Soil Biology & Biochemistry 55 (2012) 70–77

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Mineral fertilizer alters cellulolytic community structure and suppresses soil cellobiohydrolase activity in a long-term fertilization experiment

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ARTICLE INFO

Article history:
Received 8 March 2012
Received in revised form
11 June 2012
Accepted 12 June 2012
Available online 30 June 2012

Keywords:
Carbon sequestration
Cellobiohydrolase
Cellulolytic fungi
Community structure
Diversity
Long-term fertilization

ABSTRACT

Nutrient inputs to soil can alter mineralization of organic matter and subsequently affect soil carbon levels. To understand how elemental interactions affect the biogeochemistry and storage of soil C, we examined soils receiving long-term applications of mineral fertilizer and manure-containing fertilizers. As cellulose is the dominant form of carbon entering arable soils, cellulolytic communities were monitored through enzymatic analysis, and characterization of the abundance (real-time PCR) and diversity (terminal restriction fragment length polymorphism, T-RFLP) of fungal cellobiohydrolases (*cbhI*) genes. The data showed that long-term mineral fertilization increased soil organic C and crop productivity, and reduced soil heterotrophic respiration and cellobiohydrolases (CBH) activity. Correspondingly, the diversity and community structure of cellulolytic fungi were substantially altered. The variation in cellulolytic fungi is mainly attributable to shifts in the proportion of Eurotiomycetes. In addition, CBH activity was significantly correlated with the diversity and community structure of cellulolytic fungi. These results suggest that enhanced C storage by mineral fertilizer addition occurs not only from extra organic carbon input, but may also be affected through the cellulose decomposing community in arable soil.

1. Introduction

Biogeochemical cycling in soil ecosystems is directly impacted following addition of exogenous nutrients (Edmeades, 2003; Knorr et al., 2005). Not surprisingly, the most affected soils are under agricultural land use where addition of agricultural fertilizer can significantly affect soil organic matter cycling (Parton et al., 1992; Edmeades, 2003; Zhang et al., 2009). This is a critical issue, as soil C storage is related to both soil structure and fertility, and also a sink or source of the greenhouse gas CO_2 (Edmeades, 2003; Lal, 2004). The direction and extent of shift in soil C cycling following fertilization can occur via two fundamental ways. Firstly, fertilization increases plant net primary productivity thus increasing overall inputs of organic C into soil (Edmeades, 2003; Jarecki and Lal, 2003; Yu et al., 2009). Secondly, fertilization changes the rate of microbial decomposition (heterotrophic mineralization of C) which controls carbon loss from soil as CO_2 (Carreiro et al., 2000; Waldrop et al., 2004; Ding et al., 2007). Since long-term inputs of organic C to soil do not necessarily culminate in increased total C (Gill et al., 2002; Khan et al., 2007; Zhang et al., 2009), C accumulation may be more tightly coupled to microbial decomposing processes than to organic C inputs *per se*.

Knowledge of how nutrient inputs influence the microbial cycling of organic C in natural ecosystems, especially in regards to key taxa involved in this function, is rapidly accumulating. The analysis of soil extracellular enzymes associated with cycling of soil organic C, such as phenol oxidase and cellobiohydrolase, has revealed that these groups respond in a consistent manner to low level N addition in forests (Carreiro et al., 2000; Waldrop et al., 2004). In soils receiving litter of high lignin content, the residing microbial communities were dominated by Basidiomycete (white-rot) fungi. N increased C storage in these soils by down-regulating expression of ligninolytic *lcc* gene and reducing laccase activity (Carreiro et al., 2000; Waldrop et al., 2004; Blackwood et al., 2007; Edwards et al., 2011). In soil systems in which lignin content is low and other fungi are dominant (e.g. Ascomycetes), low levels of N may increase decomposition (Blackwood et al., 2007). Furthermore, the association between phenol oxidase activity and laccase gene abundance and diversity has been shown to co-vary temporally (Blackwood et al., 2007; Artz et al., 2009), indicating that associated effects on C-cycling in some

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http://dx.doi.org/10.1016/j.soilbio.2012.06.008

ecosystems are controlled by a specific component of the microbial community. However, how inputs of large amount of nutrients affect the microbial processes of organic C decomposition in agricultural ecosystems remains largely unexplored.

Compared with forest ecosystems, inputs of lignin (as a component of crop residue) to most arable soils are relatively low (Jin and Chen, 2007; Thomsen et al., 2008). As such, effects of fertilizer addition (including N) on the rate of lignin decomposition likely have minimal contribution to C storage in arable soils (Hofmann et al., 2009). On the contrary, the major forms of organic C entering agricultural soils are celluloses. For example, cellulose and hemicelluloses account for up to 63%, 72%, 60% and 56% of corn, soybean, rice and wheat biomass, respectively (Jin and Chen, 2007; Thomsen et al., 2008). This is directly supported by nuclear magnetic resonance (NMR) demonstrating that cellulose residue (O–alkyl C) represents a large component of the soil organic C pool (Leifeld and Kögel-Knabner, 2005). Although numerous studies have shown that chemical composition largely controls the decomposition rate of soil organic C (Johnson et al., 2007; Meier and Bowman, 2008), it is unknown if the nutrient-mediated C balance is regulated by cellulose mineralization through alteration of cellulolytic communities in agricultural ecosystems. To answer this question, we characterized the cellulolytic community of soils with differing C storages resulting from two long term different fertilization regimes (Yu et al., 2008), by profiling the functional genes targeting cellulose degradation.

2. Materials and methods

2.1. Experimental description

The establishment and management of the long-term field experiment have been described in detail previously (Yu et al., 2009). Briefly, the trial was initiated in 1990 on a clay loam alfisol at the experimental station of the Institute of Applied Ecology, Chinese Academy of Sciences (41°32′N, 123°23′E). The initial properties of the surface soil (depth, 0–20 cm) were as follows: clay loam texture; pH, 6.7; organic C, 22.1 g kg^{-1}; total N, 0.8 g kg^{-1}; available P, 10.6 mg kg^{-1}; soil exchangeable K, 82.5 mg kg^{-1}. Four treatments representing low input, organic, inorganic and conventional management, were used to investigate the effects of long-term mineral fertilizer inputs on the cellulose-associated microbial community. These treatments consisted of no added fertilizer (CK), recycled organic manure (M), mineral fertilizer (combination of N, phosphorus and potassium; NPK), and recycled organic manure plus mineral fertilizer (MNPK). The application rates of N, P, and K fertilizers were 150, 25 and 60 kg ha^{-1} $year^{-1}$ in the form of urea, double superphosphate, and potassium chloride. The M treatment was achieved by recycling 80% of harvested seeds, 100% of soybean straw and 50% of corn stalk as pig manure to the original plots. The cropping system was a soybean–maize–maize rotation cycle. Three replicate plots (162 m^2 per plot) were sampled for each fertilizer treatment on August 1st 2008 when maize was in flower. Five soil cores (5 cm diameter × 20 cm depth) were randomly sampled from each plot and homogenized to reduce within-plot variability. Enzymatic measurements were conducted immediately on fresh soil samples. Remaining portions of soil were set aside for measurement of respiration, frozen at −80 °C for molecular analysis or used for measurement of physicochemical properties (Pansu and Gautheyrou, 2006).

2.2. Soil heterotrophic respiration

Soil respiration was measured using the incubation method on 10 g portions of fresh soil. Soil from each replicate was placed in a 125 ml glass bottle, sealed with a rubber plug and incubated at 25 °C for 72 h. Headspace CO_2 concentrations were measured via gas chromatography (HP7890A, Agilent Technologies, CA, USA), adjusted for background CO_2, and soil respiration rates determined and expressed as μg C–CO_2 g^{-1} soil h^{-1}.

2.3. Cellobiohydrolase activity

Cellobiohydrolase (CBH; EC 3.2.1.91; alt. cellulose 1,4-beta-cellobiosidase) activity in soil solutions was measured fluorimetrically, using methylumbelliferone-labeled substrate (4-MUB-β-D-cellobioside) according to Saiya-Cork et al. (2002), except that the pH of buffer was adjusted to soil pH. Fluorescence was measured using a microplate fluorometer (TECAN Infinite 200, Crailsheim, Germany) with 365 nm excitation and 450 nm emission filters. Enzyme activity was expressed as μmol g^{-1} soil h^{-1}.

2.4. DNA extraction, PCR amplification and cloning

DNA was extracted from 0.5 g soil samples using the Fast DNA spin kit for soil (Bio101, Q-Biogene, CA, USA) and the FastPrep-24 instrument according to the manufacturer's instructions.

Genes encoding glycoside hydrolases of families 5 (*cel5*) and cellobiohydrolases of family 7 (*cbhI*) were selected as biomarkers of cellulolytic bacteria and fungi, respectively. The *cel5* gene was amplified with primers cel5_392F and cel5_754R using thermocycling conditions of 3 min at 95 °C, followed by 45 cycles of 40 s at 95 °C, 30 s at 52 °C, 30 s at 72 °C, and a final extension for 7 min at 72 °C (Pereyra et al., 2010). PCR amplification of the *cbhI* gene was based on the method of Edwards et al. (2008) with primers fungcbhIF and fungcbhIR. Thermocycling conditions for *cbhI* gene amplification were: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 48 °C for 45 s and 72 °C for 90 s, and a final extension at 72 °C for 15 min (Edwards et al., 2008). Each PCR used 1 μl of 10-fold diluted soil DNA as template, 0.5 U of TaKaRa *Ex Taq* DNA polymerase enzyme, 0.2 μM of each dNTP, and 0.5 μM of each primer in a total volume of 50 μl. Surprisingly, we were unable to amplify *cel5* genes, even after 45 PCR cycles, from the present soils. We propose that primers bias against the specific cellulose degrading bacteria present in the soil may be affecting this assay, particularly as these groups of bacteria are not numerically dominant in the upland soil tested. Thus, only the *cbhI* gene was cloned as no PCR products were obtained from all amplifications of *cel5* genes. After agarose gel purification, PCR products were ligated into the pMT19-T vector according to the manufacturer's instruction (TaKaRa, Dalian, China) at 16 °C for 5 h and then transformed into *Escherichia coli* JM109 competent cells and transformants were selected via blue–white screening. Sequencing of inserts was conducted on an ABI 3730 sequencer using BigDye terminator cycle sequencing chemistry (Applied Biosystems, CA, USA).

2.5. Terminal restriction fragment length polymorphism (T-RFLP) analysis

PCR amplifications of *cbhI* genes for T-RFLP analysis were the same as described above except that the forward primer fungcbhIF was fluorescently labeled with 6-FAM (TaKaRa). After gel-purification, PCR products were digested with TaqI which was selected via *in silico* analysis of sequences in *cbhI* clone library using program REPK (Collins and Rocap, 2007). The digested products were purified by ethanol precipitation and analyzed with an ABI 3730 sequencer using an internal size standard MapMarker® 1000 (Bioventures). Peak heights of terminal restriction fragments (TRFs) were automatically quantified using Peak Scanner Software v1.0 (Applied Biosystems). Any peaks constituting less than 1% of the total were excluded from further analyses and TRFs differing by less

than 1 bp were considered identical. Sizes of TRF were verified by T-RFLP-analysis of representative clones.

Diversity (Shannons index; H) of *cbhI* genes was calculated using the following equation:

$$H = -\sum_{i=1}^{S} p_i \ln p_i = -\sum_{i=1}^{S} (N_i/N)\ln(N_i/N) \quad (1)$$

where S is the total number of TRFs and P_i is the proportion of TRF_i to the total.

2.6. Real-time PCR

Real-time (quantitative) PCR (qPCR) of *cbhI* gene was carried out on IQ5 instrument with 2 × SYBR Green I Supermix chemistry (BioRad, CA, USA). Each 20 μl PCR contained 10 μl Supermix buffer, 0.5 μM of each primer and 1 μl 10-fold diluted template DNA. The PCR thermocycle conditions were 94 °C for 5 min, 40 cycles of 94 °C 30 s, 48 °C for 45 s and 72 °C for 60 s, with a 15-s data acquisition step at 84 °C and a melting curve. Quantification was made against standard curves, consisting of dilutions of a known amount of plasmid DNA containing a fragment of the partial *cbhI* gene. All the samples were quantified in triplicate.

2.7. Sequence analysis

Sequences were checked for chimeric regions by analyzing alignments in Ballerophon (Huber et al., 2004). The coding regions of the obtained DNA sequences were identified by aligning against known *cbhI* mRNA transcripts. Neighbor-joining trees were constructed in MEGA version 4.0 (Tamura et al., 2007) and branching tested with bootstrapping (1000 replicates). Translated amino acid sequences of partial *cbhI* gene sequences obtained from our soil and related sequences obtained by BLAST P searching within GenBank were included in the construction of the tree. The sequences determined in this study were deposited in the EMBL database under accession numbers FN688581–FN688733.

2.8. Statistical analysis

Differences in soil physicochemcial properties, respiration rates, cellobiohydrolase activities and *cbhI* gene abundances among treatments were evaluated with two-way analysis of variance (ANOVA), and correlations between variables assessed using Pearson's method. Uni-variate statistics were conducted in SAS for windows (Version 8.02). Multivariate analyses of the data were conducted with Canono version 4.5 (Ter Braak and Smilauer, 2002). Because initial analysis by detrended correspondence analysis revealed that T-RFLP data exhibited a linear response to the environmental variables, redundancy analysis (RDA) was then used to analyze the influences of different fertilizations on community structure of cellulolytic fungi and the correlation of cellobiohydrolase activities with community structures, with forward selection procedure and Monte Carlo permutation testing (499 permutations).

3. Results

3.1. Soil properties

Long-term application of mineral fertilizer significantly reduced soil pH, increased contents of soil TN, TP and organic matter ($P < 0.05$; Tables 1 and 2). Long-term application of manure significantly increased soil total nitrogen and organic matter contents, but did not change soil total P content and soil pH (Tables 1 and 2). No interactions of mineral fertilizer and manure on soil pH and contents of soil TN, TP and OM were present ($P > 0.1$; Table 1).

3.2. Soil respiration and CBH activity

Long-term application of mineral fertilizer significantly reduced soil respiration rates by 12.7% and reduced CBH activity by 36.5% (Tables 1 and 2). Long-term application of manure significantly increased soil respiration rates by 36.5% and increased CBH activity by 43.4% (Tables 1 and 2). No interactions of mineral fertilizer and manure on soil respiration rates and CBH activities were detected (Table 1).

3.3. cbhI gene abundance and diversity

The abundance of *cbhI* gene ranged from 7.1×10^6 to 11.9×10^6 copies g^{-1} soil (Table 2). Long-term application of mineral fertilizer did not significantly change *cbhI* gene abundance, but significantly reduced the diversity (Shannons index) of cellulolytic fungi (Tables 1 and 2). Long-term application of manure significantly increased diversity of cellulolytic fungi, and marginally increased *cbhI* gene abundance ($P = 0.084$, Tables 1 and 2). No interactions of mineral fertilizer and manure on *cbhI* gene abundance and the diversity of cellulolytic fungi were detected (Table 1).

3.4. T-RFLP analysis

PCR products of *cbhI* gene were grouped across 19 TRFs based on digestion with the Taq I enzyme (Fig. 1). Application of mineral fertilizer or manure significantly influenced the relative abundances of 8 TRFs (Fig. 1). TRFs of 157, 251, 257 and 425 bp were all reduced in abundance following mineral fertilizer addition, while those of 136 and 141 bp were significantly increased. Addition of manure reduced the TRF relative abundance of 136 and 257 bp, and increased those of 77 and 157 bp. Significant interactive effects between mineral fertilizer and manure on TRF relative abundance of 122 bp, 136 bp, 141 bp, 157 bp and 257 bp were detected.

3.5. Phylogenic analysis

A total of 152 clones were sequenced from the libraries of CK, NPK, M and MNPK. The length of the sequences ranged from 503 to 736 bp. Alignments against reference mRNA sequences showed that 19 sequences contained no introns, while 66 sequences contained one intron and the rest had two introns. The putative translated amino acid sequences contained 165 to 175 residues. The mean blast P matches was 81% (71–98%). Phylogenetic analysis

Table 1
Statistical summary of the effects of different long-term fertilizer treatments on pH, contents of soil organic matter (OM), soil nitrate, total nitrogen (TN) and total phosphorus (TP), respiration rate, cellobiohydrolase (CBH) activity, and *cbhI* gene abundance and diversity (Shannon index).

	pH	TN	TP	OM	Soil nitrate	Respiration rate	CBH activity	*cbhI* gene abundance	Shannon index
Mineral fertilizer	0.001	0.034	0.000	0.009	0.037	0.002	<0.001	0.471	0.001
Manure	0.450	0.002	0.085	0.006	0.846	<0.001	0.002	0.084	0.044
Mineral fertilizer × Manure	0.281	0.127	0.106	0.773	0.370	0.944	0.682	0.639	0.643

Table 2
Soil pH, contents of soil organic matter (OM), soil nitrate, total nitrogen (TN) and total phosphorus (TP), respiration rate, cellobiohydrolase (CBH) activity, and *cbhI* gene abundance and diversity (Shannon index) as affected by long-term fertilizations.

		pH	TN (g kg^{-1})	TP (g kg^{-1})	OM (g kg^{-1})	Soil nitrate (mg kg^{-1})	Maize biomass (kg hm^{-2})	Soybean biomass (kg hm^{-2})	Respiration rate (μg C–CO_2 g^{-1})	CHB activity (μmol g^{-1} soil h^{-1})	*cbhI* gene abundance ($\times 10^6$ copies g^{-1} soil)	Shannons index
NPK0[b]	M0 (CK)[c]	5.68 (0.12)[a]	0.09 (0.00)	0.06 (0.01)	16.39 (0.12)	25.79 (13.59)	8669[d]	2858[d]	4.21 (0.07)	2.12 (0.26)	7.56 (0.30)	1.55 (0.08)
	M1 (M)	5.64 (0.11)	0.10 (0.01)	0.07 (0.01)	18.29 (0.20)	38.39 (2.13)	10,918	3821	5.50 (0.09)	2.81 (0.15)	11.85 (1.64)	1.91 (0.11)
NPK1	M0 (NPK)	4.98 (0.12)	0.09 (0.01)	0.24 (0.06)	18.13 (0.15)	85.67 (24.84)	13,665	4400	3.54 (0.09)	0.85 (0.06)	7.10 (2.51)	0.94 (0.07)
	M1 (MNPK)	5.16 (0.15)	0.12 (0.01)	0.38 (0.05)	20.33 (0.92)	66.42 (25.78)	14,729	4893	5.04 (0.11)	1.44 (0.01)	9.74 (1.74)	1.18 (0.15)

[a] Data were presented as means of three replicates with standard error in the brackets.
[b] NPK0 indicates no mineral fertilizer addition, NPK1 indicates with mineral fertilizer addition. M0 indicates no manure addition. M1 indicates with manure addition.
[c] CK, no fertilizer; NPK, combination of nitrogen, phosphorus, and potassium; M, organic manure; MNPK, NPK plus M.
[d] Average aboveground biomass from 1990 to 2007 derived from Yu et al. (2009).

showed that cellobiohydrolase gene could be tentatively clustered into 5 groups with strong phylogenetic association: Eurotiomycetes, Dothideomycetes (I and II), Sordariomyteces (I and II) in Ascomyceta, Agaricomycetes in Basidiomycota and an unclassified group (Fig. 2). The average proportions of Agaricomycetes, Dothideomycetes, Eurotiomycetes, Sordariomyteces and unclassified group were 16.4%, 19.7%, 36.8%, 15.1% and 10.5%, respectively (Table 3).

The 76-bp TRF clustered in unclassified group II (Fig. 2) and the 77-bp TRF distributed in Agaricomycetes, Dothideomycetes, Eurotiomycetes, Sordariomyteces and an unclassified group. The 122-bp TRF clustered in Dothideomycetes, and the 127-bp TRF clustered in unclassified group I. The TRFs of 136, 157 and 190 bp lengths all clustered in Eurotiomycetes. The 178-bp TRF clustered in unclassified group I, while TRFs of 40, 47, 50, 51 and 231 bp length did not match any obtained sequences (i.e. were of unknown origin). TRFs of 236 and 254 bp clustered in Agaricomycetes, the 248-bp TRF distributed in Agaricomycetes and Sordariomyteces, the 251-bp TRF clustered in Agaricomycetes and Dothideomycetes, and the 257-bp TRF clustered in Dothideomycetes. TRFs of 31, 64, 65, 135, 163, 180, 202, 317, 566 and 571 bp obtained by *in silico* analysis of clones were not detected in T-RFLP analysis (Table 3).

3.6. Correlation analysis

Soil respiration was significantly correlated with CBH activity ($r = 0.745$, $P < 0.01$, Table 4). In addition, CBH activity was significantly correlated with diversity of cellulolytic fungi ($r = 0.951$, $P < 0.01$). Monte Carlo permutation test showed that CBH activity was significantly correlated with community structure of cellulolytic fungi ($r = 0.611$, $F = 15.716$, $P = 0.002$) (Fig. 3). In addition, the community structure of cellulolytic fungi was significantly correlated with soil pH ($r = 0.628$, $F = 16.905$, $P = 0.002$), soil total phosphorus content ($r = 0.488$, $F = 9.536$, $P = 0.004$) and soil nitrate content ($r = 0.340$, $F = 5.161$, $P = 0.024$) (Fig. 4).

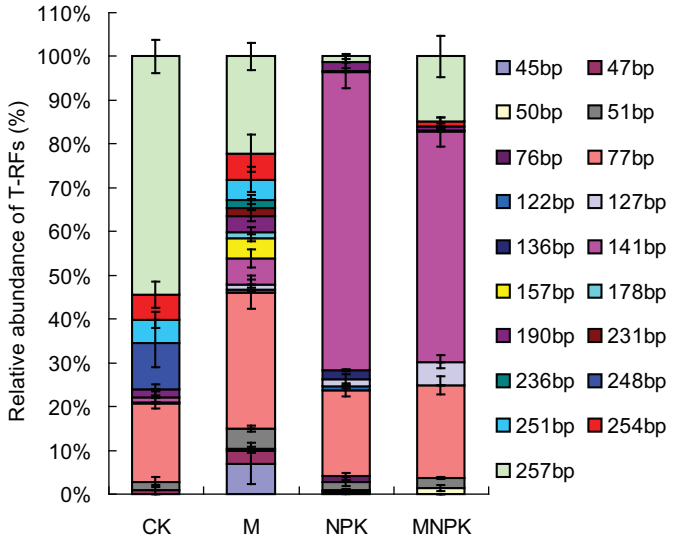

Fig. 1. Relative abundance of *cbhI* gene TRFs in soils as affected by long-term fertilizations. CK, no fertilizer; NPK, combination of nitrogen, phosphorus, and potassium; M, organic manure; MNPK, NPK plus M.

4. Discussion

Long-term application of mineral fertilizer significantly increased soil C levels (Table 2), in line with many previous studies which also show that mineral fertilizer may increase soil carbon storage by up to 120% (Anderson et al., 1990; Jarecki and Lal, 2003; Wilson and Al-Kaisi, 2008; Zhang et al., 2009). The increase in soil C following mineral fertilizer addition partially resulted from greater crop productivity and thus more residues were returned to the field (Table 2), a common phenomenon found in other agroecosystems (Zhang et al., 2009). In addition, it is shown that CO_2 emission was reduced by mineral fertilizer (Table 2), demonstrating that the increase in soil C is likely linked with the suppression of microbially-driven mineralization of soil organic C. While manure also increased soil C, the increase was likely due to direct increases in organic C as crop residues and manure but not to suppression of microbial decomposition, as we observed that manure amendment increased heterotrophic respiration rate to a greater extent than soil C storage (Table 2).

The changes in soil heterotrophic respirations are likely to be strongly associated with cellulose degradation. In our study, CBH activity was reduced in soils where respiration was reduced by mineral fertilizer addition, while CBH activity was increased in soils where respiration was increased by manure addition (Table 2). However, the reduction of CBH activity by N-containing mineral fertilizer is at odds with studies that show nutrient addition to soil increases cellulase activity through alleviation of N-limitation associated with cellulose degradation, as observed in forest ecosystems (Carreiro et al., 2000; Saiya-Cork et al., 2002). It is also inconsistent with the result obtained from an alkaline arable soil where CBH activity was enhanced by long-term application of the same type of fertilizer (Ai et al., 2012), following the approach in the present study. The different responding patterns may be attributable to the relatively high level of background mineral nitrogen (as indicated by nitrate level in CK soil) at the sampling time point (Table 2), which may originate from symbiotic nitrogen fixation by a former legume crop. Soil nitrate content was increased significantly by long-term NPK treatment and may be toxic to cellulolytic community.

To bridge the gap between fertilization practices and cellulose degradation, cellulolytic communities were explored with DNA-

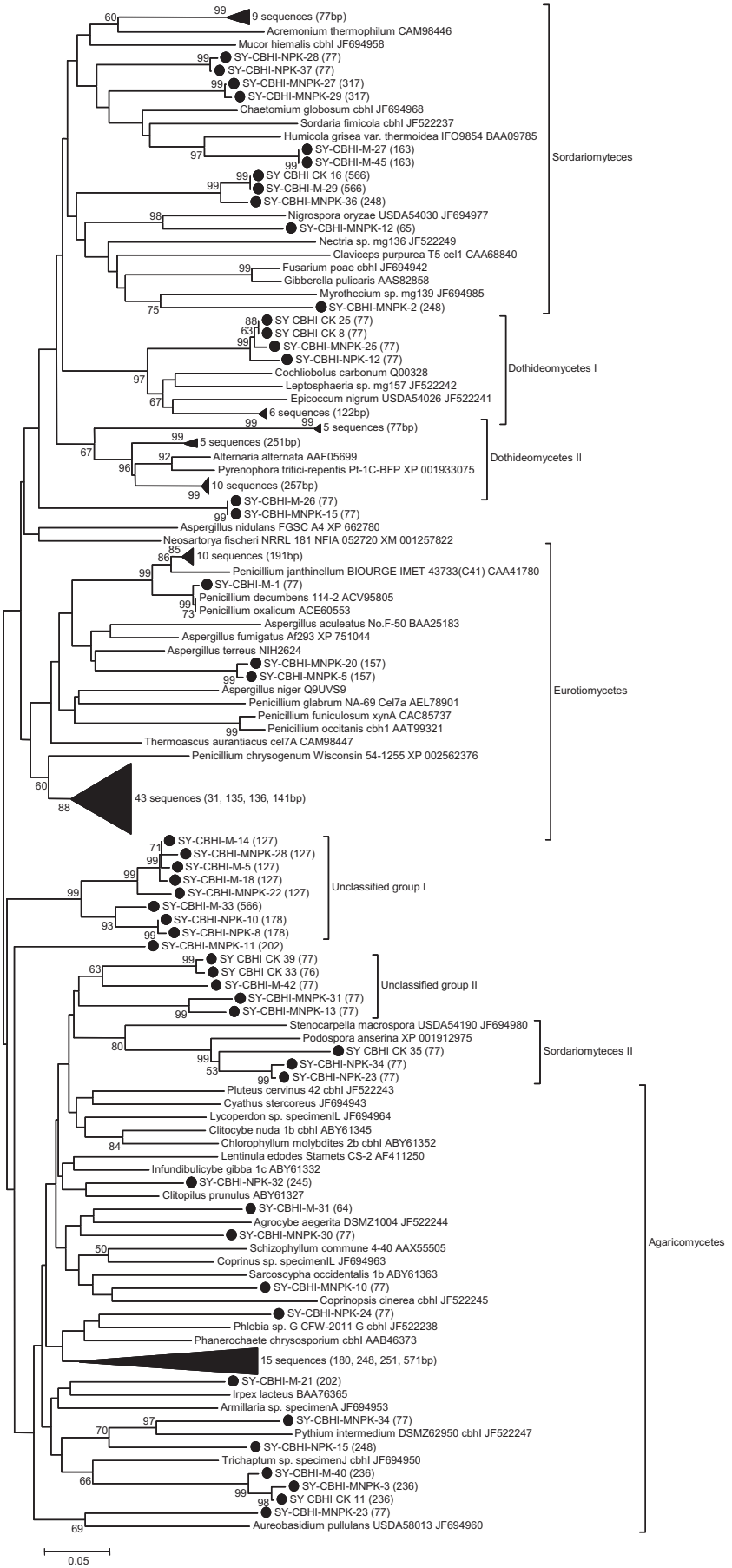

Fig. 2. Phylogenetic analysis of the partial fungal cellobiohydrolase (*cbhI*) genes. The phylogenetic tree was constructed based on *P*-distance analysis using the neighbor-joining algorithm in MEGA 4.0. Bootstrap analysis was performed with 1000 replicates. Scale bar represents substitutions per site. Sequences from this experiment were highlighted as a close circle with TRF length (bp) in the following bracket.

Table 3
Phylogenetic affiliations, TRF length and numbers of *cbhI* gene clones retrieved in libraries of different long-term fertilization treatments.

Affiliation	TRF length (bp)	Detection in T-RFLP analysis?	CK[a]	M	NPK	MNPK
Agaricomycetes	64	No		1		
	77	Yes			1	4
	180	No	2			
	202	No		1		
	236	Yes	1	1		1
	245	No			1	
	248	Yes	1	6	1	
	251	Yes	2			
	254	Yes		1		
	571	No		3		
Dothideomycetes	77	Yes	3	3	2	1
	122	Yes	2	4		
	251	Yes	1	1	2	1
	257	Yes	8	1	1	
Eurotiomycetes	31	No			1	
	77	Yes		1		
	135	No			1	
	136	Yes			2	
	141	Yes	3	2	17	17
	157	Yes				2
	190	Yes	5	2	1	2
Sordariomyteces	65	No				1
	77	Yes	4	4	5	1
	163	No		2		
	248	Yes				2
	317	No				2
	566	No	1	1		
Unclassified group	76	Yes	1			
	77	Yes	1	2		3
	127	Yes		3		2
	178	Yes			2	
	202	No				1
	566	No		1		
Total			35	40	37	40

[a] CK, no fertilizer; NPK, combination of nitrogen, phosphorus, and potassium; M, organic manure; MNPK, NPK plus M.

based molecular methods targeting the *cel5* gene in bacteria and *cbhI* gene in fungi (Edwards et al., 2008; Pereyra et al., 2010). Surprisingly, we were unable to amplify *cel5* genes (bacterial origin) from the soil studied, probably due to the reasons mentioned in the materials and methods section. Further development of these primers may solve this problem in the future. On the contrary, *cbhI* genes were found to be 7.1×10^6 to 11.9×10^6 copies g^{-1} soil (Table 2). In addition, the primer pair fungcbhIF/fungcbhIR was capable of recovering *cbhI* genes of major soil cellulolytic fungi groups (Moubasher and Mazen, 1991; Luque et al., 2005). As such, this DNA tool is useful in assessing cellulolytic fungi community in arable soil despite these primers being originally tested in forested ecosystems (Edwards et al., 2008).

The cellulolytic fungi community was highly responsive to long-term mineral fertilizer treatment. Diversity of cellulolytic genes decreased by 39% in mineral fertilizer treatment relative to no mineral fertilizer treatment (Table 2). Consistent results have been reported for forest soils where the diversity of mycorrhiza and general fungi decrease after N deposition (Lilleskov et al., 2002; Allison et al., 2007). In addition, the structure of cellulolytic fungi was altered by mineral fertilization (Figs. 1 and 3). Similar results have been documented for responses of soil cellulolytic fungal communities to elevated atmospheric CO_2 in some natural ecosystems (Weber et al., 2011). Furthermore, a significant correlation existed between CBH activity and the diversity and community structure of cellulolytic fungi (Table 4, Fig. 3), suggesting that shifts in diversity and structure of cellulolytic fungi are of functional relevance in terms of carbon transformation in agricultural ecosystems.

The most striking change in community structure was that of the Eurotiomycetes. In NPK-treated soils (NPK and MNPK), Eurotiomycetes abundances were more than doubled compared with the other treatments (CK and M). Eurotiomycetes consisted mainly of '*Penicillium*- and *Aspergillus*-like' taxa in the present soils, with *Penicillium chrysogenum*-like phylotype accounting for 86% and 81% in the clone libraries of NPK and MNPK treatments, respectively (Table 3). This is in agreement with a report on a red soil in which numbers of *Penicillium* isolates were enhanced by mineral fertilizer treatments (He et al., 2008). It seems that *Penicillium* is copiotrophs and may be able to outcompete other fungal types with NPK addition. This taxa may produce antagonist secondary metabolites and reduce activity of other cellulolytic microorganisms (Santamarina et al., 2002). In addition to Eurotiomycetes, mineral fertilizer treatment decreased the relative abundance of Dothideomycetes by up to 66% and 78% in the soils amended with and without organic manure, respectively (Table 3). The mineral fertilizer-induced reduction was also observed for the relative abundance of Sordariomycetes. The same pattern of relative abundance of Dothideomycetes and Sordariomycetes as soil CBH activity variation suggests the reduction is of functional importance. Considering diversity and community structure of cellulolytic fungi were significantly correlated with several soil properties (e.g. soil pH and nitrate, Table 4, Fig. 4), it seems that fertilization regimes change cellulose degradation through altering abiotic habitat to shape a cellulolytic community of low activity.

Although shifts in cellulolytic fungi were found to be associated with C biogeochemistry, they might interact with many other processes to control the long-term soil C balance. For instance, nutrient amendment may alter the physiological status of the crop and reduce the carbon allocation to root tissue and the rhizosphere as exudates. This change in carbon input may interact with soil nutrient status to differentially prime C decomposition of soil native organic carbon (Kuzyakov, 2002; Fontaine et al., 2004),

Table 4
Correlations among soil respiration, cellobiohydrolase (CBH) activity, abundance and diversity (Shannon index) of cellulolytic fungi and soil properties.

	pH	Total nitrogen	Total phosphorus	Organic matter	Nitrate	C:N	Respiration rate	CBH activity	*cbhI* gene abundance
Total nitrogen	−0.240								
Total phosphorus	−0.706[a]	0.702[a]							
Organic matter	−0.389	0.891[b]	0.822[b]						
Nitrate	−0.349	0.007	0.365	0.374					
C:N	−0.085	−0.775[b]	−0.252	−0.410	0.497				
Respiration rate	0.448	0.582[a]	−0.114	0.394	−0.239	−0.649[a]			
CBH activity	0.686[a]	−0.024	−0.662[a]	−0.242	−0.491	−0.315	0.745[b]		
cbhI gene abundance	−0.039	0.547	0.068	0.292	−0.586[a]	−0.714[b]	0.608[a]	0.471	
Shannon index	0.770[b]	−0.060	−0.657[a]	−0.183	−0.311	−0.178	0.714[b]	0.951[b]	0.313

[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

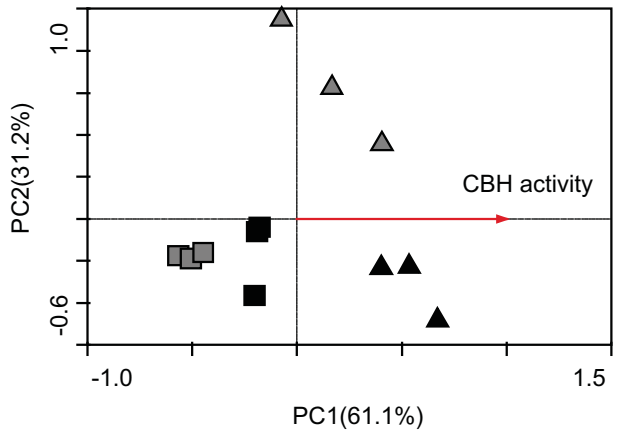

Fig. 3. Redundancy analysis of the correlation of cellulolytic fungi community structure with CBH activity. Community structures are presented as the relative abundance of each TRF. Gray triangles indicate no fertilizer (CK), gray squares indicate combination of nitrogen, phosphorus, and potassium (NPK), filled triangles indicate organic manure (M) and filled squares indicate NPK plus M (MNPK).

which is not the focus in the present study. In addition, since microbial community and carbon transformation show strong seasonal dynamics (Fan et al., 2008; Carbone et al., 2011), it is currently unknown how the mechanism found in the present study operates to determine long-term carbon storage. More detailed studies employing sensitive analytical methods such as catalyzed reporter deposition (CARD)-FISH at mRNA level (Pratscher et al., 2011) may help to resolve the complicated picture of microbial (especially bacterial) mechanisms underpinning soil carbon transformation in agroecosystems.

In conclusion, we found that long-term mineral fertilizer increased soil organic C and crop productivity, and reduced soil heterotrophic respiration and CBH activity. Correspondingly, diversity and community structure of cellulolytic fungi were substantially altered. In addition, CBH activity was significantly correlated with diversity and community structure of cellulolytic fungi. These results suggest that NPK-enhanced C storage, apart from extra organic carbon input, may be mediated by cellulolytic fungal communities in cellulose-rich arable soil. This is probably the first report describing a significant effect of agricultural management on a functional microbial group mediating organic carbon transformation using culture-independent method.

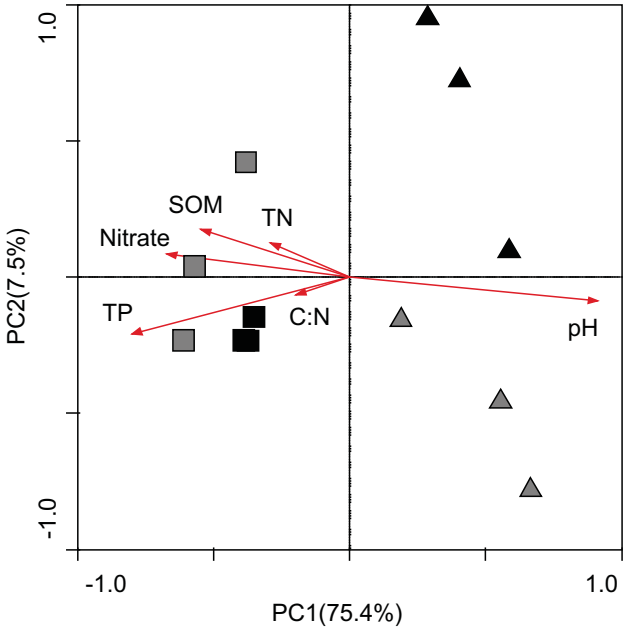

Fig. 4. Redundancy analysis of the correlation of cellulolytic fungi community structure with soil properties. Community structures are presented as the relative abundance of each TRF. Gray triangles indicate no fertilizer (CK), gray squares indicate combination of nitrogen, phosphorus, and potassium (NPK), filled triangles indicate organic manure (M) and filled squares indicate NPK plus M (MNPK).

Acknowledgments

This work was jointly supported by National Natural Science Foundation of China, National Key Technology R&D Program and The Funding from Institute of Agricultural Resources and Regional Planning of Chinese Academy of Agricultural Sciences. We are also grateful to the two anonymous reviewers for their helpful comments on the manuscript.

References

Ai, C., Liang, G., Sun, J., Wang, X., Zhou, W., 2012. Responses of extracellular enzyme activities and microbial community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil. Geoderma 173–174, 330–338.

Allison, S.D., Hanson, C.A., Treseder, K.K., 2007. Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems. Soil Biology and Biochemistry 39, 1878–1887.

Anderson, S.H., Gantzer, C.J., Brown, J.R., 1990. Soil physical properties after 100 years of continuous cultivation. Journal of Soil and Water Conservation 45, 117–121.

Artz, R.R.E., Reid, E., Anderson, I.C., Campbell, C.D., Cairney, J.W.G., 2009. Long term repeated prescribed burning increases evenness in the basidiomycete laccase gene pool in forest soils. FEMS Microbiology Ecology 67, 397–410.

Blackwood, C.B., Waldrop, M.P., Zak, D.R., Sinsabaugh, R.L., 2007. Molecular analysis of fungal communities and laccase genes in decomposing litter reveals differences among forest types but no impact of nitrogen deposition. Environmental Microbiology 9, 1306–1316.

Carbone, M.S., Still, C.J., Ambrose, A.R., Dawson, T.E., Williams, A.P., Boot, C.M., Schaeffer, S.M., Schimel, J.P., 2011. Seasonal and episodic moisture controls on plant and microbial contributions to soil respiration. Oecologia 167, 265–278.

Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A., Parkhurst, D.F., 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81, 2359–2365.

Collins, R.E., Rocap, G., 2007. REPK: an analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Research 35, W58–W62.

Ding, W., Meng, L., Yin, Y., Cai, Z., Zheng, X., 2007. CO_2 emission in an intensively cultivated loam as affected by long-term application of organic manure and nitrogen fertilizer. Soil Biology and Biochemistry 39, 669–679.

Edmeades, D.C., 2003. The long-term effects of manures and fertilisers on soil productivity and quality: a review. Nutrient Cycling in Agroecosystems 66, 165–180.

Edwards, I.P., Upchurch, R.A., Zak, D.R., 2008. Isolation of fungal cellobiohydrolase I genes from sporocarps and forest soils by PCR. Applied and Environmental Microbiology 74, 3481–3489.

Edwards, I.P., Zak, D.R., Kellner, H., Eisenlord, S.D., Pregitzer, K.S., 2011. Simulated atmospheric N deposition alters fungal community composition and suppresses ligninolytic gene expression in a northern Hardwood Forest. PLoS One 6, 1–10.

Fan, F., Zhang, F., Qu, Z., Lu, Y., 2008. Plant carbon partitioning below ground in the presence of different neighboring species. Soil Biology and Biochemistry 40, 2266–2272.

Fontaine, S., Bardoux, G., Abbadie, L., Mariotti, A., 2004. Carbon input to soil may decrease soil carbon content. Ecology Letters 7, 314–320.

Gill, R.A., Polley, H.W., Johnson, H.B., Anderson, L.J., Maherali, H., Jackson, R.B., 2002. Nonlinear grassland responses to past and future atmospheric CO_2. Nature 417, 279–282.

He, J.Z., Zheng, Y., Chen, C.R., He, Y.Q., Zhang, L.M., 2008. Microbial composition and diversity of an upland red soil under long-term fertilization treatments as revealed by culture-dependent and culture-independent approaches. Journal of Soils and Sediments 8, 349–358.

Hofmann, A., Heim, A., Gioacchini, P., Miltner, A., Gehre, M., Schmidt, M.W.I., 2009. Nitrogen fertilization did not affect decay of old lignin and SOC in a 13 C-labeled arable soil over 36 years. Biogeosciences Discussions 6, 1657–1675.

Huber, T., Faulkner, G., Hugenholtz, P., Huber, T., Faulkner, G., Hugenholtz, P., 2004. Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20, 2317–2319.

Jarecki, M.K., Lal, R., 2003. Crop management for soil carbon sequestration. Critical Reviews in Plant Sciences 22, 471–502.

Jin, S., Chen, H., 2007. Near-infrared analysis of the chemical composition of rice straw. Industrial Crops & Products 26, 207–211.
Johnson, J.M.F., Barbour, N.W., Weyers, S.L., 2007. Chemical composition of crop biomass impacts its decomposition. Soil Science Society of America Journal 71, 155–162.
Khan, S.A., Mulvaney, R.L., Ellsworth, T.R., Boast, C.W., 2007. The myth of nitrogen fertilization for soil carbon sequestration. Journal of Environmental Quality 36, 1821–1831.
Knorr, M., Frey, S.D., Curtis, P.S., 2005. Nitrogen additions and litter decomposition: a meta-analysis. Ecology 86, 3252–3257.
Kuzyakov, Y., 2002. Review: factors affecting rhizosphere priming effects. Journal of Plant Nutrition and Soil Science 165, 382–396.
Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. Science 304, 1623–1627.
Leifeld, J., Kögel-Knabner, I., 2005. Soil organic matter fractions as early indicators for carbon stock changes under different land-use? Geoderma 124, 143–155.
Lilleskov, E.A., Fahey, T.J., Horton, T.R., Lovett, G.M., 2002. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. Ecology 83, 104–115.
Luque, A.G., Pioli, R., Bonel, B., Alvarez, D.P., 2005. Cellulolytic fungi populations in stubble and soil as affected by agricultural management practices. Biological Agriculture and Horticulture 23, 121–142.
Meier, C.L., Bowman, W.D., 2008. Links between plant litter chemistry, species diversity, and below-ground ecosystem function. Proceedings of the National Academy of Sciences, United States of America 105, 19780–19785.
Moubasher, A.H., Mazen, M.B., 1991. Assay of cellulolytic activity of cellulose-decomposing fungi isolated from Egyptian soils. Journal of Basic Microbiology 31, 59–68.
Pansu, M., Gautheyrou, J., 2006. Handbook of Soil Analysis: Mineralogical, Organic and Inorganic Methods. Springer Verlag, Berlin.
Parton, W.J., Paustian, K., Persson, J., 1992. Modeling soil organic matter in organic-amended and nitrogen-fertilized long-term plots. Soil Science Society of America Journal 56, 476–488.
Pereyra, L.P., Hiibel, S.R., Prieto Riquelme, M.V., Reardon, K.F., Pruden, A., 2010. Detection and quantification of functional genes of cellulose-degrading, fermentative, and sulfate-reducing bacteria and methanogenic archaea. Applied and Environmental Microbiology 76, 2192–2202.
Pratscher, J., Dumont, M.G., Conrad, R., 2011. Ammonia oxidation coupled to CO_2 fixation by archaea and bacteria in an agricultural soil. Proceedings of the National Academy of Sciences, United States of America 108, 4170–4175.
Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biology and Biochemistry 34, 1309–1315.
Santamarina, M.P., Roselló, J., Llacer, R., Sanchis, V., 2002. Antagonistic activity of *Penicillium oxalicum* Corrie and Thom, *Penicillium decumbens* Thom and *Trichoderma harzianum* Rifai isolates against fungi, bacteria and insects in vitro. Revista iberoamericana de micología 19, 99–103.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4. 0. Molecular Biology and Evolution 24, 1596–1599.
Ter Braak, C.J.F., Smilauer, P., 2002. CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5). Microcomputer Power, Ithaca, NY, pp. 1–500.
Thomsen, M.H., Thygesen, A., Thomsen, A.B., 2008. Hydrothermal treatment of wheat straw at pilot plant scale using a three-step reactor system aiming at high hemicellulose recovery, high cellulose digestibility and low lignin hydrolysis. Bioresource Technology 99, 4221–4228.
Waldrop, M.P., Zak, D.R., Sinsabaugh, R.L., Gallo, M., Lauber, C., 2004. Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. Ecological Applications 14, 1172–1177.
Weber, C.F., Zak, D.R., Hungate, B.A., Jackson, R.B., Vilgalys, R., Evans, R.D., Schadt, C.W., Megonigal, J.P., Kuske, C.R., 2011. Responses of soil cellulolytic fungal communities to elevated atmospheric CO_2 are complex and variable across five ecosystems. Environmental Microbiology 13, 2778–2793.
Wilson, H.M., Al-Kaisi, M.M., 2008. Crop rotation and nitrogen fertilization effect on soil CO_2 emissions in central Iowa. Applied Soil Ecology 39, 264–270.
Yu, W.T., Zhao, X., Ma, Q., Zhou, H., 2008. Effect of long-term fertilization on available carbon pool and carbon pool management index in an aquic brown soil. Chinese Journal of Soil Science 39, 539–544.
Yu, W.T., Jiang, Z.S., Zhou, H., Ma, Q., 2009. Effects of nutrient cycling on grain yields and potassium balance. Nutrient Cycling in Agroecosystems 84, 203–213.
Zhang, W.J., Wang, X.J., Xu, M.G., Huang, S.M., Liu, H., Peng, C., 2009. Soil organic carbon dynamics under long-term fertilizations in arable land of northern China. Biogeosciences Discussions 6, 6539–6577.