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

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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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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