Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Phosphorus status and microbial community of paddy soil with the growth of annual ryegrass (*Lolium multiflorum* Lam.) under different phosphorus fertilizer treatments*

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Received Apr. 12, 2009; Revision accepted June 29, 2009; Crosschecked Sept. 17, 2009

Abstract: Annual ryegrass (*Lolium multiflorum* Lam.) was grown in paddy soil in pots under different phosphorus (P) fertilizer treatments to investigate changes of P fractions and microbial community of the soil. The treatments included Kunyang phosphate rock (KPR) applications at 50 mg P/kg (KPR_{50}) and 250 mg P/kg (KPR_{250}), mono-calcium phosphate (MCP) application at 50 mg P/kg (MCP_{50}), and the control without P application. The results showed that KPR_{50}, KPR_{250}, and MCP_{50} applications significantly increased the dry weight of the ryegrass by 13%, 38%, and 55%, and increased P uptake by 19%, 135%, and 324%, respectively. Compared with MCP_{50}, the relative effectiveness of KPR_{50} and KPR_{250} treatments in ryegrass production was about 23% and 68%, respectively. After one season of ryegrass growth, the KPR_{50}, KPR_{250}, and MCP_{50} applications increased soil-available P by 13.4%, 26.8%, and 55.2%, respectively. More than 80% of the applied KPR-P remained as HCl-P fraction in the soil. Phospholipid fatty acid (PLFA) analysis showed that the total and bacterial PLFAs were significantly higher in the soils with KPR_{250} and MCP_{50} treatments compared with KPR_{50} and control. The latter had no significant difference in the total or bacterial PLFAs. The KPR_{50}, KPR_{250}, and MCP_{50} treatments increased fungal PLFA by 69%, 103%, and 69%, respectively. Both the principal component analysis and the cluster analysis of the PLFA data suggest that P treatments altered the microbial community composition of the soils, and that P availability might be an important contributor to the changes in the microbial community structure during the ryegrass growth in the paddy soils.

Key words: Phosphorus fractionation, Phospholipid fatty acid (PLFA), Ryegrass, Phosphate rock
doi:10.1631/jzus.B0920101 **Document code:** A **CLC number:** S143

INTRODUCTION

Phosphorus (P) is considered the prime limiting factor on plant growth in many areas, because it is the least mobile and available essential nutrient in soil (Hinsinger, 2001). P fertilizer application is needed to sustain optimum plant production and quality (Zapata and Zaharah, 2002). The main objective of P management is to prevent P deficiency rather than to alleviate P-deficiency symptoms. If soil P supply is low, management must be focused on the buildup and maintenance of adequate soil-available P levels to ensure that P supply does not limit crop growth and N-use efficiency (Fairhurst and Witt, 2002). In the past, numerous studies were focused on possible substitution of phosphate rock (PR) for water-soluble P fertilizers mainly based on agronomic and economic considerations. Results have shown that PR can be as effective as superphosphate in increasing plant yield and improving soil P status in many tropic and subtropical areas (Chien *et al*., 1980; Bolan *et al*., 1990). PR applied to upland crops is more effective

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* Project supported by Zhejiang Provincial Key Laboratory of Subtropical Soil and Plant Nutrition, and the Key Laboratory of Polluted Environment Remediation and Ecological Health of Ministry of Education, Zhejiang Province, China

for rice in rice-upland crop rotation than for that in rice-rice system. It was suggested that the PRs be applied only to the upland crops so that the flooded rice could utilize residual available P (Meng *et al*., 2006). Recently, eutrophication of surface water bodies caused by excessive P from agricultural soil surface run-off or leaching has drawn many researchers to find strategies to mitigate the P pollution problem. It has been suggested that the use of reactive PR not only can sustain crop productivity but also may minimize eutrophication problems because of lower availability of PR for algal growth (Hart *et al*., 2004; Shigaki *et al*., 2006). However, more work including field studies is needed to validate this supposition.

When a PR is applied to acid soil, dissolution of PR releases P to soil solution (Chien *et al*., 1980). Part of the released P can be absorbed directly by the plant roots, and part of the released P reacts with soil components such as Fe-Al-oxides to form reaction products with different availabilities, which can provide available P later through different release processes. Thus, both the reaction products and undissolved PR can provide available P to the plant (Chien, 1978; Chien and Menon, 1995). However, the detail of the transformation process of the PR-P in paddy soil is still not very clear.

It is known that plant rhizosphere process may enhance the dissolution of PR (Hoffland *et al*., 1989; Bolan *et al*., 1997), and that the soil microbial community-inhabitants can accelerate dissolution of PR by producing organic acids, phenolic compounds, protons, and siderophores (Drever and Vance, 1994). Several research studies have identified microbial groups that could solubilize P minerals and improve plant P nutrition (Duponnois *et al*., 2005). Numerous studies have been conducted on the impact of nitrogen fertilizer, manure, and different management practices on soil microbial community (Lovell *et al*., 1995; Murray *et al*., 2006; Toyota and Kuninaga, 2006). However, there are few studies conducted on the impact of P fertilizer, particularly PR application on the composition of soil microbial community (Rooney and Clipson, 2009).

The objective of this study was to investigate the changes of P fractions and microbial community of soil planted with annual ryegrass (*Lolium multiflorum* Lam.) under different P fertilizer applications.

MATERIALS AND METHODS

Soil samples

A surface paddy soil sample (0~15 cm in depth) of alluvial deposit was taken from a long term site-specific nutrient management trial in Jinhua City (29°01′ N, 119°37′ E), Zhejiang Province, China. The soil was acidic (pH 4.81), containing 278 g/kg sand, 562 g/kg silt, 160 g/kg clay, 255 mg/kg total P, 4.35 mg/kg Olsen-P, and 2.5 g/kg Fe_{DCB}. Soil pH was measured in deionized water at a soil:solution ratio of 1:1. Total P was digested with H_2SO_4-$HClO_4$, available P was extracted with the method of Olsen *et al*. (1954), and total free iron oxide was extracted with the method of Mehra and Jackson (1960). The soil was air-dried and ground to pass through a 2-mm sieve before potting.

Phosphorus sources

Kunyang phosphate rock (KPR), collected from Kunyang, Yunnan Province, China, was ground to pass through a 0.149-mm sieve. The KPR (pH 7.0 at solid:water=1:5 (w/v)) contained 138.5 g/kg total P and 29.1 g/kg of 2% (w/v) citric acid extractable P. The minerals of the KPR were identified by standard X-ray diffraction (Phillips-PW1732 X-diffractometer using nickel filter and Cu radiator with intensity of scan at (2°)/min at 40 kV and 20 mA). The empirical formula of apatite in Kunyang phosphate rock (KPR), was $Ca_{9.83}Na_{0.12}Mg_{0.05}(PO_4)_{5.50}(CO_3)_{0.50}F_{2.20}$. Monocalcium phosphate (MCP, $Ca(H_2PO_4)_2{\cdot}H_2O$) of analytical grade was used as water-soluble P fertilizer.

Pot experiment

A pot experiment was conducted in the greenhouse of Zhejiang University (China) in 2008. Treatments included applications of KPR at 50 and 250 mg P/kg (KPR_{50} and KPR_{250}), application of MCP at 50 mg P/kg (MCP_{50}), and the control (without P application). All the pots received 100 mg N/kg with urea and 50 mg K/kg with KCl. Fertilizer-P and Fertilizer-K were incorporated in the soil at the beginning of the experiment (basal). Fertilizer-N was applied with 50% as basal and 50% top-dressed 30 d after seeding. The pots were arranged in a randomized complete block design with three replicates. Approximately 30 seeds of ryegrass were sown per pot. After one-week growth, the ryegrasses were thinned

to 20 plants per pot. All pots were irrigated with deionized water to maintain 80% soil water-holding capacity during the entire experiment.

The ground ryegrass plants were cut 70 d after seeding when the plants were about 30 cm high. They were then oven-dried, weighed, and ground to pass through a 2-mm sieve. The concentration of P in the plants was determined after digestion with H_2SO_4-H_2O_2 mixture. Soil sample was taken from each pot after harvest. One portion of the soil sample was air-dried and ground to pass through a 0.148-mm sieve for P fractionation, and another portion of the soil sample was freeze-dried immediately at −50 °C, and then stored at −20 °C for microbial community analysis. The root material was removed from the sieved soil samples before lipid extraction.

Phosphorus fractionation

Soil P was sequentially fractionated following the method of Hedley *et al*.(1982). A 0.50 g soil sample (<0.148 mm) was shaken for 16 h with one HCO_3^- saturated resin capsule (purchased from UNIBEST Company, USA) enclosed in a nylon mesh-bag and immersed in 30 ml deionized water. After recovering the resin capsule, it was then placed in 30 ml of 2.0 mol/L HCl for P desorption. After centrifugation of the soil-water suspension, the supernatant was discarded. The remaining soil was then successively extracted with 0.5 mol/L $NaHCO_3$ (pH 8.5), 0.1 mol/L NaOH, and 1 mol/L HCl each for 16 h. The inorganic P (P_i) concentration of each extract was determined and referred to as resin-, $NaHCO_3$-, NaOH-, and HCl-extractable P_i fractions. Organic P (P_o) in the resin and 1 mol/L HCl was not determined due to its negligible amount. Total P concentrations of $NaHCO_3$ and NaOH extracts were determined after acid ammonium persulfate digestion (EPA, 1971). P_o concentration of these extracts was calculated as the difference between the total and inorganic P, referred to as $NaHCO_3$- and NaOH-extractable P_o fractions. The soil residue was digested with 5 ml of concentrate H_2SO_4 and 0.5 ml of $HClO_4$. All P determinations were done in duplicates using a colorimetric method (Murphy and Riley, 1962).

Phospholipid fatty acid analysis

Lipid extraction and phospholipid fatty acid (PLFA) analysis were performed with the method of Bossio *et al*.(1998). A 3-g soil sample (equivalent dry weight) was extracted with a chloroform-methanol-citrate buffer mixture, and the phospholipids were separated from other lipids on a silica-bonded phase column (SPE-Si, Supelco, Poole, UK). The phospholipid fraction was subjected to mild alkaline hydrolysis for producing fatty acid methyl esters before analysis. *c*19:0 was used as the internal standard. Fatty acids were analyzed by Agilent 6890 gas chromatography with a flame ionization detector carried out by an MIDI Sherlocks microbial identification system (Version 4.5, MIDI, Newark, NJ, USA).

The fatty acid nomenclature used in this study included total number of carbon atoms:number of double bonds, followed by the position of the double bond (ω) from the methyl end of the molecule. *Cis* and *trans* geometries were indicated by the suffixes *c* and *t*, respectively. The prefixes *a* and *i* referred to anteiso- and iso-branching, respectively. 10Me indicated a methyl group on the tenth carbon atom from the carboxyl end of the molecule, position of hydroxyl (OH) groups was noted, and *cy* indicated cyclopropane fatty acids (Bossio *et al*., 2006). Each value was represented by mean of three replicates.

Bacterial PLFAs were represented by *i*15:0, *a*15:0, 15:0, *i*16:0, 16:1ω7*c*, *i*17:0, *a*17:0, *cy*17:0, 17:0, 18:1ω7*c*, and *cy*19:0ω8*c* (Bossio *et al*., 1998), fungal PLFAs were represented by 18:2ω6,9*c* and 18:3ω6*c* (6,9,12) (Myers *et al*., 2001; Vestal and White, 1989), and actinomycetic PLFAs were represented by 16:0 (10Me), 17:0 (10Me), 18:0 (10Me) (Turpeinen *et al*., 2004).

Statistical analysis

Statistical analysis was carried out with SPSS Version 13.0. Analysis of variance (ANOVA) was performed by using Fisher's least significant difference comparison of means (LSD). Principal component analysis (PCA) and cluster analysis of the PLFA data were carried out with the correlation matrix and Ward's method, respectively.

RESULTS

Yield response and phosphorus uptake of ryegrass

The soil was acidic (pH 4.81) with low P status (4.35 mg/kg Olsen-P), which might favor PR

dissolution. The pot experiment showed that KPR_{50}, KPR_{250}, and MCP_{50} applications significantly increased ryegrass dry weight by 13%, 38%, and 55%, and increased P uptake by 19%, 135%, and 324%, respectively (Table 1). Compared with MCP_{50}, the effectiveness of KPR_{50} and KPR_{250} treatments was about 73% and 89% in dry weight and 28% and 55% in P uptake, respectively.

Phosphorus fractions

After one season of ryegrass growth, KPR_{50}, KPR_{250}, and MCP_{50} applications increased soil available P (summation of resin-P, $NaHCO_3$-P_i, and $NaHCO_3$-P_o) by 13.4%, 26.8%, and 55.2%, respectively. NaOH-P_i also significantly increased while soil NaOH-P_o was relatively stable and less affected by P additions (Table 2). Soil HCl-P substantially increased after KPR application. The HCl-P is defined as Ca-associated P (Cross and Schlesinger, 1995), so the amounts of the undissolved apatite mineral of KPR in the soil can be estimated from the increases in the amounts of HCl-P in the KPR-treated soil over control soil (Tambunan *et al*., 1993). More than 80% of the applied KPR-P remained in the soil during one season of ryegrass growth. The MCP_{50} application did not show a significant increase of HCl-P or residual-P.

Table 1 Dry weight of ryegrass and uptake of P

Treatment	Dry weight (g/pot)	P uptake (mg/pot)
Control	0.80 ± 0.02^{d}	0.86 ± 0.09^{c}
KPR_{50}	0.90 ± 0.02^{c}	1.02 ± 0.10^{c}
KPR_{250}	1.10 ± 0.01^{b}	2.02 ± 0.20^{b}
MCP_{50}	1.24 ± 0.01^{a}	3.65 ± 0.48^{a}

Data are expressed as mean±SD of three replicates. Means with the same letter within the same column are not significantly different at 0.05 level from each other

Phospholipid fatty acid

The predominant PLFAs in the soil were 16:0, *i*15:0, 18:1ω9*c*, 18:1ω7, *cy*19:0ω8*c*, and 16:0 (10Me) (Fig.1). P application significantly (P<0.05) increased PLFA 18:2ω6,9*c*. PLFA *i*15:0 was significantly (P<0.05) higher in soil with MCP_{50} application than in soil with KPR application and the control. KPR application produced more (P<0.05) PLFA 16:1ω7*c* compared with MCP_{50} and control.

Table 2 Soil P fractions (mg/kg) with different P fertilizations after ryegrass planting

P treatment	Resin-P	$NaHCO_3$-P_i	$NaHCO_3$-P_o	NaOH-P_i	NaOH-P_o	HCl-P	Residual-P
Control	10.62^{c}	5.02^{d}	20.17^{b}	26.04^{d}	67.03^{a}	34.95^{c}	83.59^{a}
KPR_{50}	13.16^{b}	5.60^{c}	21.81^{b}	27.38^{c}	66.60^{a}	79.15^{b}	82.19^{a}
KPR_{250}	14.42^{b}	7.04^{b}	23.85^{a}	29.77^{b}	67.59^{a}	230.58^{a}	86.87^{a}
MCP_{50}	20.76^{a}	10.27^{a}	24.50^{a}	33.58^{a}	66.89^{a}	35.29^{c}	84.27^{a}

Means with the same letter within the same column are not significantly different at 0.05 level from each other

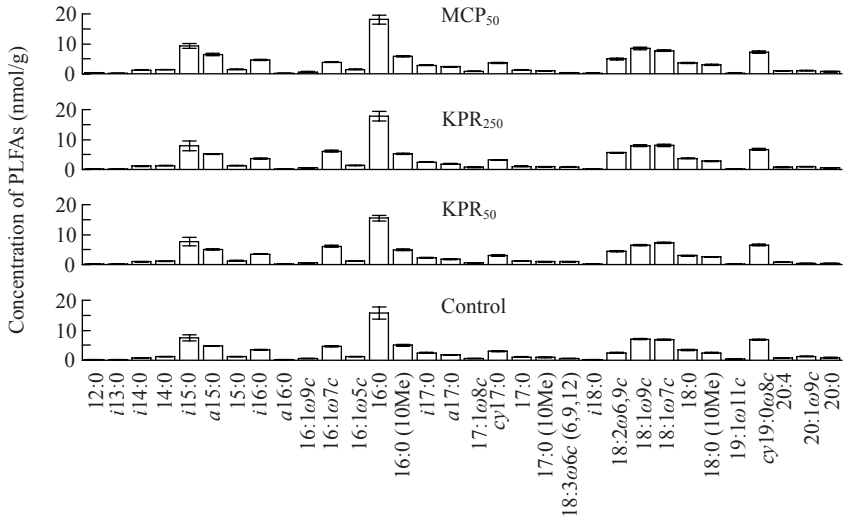

Fig.1 Concentrations of PLFAs in different P treatments

Table 3 shows PLFA concentrations of microbial groups in the soil, following the order of bacteria ($PLFA_{bact}$)>actinomycete ($PLFA_{acti}$)>fungi ($PLFA_{fung}$). The total PLFA ranged from 90 to 106 nmol/g in the soil with different P treatments. The total PLFA and bacterial PLFA were significantly higher in the soil with KPR_{250} and MCP_{50} treatments compared with KPR_{50} and control while the latter two treatments were not statistically different from each other. The KPR_{50}, KPR_{250}, and MCP_{50} treatments increased fungal PLFAs by 69%, 103%, and 69%, respectively. P application also increased the ratio of $PLFA_{fung}$/$PLFA_{bact}$, which might be a good indicator for a shift in soil microbial community. $PLFA_{acti}$ did not change significantly with KPR applications while MCP_{50} significantly increased $PLFA_{acti}$.

Changes of soil microbial community

The analysis of principal components (PCs) of the PLFA data indicated that different P treatments were significantly discriminated by their PLFA profile (Fig.2). The variations of the first two principal components, PC1 and PC2, were 71.23% and 17.84%, respectively. MCP_{50} significantly discriminated the other treatments on PC1. The control had the lowest ordinate scores on PC2, and it was clearly discriminated from the other treatments.

Cluster analysis of the PLFA data was also performed by using Ward's method (Fig.3). The results show that the microbial community structure in the four treatments could be classified into two large clusters: (1) Control, KPR_{50}, and KPR_{250} and (2) MCP_{50}. The first cluster could be subdivided into two clusters: (1) Control and KPR_{50} and (2) KPR_{250}.

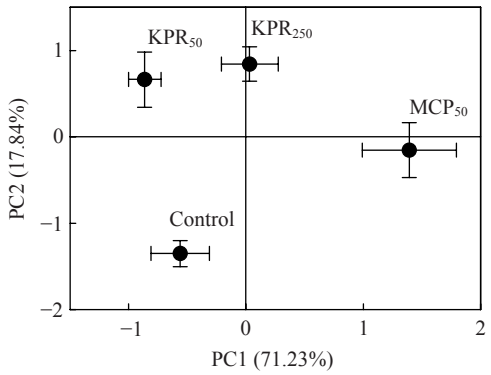

Fig.2 Principal component analysis of the PLFA profile in different P treated soils

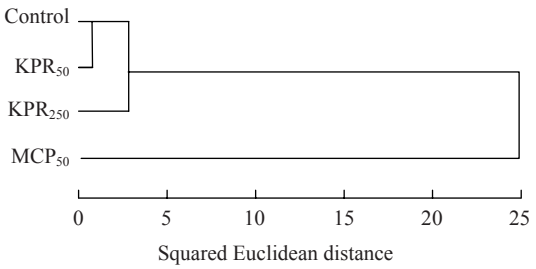

Fig.3 Cluster analysis of PLFA profile in different P treated soils

Table 3 Distribution of PLFA profiles (nmol/g) in different P treated soils

P treatment	$PLFA_{bact}$	$PLFA_{fung}$	$PLFA_{fung}/PLFA_{bact}$	$PLFA_{acti}$	$PLFA_{total}$
Control	43.66^{c}	3.15^{c}	0.07	8.36^{b}	90.00^{b}
KPR_{50}	45.47^{bc}	5.41^{b}	0.12	8.43^{b}	91.31^{b}
KPR_{250}	47.61^{ab}	6.50^{a}	0.14	8.94^{ab}	101.19^{a}
MCP_{50}	50.83^{a}	5.39^{b}	0.11	9.90^{a}	106.00^{a}

$PLFA_{bact}$, $PLFA_{fung}$, $PLFA_{acti}$, and $PLFA_{total}$ are bacterial, fungal, actinomycetic, and total PLFAs, respectively; Means with the same letters within the same column are not significantly different at 0.05 level from each other; n=6

DISCUSSION

The sequential chemical extraction method developed by Hedley *et al*.(1982) has been widely used to chemically fractionate the continuum of soil P. It is generally assumed that P availability to plants decrease with increasing strength of the chemicals used in the fractionation procedure. Measurable P fractions such as resin- and bicarbonate-extractable P are thought to be labile-P that contributes most to plant available-P, while hydroxide- and acid-extractable P fractions are thought to be P forms of moderate or low availability to plants (Cross and Schlesinger, 1995). However, recent research results have shown that lowland rice drew P from nearly the entire continuum of alkali- and acid-soluble inorganic soil P fractions under P exhaustion conditions (Zhang *et al*., 2006). In the current experiment, PR application not only

significantly increased ryegrass dry weight and P uptake by providing nutrient P during the ryegrass season, but also significantly increased soil available P and NaOH-P fractions after the ryegrass season, which could supply P to rice in next season. Most of the PR-P applied went to HCl-P and residual-P fractions in the soil, which could gradually provide available P to the plants of late seasons (Chien and Menon, 1995). To be practical, the rate of KPR-P application is usually about 5 times that of water-soluble P fertilizers (Meng *et al*., 2006). The relative agronomic effectiveness (RAE) of KPR_{250} treatment was about 68% in dry weight and 42% in P uptake with respect to MCP_{50} by taking RAE of control as 0%. From a practical perspective to guarantee no yield loss in the first several crop seasons, application of PR combined with certain amount of water-soluble P fertilizer might be necessary to maintain a suitable soil available P level. When the soil P status is high, there is no yield response to P application in the first two to three rice crop seasons as in the case of many paddy fields in Zhejiang Province (Zhang and Wang, 1999). Substitution of suitable PR for water-soluble P fertilizers to maintain P status or replenish P uptake and at the same time to reduce eutrophication due to high soil available P was tested for rice production in Zhejiang Province (Wu *et al*., 2002). It also has been used for pasture production in New Zealand (Bolan and Hedley, 1997). The benefits of this technique need to be further evaluated locally.

The paddy soil used in this study was taken from a long-term field experiment with double rice cropping after seven years of no P treatment, which resulted in low available P. Deficiencies of P limit both plant growth and microbial growth, so its availability is a major determinant of overall soil productivity (Zhang *et al*., 2005). The results show that ryegrass yield and soil total microbial biomass ($PLFA_{total}$) significantly increased after high rate of KPR (KPR_{250}) and water-soluble P fertilizer (MCP_{50}) applications compared with the low rate of KPR (KPR_{50}) and the control.

PLFAs are widely accepted as biomarkers that indicate viable components of soil microbial biomass and can provide a microbial community "fingerprint" (Liang *et al*., 2008). PLFA analysis can provide more detailed information of the "active" soil microbial community compared with the culture method (Vestal and White, 1989; Yao *et al*., 2000; Liang *et al*., 2008). Application of KPR significantly ($P<0.05$) increased fungal PLFA as well as the ratio of $PLFA_{fung}/PLFA_{bact}$ in the soil as water-soluble P did. Increased P nutrient availability, enhancement of root growth and exudation may have stimulated fungi (with a wide range of catabolic pathways) to utilize carbon sources unavailable to other microorganisms (Steer and Harris, 2000).

Both the principal component analysis and the cluster analysis of the PLFA data indicated a shift in microbial community under different P treatments compared with the control (no P application). Rooney and Clipson (2009) reported that phosphate addition to grassland soil influenced both fungal and bacterial communities. It was thought that phosphate was an important factor governing overall microbial communities in soil (Rooney and Clipson, 2009; Stutter *et al*., 2009). In this study, the microbial community changes could be related to changes in the availability of resources, particularly root exudates, and quantitative and qualitative changes in the input of organic substrates that were caused by the improvement of soil P availability (Yeates *et al*., 1997).

A different soil microbial community was established after water-soluble P fertilizer application compared with KPR and control. Water-soluble P application can immediately produce a higher soil available P level compared with PR because of poor solubility and slow release natures of PR. It was found that the increased P nutrient availability might increase C input to soils through enhanced plant growth and increased fine root turnover (King *et al*., 2002; Dawson *et al*., 2003). Relative utilization of this C source is an important driver of microbial group abundance (Paterson *et al*., 2007). It seems that P availability is an important contributor to the soil total microbial biomass and community diversity.

ACKNOWLEDGEMENT

The authors would like to thank Dr. S. H. Chien, Principal Soil Scientist of International Fertilizer Development Center (IFDC), USA, for his kindly revising and editing the manuscript. Thanks also go to Ms. Na Ding of Zhejiang University, China for her assistance in PLFA analysis.

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