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Evaluation of an optimal extraction method for measuring D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) in agricultural soils and its association with soil microbial CO_2 assimilation

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ABSTRACT

Assimilating atmospheric carbon (C) into terrestrial ecosystems is recognized as a primary measure to mitigate global warming. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is the dominant enzyme by which terrestrial autotrophic bacteria and plants fix CO_2. To investigate the possibility of using RubisCO activity as an indicator of microbial CO_2 fixation potential, a valid and efficient method for extracting soil proteins is needed. We examined three methods commonly used for total soil protein extraction. A simple sonication method for extracting soil protein was more efficient than bead beating or freeze–thaw methods. Total soil protein, RubisCO activity, and microbial fixation of CO_2 in different agricultural soils were quantified in an incubation experiment using ^{14}C-CO_2 as a tracer. The soil samples showed significant differences in protein content and RubisCO activity, defined as nmol CO_2 fixed g^{-1} soil min^{-1}. RubisCO activities ranged from 10.68 to 68.07 nmol CO_2 kg^{-1} soil min^{-1}, which were closely related to the abundance of *cbbL* genes ($r = 0.900$, $P = 0.0140$) and the rates of microbial CO_2 assimilation ($r = 0.949$, $P = 0.0038$). This suggests that RubisCO activity can be used as an indicator of soil microbial assimilation of atmospheric CO_2.

Introduction

The anthropogenic increase in atmospheric carbon dioxide (CO_2) is generally believed to significantly contribute to global warming (Lacis et al., 2010). Transferring carbon (C) from the atmosphere to terrestrial ecosystems is a major C sequestration measure (Midgley et al., 2010). Carbon sequestration in terrestrial ecosystems is a result of the assimilation of CO_2 by autotrophic bacteria, algae, and plants (Tabita, 1999) via the Calvin–Benson–Basham (CBB) cycle. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is the most abundant protein on Earth (Raven, 2013) and an essential enzyme in the CBB cycle. It fixes CO_2 through the reductive pentose phosphate pathway by combining with CO_2 to form 3-phosphoglycerate (Siegenthaler and Sarmiento, 1993; Sato et al., 2010). RubisCO is a major autotrophic carboxylase in all photosynthetic organisms, and more than 99.5% of the inorganic C assimilated by primary producers (chemolithotrophs as well as photolithotrophs) involves RubisCO (Raven, 2009).

RubisCO enzymes, which exist in a variety of autotrophic organisms including *Proteobacteria* and *Acinobacteria* as well as *Cyanobacteria*, *Firmicutes* and *Chloroflexi* (Hügler and Sievert, 2010), have been widely investigated in aquatic ecosystems or laboratory microbial cultivation (Ezaki et al., 1999; Chakrabarti et al., 2002; Takai et al., 2005; Hügler and Sievert, 2010; Tourova et al., 2010) because of the enzymatic properties of RubisCO that link directly to CO_2 fixation rates. However, relative few studies are concerned with the RubisCO of microbes involved in soil CO_2 assimilation. Carbon fixation genes (*cbbL*), which encode the RubisCO enzyme, are numerous and widespread in diverse soil ecosystems (Selesi et al.,

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Table 1
Characteristics of paddy and upland soils used in this study.

Soil	Soil type	Dominant crop	pH	TC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	CEC (cmol kg^{-1})	Clay content (%)	MBC (mg kg^{-1})
P1	Fluvisol	Rice	5.8 ± 0.01	20.9 ± 1.03	2.7 ± 0.02	1.2 ± 0.00	12.8 ± 0.32	39.8 ± 1.40	1029.8 ± 27.8
P2	Fluvisol	Rice	5.2 ± 0.00	18.5 ± 0.16	2.4 ± 0.03	0.7 ± 0.02	5.8 ± 0.05	22.1 ± 0.55	875.6 ± 15.7
P3	Ultisol	Rice	6.4 ± 0.01	19.84 ± 0.40	2.1 ± 0.01	0.8 ± 0.00	16.1 ± 0.24	24.5 ± 0.39	898.9 ± 9.6
U1	Fluvisol	Maize	6.0 ± 0.04	11.4 ± 0.28	1.8 ± 0.01	1.4 ± 0.06	12.4 ± 0.41	21.0 ± 0.56	382.6 ± 11.6
U2	Fluvisol	Soybean	7.5 ± 0.00	9.07 ± 0.13	1.3 ± 0.00	0.4 ± 0.06	22.5 ± 0.13	36.3 ± 0.99	156.4 ± 6.8
U3	Ultisol	Vegetables	6.7 ± 0.01	18.26 ± 0.10	2.9 ± 0.02	3.9 ± 0.05	14.2 ± 0.10	26.1 ± 1.84	192.3 ± 13.8

Note: P1–P3 were paddy soils; U1–U3 were upland soils. All values are expressed on a dry weight basis and represent means of three replicates. TC: total carbon. TN: total nitrogen. TP: total phosphorus. CEC: cation exchange capacity. MBC: microbial biomass carbon.

2007; Videmšek et al., 2009; Yuan et al., 2012; Wu et al., 2013; Xiao et al., 2014).

In comparison to functional gene studies, the analysis of proteins can provide more information about active metabolic pathways (Singleton et al., 2003; Tyson et al., 2004; Ram et al., 2005; Schulze et al., 2005; Wilmes and Bond, 2006; Benndorf et al., 2007; Renella et al., 2014). This protein-based technique can characterize soil enzymes involved in biogeochemical cycling in both natural and polluted soil ecosystems, such as rhizosphere soil, grassland soil, forest soil and contaminated soil (Renella et al., 2002; Singleton et al., 2003; Schulze et al., 2005; Benndorf et al., 2007; Fornasier and Margon, 2007; Wang et al., 2011; Wu et al., 2011). Enzymes are protein molecules with catalytic activities (Ogunseitan, 1997), therefore, the accuracy of the RubisCO activity measurements of field samples depends on obtaining representative extracts of bioactive protein. In the initial efforts to extract protein from sediments and soils, researchers used either cell extraction (recovery of cells from the soil matrix prior to cell lysis) or direct lysis within the soil matrix (Singleton et al., 2003; Barzaghi et al., 2004; Benndorf et al., 2007). Direct extraction of soil protein provides a less biased sample of the soil microbial communities (Singleton et al., 2003; Ogunseitan, 2006).

Bead beating, freeze–thaw and sonication are three commonly used methods for breaking aggregated soil particles and for lysing cells to release protein molecules (Singleton et al., 2003; Ogunseitan et al., 2004; Bastida et al., 2009). However, these soil protein extraction methods are inefficient in the various individual step, with problems of incomplete cell lysis, protein sorption on soil surfaces, and loss or degradation of the protein. Therefore, the primary objective of this work was to establish an optimal method for rapid and efficient soil protein extraction. The extraction method we propose here preserves protein function as much as possible and extracts protein from the entire microbial community, representing the contributions from both prokaryotic and eukaryotic microorganisms. Once the method was established, it was used to examine the activity of soil RubisCO in response to changes in soil microbial CO_2 assimilation rates in different types of agricultural soils using a ^{14}C-CO_2 tracer experiment. We hypothesized that the measurement of soil RubisCO activity following appropriate calibration can provide a reasonable estimate of the microbial CO_2 fixation potential in the soil.

Materials and methods

Soils

Three separate bulked soil samples from paddy fields P1–P3 and three soils samples from upland crop fields U1–U3, which covered the two typical (Fluvisol, Ultisol) paddy and upland soils in the subtropical region of China, were collected from the Ap horizon (0–20 cm) in different geographical regions of China (28°12′–29°50′ N, 111°13′–113°45′ E) (Table 1). Mean annual temperatures of the sampling sites range from approximately 8.1 °C to 16.8 °C and mean annual rainfall range from approximately 721 mm to 1400 mm. Soil physiochemical properties are shown in Table 1. After removing visible plant residues, the soil samples were sieved to <5 mm. The paddy soils were flooded with distilled water, and the upland soils were adjusted to 45% water-holding capacity (WHC) (Priha and Smolander, 1999). All soils were pre-incubated for 2 weeks at 25 °C after flooding or rewetting prior to incubation.

Incubation experiment with $^{14}CO_2$

Each soil sample was divided into four replicates. For each replicate, 1 kg moist soil (dry soil equivalent) was added to a PVC container (10 cm diameter, 20 cm height). The pots were then incubated for 110 days in an airtight glass chamber (80 cm × 250 cm, height 120 cm), as previously described by Ge et al. (2012, 2013). This chamber was capable of generating $^{14}CO_2$ (concentration maintained at ≈350 μLL^{-1}) through a reaction between $Na_2{}^{14}CO_3$ (at a radioactivity of 1.65×10^4 Bq mL^{-1}) and HCl (2 M). The artificial light intensity was kept at 500 mmol photons m^{-2} s^{-1} photosynthesis active radiation (PAR) from 8:00 am to 8:00 pm, and the relative humidity was maintained at 80–90% with day/night temperatures of 31 ± 1 °C/24 ± 1 °C during the incubation period. Following the incubation (after labeling for 110 days), the soil in each pot was divided into two sub-samples. One sub-sample was analyzed for fixed ^{14}C to trace the incorporation of ^{14}C-CO_2 into soil organic matter through microbial assimilation. The other sub-sample (about 50 g) was freeze-dried and stored in 10 mL sterile centrifuge tubes at −70 °C for further analysis of microbial RubisCO activity. Gravimetric water content was determined at different sampling times.

Soil protein extraction

We tested three methods for soil protein extraction: bead beating, freeze–thaw and sonication. These methods were modified from those proposed by Singleton et al. (2003) and Takai et al. (2005).

Bead beating-Fastprep

Briefly, 0.5 g (freeze-dried) soil samples were weighed into microcentrifuge tubes (2.0 mL capacity) to which 0.2 g 150–212 μm sterile glass beads, 100 μL of protease inhibitor (2.0 μg mL^{-1}, Sigma) and 1 mL of extraction buffer (50 mM Tris–HCl, 10% sucrose, 2 mM dithiothreitol (DTT), 4 mM EDTA, 0.1% Brij 58, pH 7.58) were added. The samples were then prepared following the protocols listed in Table 2 by shaking at 5.5 ms^{-1} in a FastPrep^{TM} instrument (Pri-Eco, Beijing, China).

Freeze–thaw

Freeze-dried soil samples (0.5 g) were weighed into microcentrifuge tubes (2.0 mL capacity) to which 100 μL of protease inhibitor (2.0 μg mL^{-1}, Sigma) and 1 mL of extraction buffer were added. After addition of the buffer solution, the microcentrifuge tubes were sealed and spun for 10 s in a vortex mixer. The lids were then