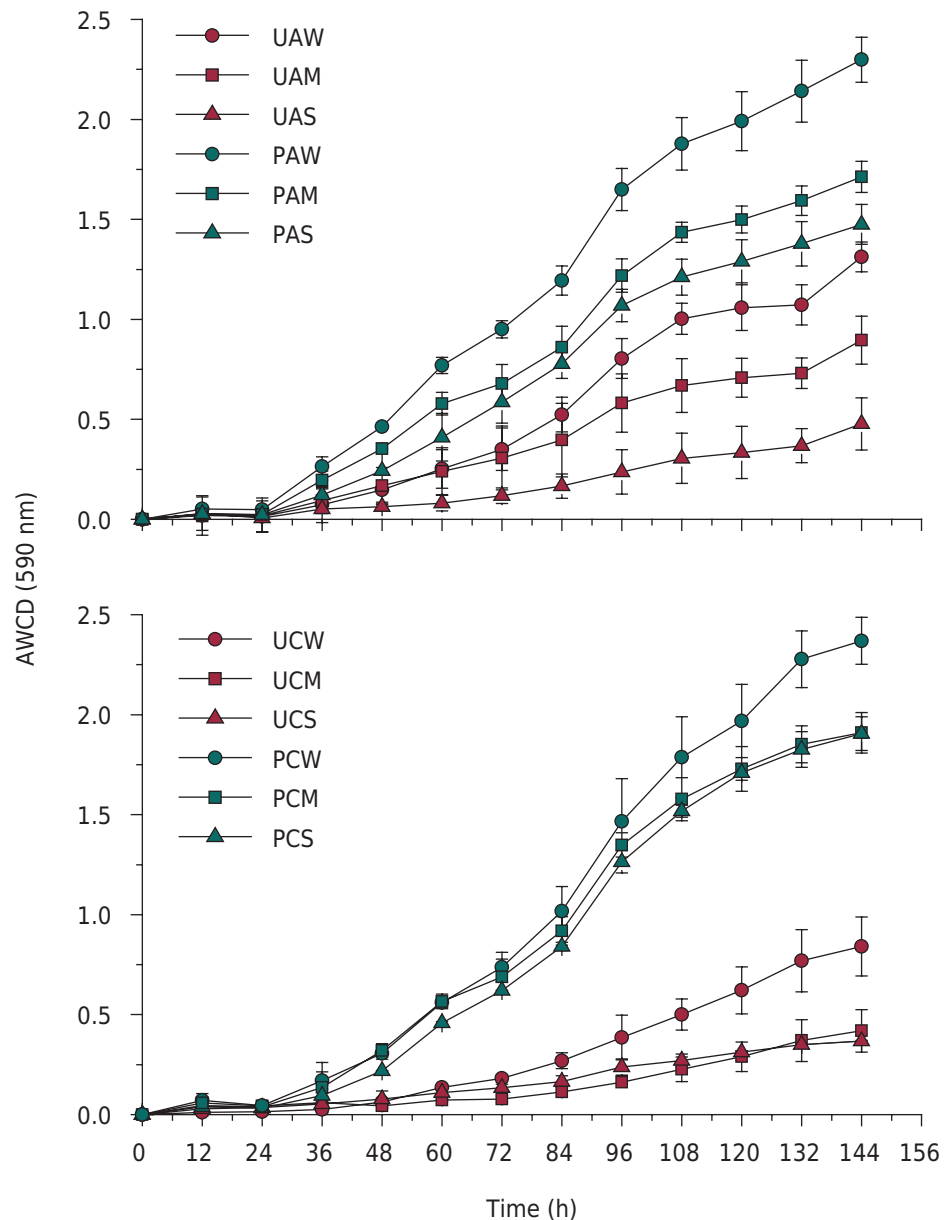


Figure 3. Average well color development (AWCD) of the BIOLOG EcoPlate for different treatments. UAW: unplanted treatment with ambient CO₂ and well-watered condition; UAM: unplanted treatment with ambient CO₂ and mild drought condition; UAS: unplanted treatment with ambient CO₂ and severe drought condition; PAW: planted treatment with ambient CO₂ and well-watered condition; PAM: planted treatment with ambient CO₂ and mild drought condition; PAS: planted treatment with ambient CO₂ and severe drought condition; UCW: unplanted treatment with elevated CO₂ and well-watered condition; UCM: unplanted treatment with elevated CO₂ and mild drought condition; UCS: unplanted treatment with elevated CO₂ and severe drought condition; PCW: planted treatment with elevated CO₂ and well-watered condition; PCM: planted treatment with elevated CO₂ and mild drought condition; PCS: planted treatment with elevated CO₂ and severe drought condition.

Table 3 showed the diversity index based on the BIOLOG patterns of the different treatments. In the cultivated soil treatments, the functional microbial diversity was larger than in bare soil ($p < 0.05$). Root microbes had the highest metabolic activity and all carbon sources were used. This could be explained by DGGE analysis, which showed the highest bio-diversity and abundance for root microbes. In bare soil, elevated CO₂ concentration decreased microbial functional diversity ($p > 0.05$). Soil moisture affected microbial functional diversity significantly ($p < 0.05$). The highest functional diversity was observed in wet soil. The effect of elevated CO₂ concentration on microbial functional diversity was insignificant in the cultivated soil treatments. In the literature, protein, amino acids, and water-soluble sugar were described as the predominant constituents

of the dissolved organic carbon produced by crops (Zhai et al., 2013). The dissolved organic carbon release rate of *Iris pseudacorus* species was in the mean $12.2 \pm 0.7 \mu\text{g g}^{-1}$ root dry mass per h (Zhai et al., 2013). In addition, these compounds can be easily used as carbon resource for the growth and metabolism of microorganisms. Therefore, no significant microbial functional diversity was found among the cultivated soil treatments.

There was variance contribution of microbial carbon source utilization (Figure 5). Amine, carboxylic acid, and phenol had a significant positive correlation with PC1, whereas esters and saccharides were positively related to PC2. The PC1 could be explained by soil water content and PC2 indicated the differences in CO₂ concentration. Drought inhibited the utilization of carbon sources, but the functional diversity was not changed. Due to the overlapping microbial function, microbes of similar function were probably exchanged especially in the crop treatments (Kohler et al., 2010). The enzyme activities of microorganisms were found to be significantly affected by elevated CO₂ (Kandeler et al., 2006). In the treatments with elevated CO₂, microbes tended to utilize saccharides and esters when the metabolic activity was intensified. Moreover, elevated CO₂ concentrations

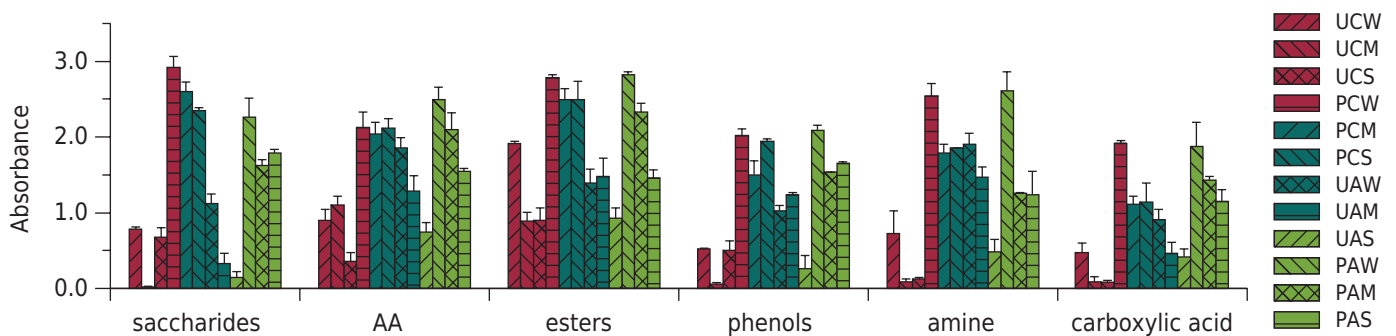


Figure 4. Carbon utilization profile of soil microbes on BIOLOG EcoPlate for different treatments. The bars above the columns indicate standard deviation. UCW: unplanted treatment with elevated CO₂ and well-watered condition; UCM: unplanted treatment with elevated CO₂ and mild drought condition; UCS: unplanted treatment with elevated CO₂ and severe drought condition; PCW: planted treatment with elevated CO₂ and well-watered condition; PCM: planted treatment with elevated CO₂ and mild drought condition; PCS: planted treatment with elevated CO₂ and severe drought condition; UAW: unplanted treatment with ambient CO₂ and well-watered condition; UAM: unplanted treatment with ambient CO₂ and mild drought condition; UAS: unplanted treatment with ambient CO₂ and severe drought condition; PAW: planted treatment with ambient CO₂ and well-watered condition; PAM: planted treatment with ambient CO₂ and mild drought condition; PAS: planted treatment with ambient CO₂ and severe drought condition.

Table 3. Diversity index calculated from the BIOLOG pattern under different treatment of soil moisture, elevated CO₂ level, and soil cover (with or without *Glycine max* plant)

CO ₂	Cover	WHC	H' function	D function	E function	D _{Mc} function
700 ppm	bare	80 %	2.831	0.931	0.824	0.916
		60 %	2.595	0.901	0.756	0.811
		40 %	2.046	0.816	0.636	0.950
	soybean	80 %	3.313	0.961	0.965	0.910
		60 %	3.183	0.955	0.927	0.906
		40 %	3.170	0.955	0.923	0.904
350 ppm	bare	80 %	3.007	0.943	0.893	0.904
		60 %	2.706	0.920	0.796	0.884
		40 %	2.553	0.903	0.743	0.930
	soybean	80 %	3.232	0.958	0.941	0.903
		60 %	3.062	0.949	0.900	0.898
		40 %	3.147	0.952	0.916	0.916

Shannon-Wiener (H') and Simpson (D) indices were used to estimate the microbial diversity; Pielou (E) and McIntosh (DMc) indices were used to estimate the microbial diversity. WHC: water holding capacity.

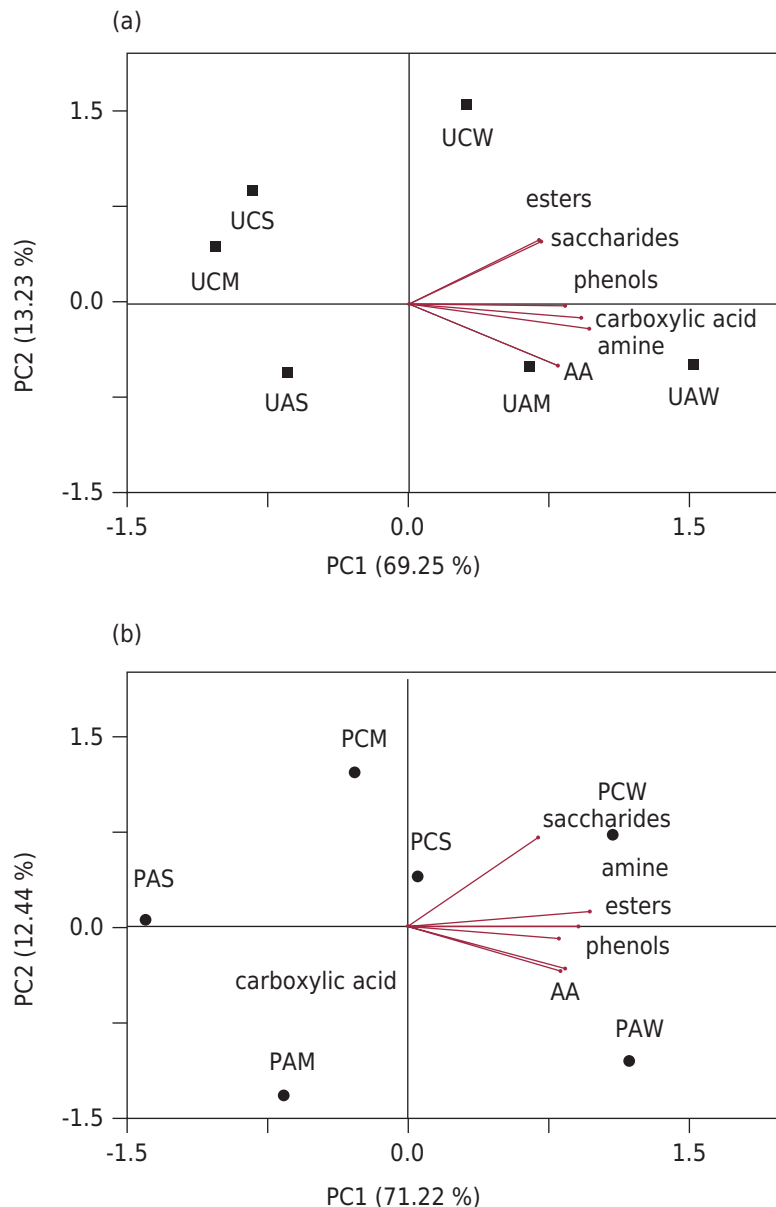


Figure 5. The PCA analysis of average well color development (AWCD) over the soil microbial carbon utilization for soybean (a) and bare (b) soil treatments. UCW: unplanted treatment with elevated CO₂ and well-watered condition; UCM: unplanted treatment with elevated CO₂ and mild drought condition; UCS: unplanted treatment with elevated CO₂ and severe drought condition; PCW: planted treatment with elevated CO₂ and well-watered condition; PCM: planted treatment with elevated CO₂ and mild drought condition; PCS: planted treatment with elevated CO₂ and severe drought condition; UAW: unplanted treatment with ambient CO₂ and well-watered condition; UAM: unplanted treatment with ambient CO₂ and mild drought condition; UAS: unplanted treatment with ambient CO₂ and severe drought condition; PAW: planted treatment with ambient CO₂ and well-watered condition; PAM: planted treatment with ambient CO₂ and mild drought condition; PAS: planted treatment with ambient CO₂ and severe drought condition.

inhibited the utilization of organic nitrogen compounds (Nie et al., 2013). These findings suggest that elevated CO₂ can promote the enzyme activities using saccharides and esters as carbon sources, but inhibit the enzyme activities by using organic nitrogen compounds as nutrients.

CONCLUSIONS

Soil microbial biomass and community structure were significantly affected by drought. The relative abundance and diversity of bacteria were decreased by drought, while fungi were more tolerant to mild drought. Severe drought decreased microbial functional

diversity and restrained the metabolic activity of soil microbes. Elevated CO₂ concentration insignificantly enhanced the metabolic activity in the cultivated soil. In bare soil, the interactive effect between CO₂ and drought was insignificant. Plants have major impacts on the soil microbial community. Although the influence of elevated CO₂ concentration was not significant, it could minimize the impacts of drought on soil microbes.

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