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Phosphate levels influence the utilisation of rice rhizodeposition carbon and the phosphate-solubilising microbial community in a paddy soil

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ABSTRACT

To fulfil the demand of a growing population for rice food, a better understanding of soil biological systems is urgently required to improve the efficient use of chemical fertilisers, especially phosphate (P)-based fertilisers. Microorganisms play an important role in the decomposition of organic compounds and solubilisation of inorganic P. Presently, whether P availability affects the incorporation of rice root-derived carbon and the activity of P-solubilising bacteria remains uncertain. Herein, stable isotope probing of phospholipid fatty acids and DNA and high-throughput sequencing methods were used to investigate active P-solubilising bacteria and the microbial community in a paddy soil treated at 0 (controls), 50, and 200 mg P_2O_5 kg^{-1}. Compared with controls (23.4–62.2%), the $\delta^{13}C$ values of 16:1ω7c, 18:1ω7c, and 18:1ω9c (Gram-negative bacteria) increased to 69.2–167.4% with low P input, but decreased to 6.6–58.6% under high P input. Similarly, higher ^{13}C enrichment (1.04–1.47 ng g^{-1} dry weight soil) was observed in phospholipid fatty acids (16:1ω7c, 18:1ω7c and 18:1ω9c) with low P input. High-throughput sequencing indicated that low rather than high P input promoted the growth of P-solubilising *Bacillus*, *Alicyclobacillus*, and *Clostridium*. The relative abundance of these organisms increased significantly in light DNA fractions following both ^{12}C and ^{13}C treatments, suggesting they seldom use rice root-derived carbon as carbon sources. By contrast, P-solubilising *Rhizobiales*, *Rhodospirillales*, and *Myxococcales* preferred root-derived carbon under P input conditions. Community and phylogenetic analysis of genes related to P solubilisation (alkaline phosphatase phoD and pyrroloquinoline quinone pqqC) indicated a dominant role for *Rhizobiales* and *Actinomycetales* in paddy soil P solubilisation.

1. Introduction

Paddy soil areas around the world cover approximately 135 million ha (IRRI, 2002). To increase the rice yield and meet the food demand of growing populations, chemical fertilisers are regularly applied to rice fields. Phosphorus deficiency is a major factor limiting rice yield, since P is often not readily available due to tight binding in Fe-P or Al-P forms. Although fertiliser application can alleviate P deficiency, increasing fertiliser costs and environmental degradation associated with N and P runoff are serious issues that need confronting. Hence, there is an imperative need to better comprehend the P cycle in plant-soil-microbe systems to reduce the demand for mineral fertilisers. Increasing interest in this regard can lead to the utilisation of microorganisms to support P cycling in agroecosystems (Pii et al., 2015).

Many soil bacteria and fungi play significant roles in converting soil P into forms that are more bioavailable to plants. Phosphate-solubilising microorganisms (PSMs), which are ubiquitous in soil, account for ~1–50% of bacteria and ~0.1–0.5% of fungi (Chen et al., 2006). The solubilisation of inorganic P is determined by the ability of the microbial community to produce organic (gluconic, oxalic, and citric) acids and extrude protons, which releases phosphate into the soil by decreasing the rhizosphere pH, chelating cations (Al, Fe, Ca), and competing for adsorption sites (Khan et al., 2014; Uroz et al., 2009), in addition to the mineralization of organic P. Generally, soil insoluble organic P can be mobilised by either cell-wall-bound or free enzymes, such as phosphomonoesterases, phosphoprotein phosphatases, phytases, and nucleotidases (Nannipieri et al., 2011). There is no evidence of alkaline phosphomonoesterase (ALP) activity in plants (Juma and

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Tabatabai, 1988), and soil ALPs are presumed to be derived mostly from microorganisms (Tabatabai, 1994).

Bacterial ALPs, especially those encoded by putative Pho regulon genes (phoA, phoD, and phoX), have been most thoroughly investigated in soil and aquatic ecosystems (Ferrera et al., 2015; Lidbury et al., 2016; Ragot et al., 2017). Although prokaryotic genome analysis suggests 32% of soil microbes carry at least one of either phoA, phoD, or phoX (Zimmerman et al., 2013), phoD was found to be the dominant ALP gene in pasture soils (Tan et al., 2012). As a cofactor in the production of gluconic acid, which is the key driver of phosphate solubilisation for Gram-negative bacteria, pyrroloquinoline quinine (PQQ) is an alternative bio-indicator for PSMs (Rodríguez et al., 2006). The activity and microbial community of P-solubilising bacteria can be significantly affected by multiple environmental factors, including soil pH (Ragot et al., 2015), vegetation (Wang et al., 2012), land-use, nutrient concentration (TOC, TN, TP and organic P) (Ragot et al., 2017) and fertiliser management (Fraser et al., 2015; Tan et al., 2012). However, previous studies have primarily focused on P-solubilising bacteria in dry land ecosystems.

Paddy soils are characterised by alternating redox cycles that create a special environment for nutrient transformation. A shift from oxidising to reducing conditions alters the P equilibrium of Mn and/or Fe oxides in paddy soils, and increases the release of P from organic fractions and the exchange between organic anions and phosphate ions in Fe-P and AI-P compounds. In general, the availability of soil native or added P increases under flood conditions (Sanyal and Datta, 1991). However, due to long-term chemical fertiliser P input, excess P has accumulated in paddy soils, raising environmental concerns. Application of PSMs in paddy soils not only promotes the rice yield, but also favours the P balance between soil and plants (Datta et al., 1982). A previous study revealed the occurrence of both anaerobic and aerobic PSMs in the rice rhizosphere, and anaerobic species outnumbered their aerobic counterparts (Raghu and MacRae, 1966). However, the effects of P input on the rice rhizosphere and the PSMs in paddy soils remain unclear.

To investigate the effects of P application on PSMs and the soil microbial community, a continuous ^{13}C-CO_2 labelling experiment was carried out on root rhizodeposition carbon from a long-term P-deficient paddy soil. DNA stable isotope probing in combination with phospholipid fatty acid (PLFA) analysis and high-throughput sequencing were used to determine the microbial composition. Functional genes (phoD and pqqC) of PSMs were examined by terminal restriction fragment length polymorphism (T-RFLP) using labelled DNA fractions. We hypothesised that (1) P inputs would alter rice rhizodeposition, and (2) P inputs would influence microbial processes involved in P cycling in the paddy soil.

2. Materials and methods

2.1. Soil sampling and microcosm incubation

Paddy soil was collected during August 2014 from a long-term fertiliser experiment site located at the Taoyuan Station of Agro-ecology Research (111°27′E, 28°55′N), Chinese Academy of Sciences, Hunan Province, China. The site has a subtropical monsoon climate with a mean annual temperature of 16.5 °C and average annual precipitation of 1447 mm. The soil is a hydragric anthrosol with a silt loam texture. This fertiliser experiment site was established in 1990. Sampling plots were treated with N (180 kg ha^{-1}), P (18 kg ha^{-1}), and K (56 kg ha^{-1}) from 1990 to 2004, and no further fertiliser has been added since 2004.

Topsoil samples (0–20 cm) were collected using a 7 cm diameter auger and a total of 10–12 cores were taken in each plot. Air-dried soils were sieved through a 2 mm sieve to remove plant debris and stones, and stored at room temperature.

The experiment was set up in September 2014. Three treatments were performed: controls (P0), low P (P50) and high P (P200). For all treatments, the equivalent of 100 mg N kg^{-1} and 100 mg K_2O kg^{-1} were added to all paddy soil samples using urea and KCL. Additionally, the equivalent of 50 mg and 200 mg P_2O_5 kg^{-1} were added to P50 and P200 treatments, respectively, using $NaH_2PO_4{\cdot}2H_2O$. For each treatment, 300 g of soil was added to a PVC pot (5.3 cm diameter, 13.2 cm height) and irrigated with approximately 200 mL of distilled water. Rice (*Oryza sativa* L.) seeds were presoaked with 6% H_2O_2 solution and rinsed with distilled water before incubation at room temperature. Three seedlings (~3 cm tall) were transplanted in each pot. Soils were flooded with water to a 2 cm depth above the soil surface at the three leaf stage. All pots were transferred to the continuous labelling chamber after growing in the greenhouse for 25 days. For PLFA-SIP analysis, three replicates of each treatment were collected at the first day and after one day of ^{13}C-CO_2 labelling, respectively. The remaining 18 pots (three replicates for each treatment under ^{12}C-CO_2 and ^{13}C-CO_2 conditions) were continuously labelled in the chamber for 20 days before soil sampling. Detailed information concerning the ^{13}C-CO_2 labelling chamber can be found in a previous study (Wang et al., 2016). Soil was separated from roots by washing with deionised water. The soil slurries were then freeze-dried for physicochemical and molecular analysis.

2.2. Microbial PLFA extraction and $\delta^{13}C$ analysis

Soil PLFAs were extracted from 2 g of freeze-dried sample as described previously (Bligh and Dyer, 1959; White et al., 1979). Briefly, samples were extracted with a solution of methanol, chloroform and phosphate buffer (2:1:0.8 v/v/v). Phospholipids were separated from glycol- and neutral-lipids on a silicic acid column by sequential washes with chloroform, acetone and methanol. Then, PLFAs were converted into fatty acid methyl esters by mild alkaline methanolysis. Fatty acid methyl esters contained the methyl nonadecanoate fatty acid (19:0) as an internal concentration standard to quantify phospholipids. Finally, methylated phospholipids were dissolved in hexane. PLFA patterns were determined using Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA) and a 25 m × 0.25 mm fused silica capillary column (HP-5). PLFAs $\delta^{13}C$ values were measured by a Flash 2000 HT Elemental Analyser connected via a GC/combustion/isotope ratio mass spectrometry system (GC-C-IRMS) (Thermo Fisher Scientific, Wilmington, DE, USA).

2.3. Soil DNA extraction and SIP gradient fractionation

Soil DNA was extracted from 0.5 g labelled samples using a FastDNA SPIN kit for soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions. Extracted DNA content was measured with a Nanodrop ND-1000 UV/vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 5 μL were loaded on a 1% agarose gel to check DNA quality. The DNA-SIP fractionation procedure was conducted in accordance with our previous study (Long et al., 2015). Briefly, for each sample, ~2–3.0 μg of DNA was gently mixed with a stock CsCl solution (7.163 M), and a final density of 1.6914 g mL^{-1} was obtained as determined using an AR200 hand-held refractometer (Reichert, Inc., Buffalo, NY, USA) from the infractive index (1.3999) reading. Ultracentrifugation was performed at 180,300 g (42,800 rpm) for 40 h at 20 °C in a Vti 65.2 vertical rotor (Beckman Coulter, Brea, CA, USA) using 5.1 mL tubes (Beckman, cat. no. 342412). DNA fractions (16 × 300 μL) were collected with an LSP01-1A single-channel syringe pump (Longer Precision Pump Co., Ltd., Baoding, Hebei, China) by replacing the gradient medium with sterile water. The buoyant density of each fraction was determined by the refractive index reading on the AR200 hand-held refractometer. All DNA fractions were purified with PEG solution (30% PEG 6000 and 1.6 M NaCl) and 70% ethanol, then dissolved in 35 μL of sterile water for further analysis.