Research article

Comparison of Microbial Diversity of Paddy Soils in Sustainable Organic Farming

CHUNCHARA THUITHAISONG
School of Environment, Resources and Development, Asian Institute of Technology, Phatum Thani, Thailand
Email: Chunchara.Thuithaisong@ait.ac.th

PREEDA PARKPIAN AND OLEG V. SHIPIN
School of Environment, Resources and Development, Asian Institute of Technology, Phatum Thani, Thailand

RAJENDRA P. SHRESTHA
School of Environment, Resources and Development, Asian Institute of Technology, Phatum Thani, Thailand

KUNNIKA NAKLANG
Surin Rice Research Center, Bureau of Rice Research and Development, Rice Department, Bangkok, Thailand

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Abstract Agricultural management significantly influences soil microbiological properties, such as microbial biomass carbon, microbial biomass nitrogen and respiration rate. Besides soil parameters, microbial diversity is useful for monitoring changes in soil quality to evaluate sustainable agriculture. This study aims to develop and use microbial diversity in rice soil as an indicator of soil quality for sustainable organic rice farming. An experiment was carried out using the existing rice fields (Kao Dawk_Mali 105 variety) in Surin Rice Research Center, Thailand. Four plots of rice received different management practices for over 11 years, including (1) conventional farming (CF) with a normal rate of chemical fertilizer applied, (2,3) two plots of organics: one with green manure (GM) and the other with rice straw (RS) and (4) a control plot (CT) without external sources of plant nutrients. Soil microbial communities were determined by cultural and molecular methods such as total plate counts, community level physiological profiling (CLPP) with BIOLOG^{TM} Ecoplate, and PCR-amplified (16S rDNA) and analysed by denaturing gradient gel electrophoresis (DGGE). Two-way ANOVA of results revealed that total plate counts were significantly ($P<0.05$) affected by the four different management practices. However, the GM plot ($2.3x10^6$ CFU g^{-1} of dry soil) and CF plot ($1.8x10^6$ CFU g^{-1} of dry soil) were not significantly different ($P>0.05$). Likewise soil management practices influenced the microbial diversity, both in functional and genetic diversity in the rice plots studied. Further a narrow range of Shannon-Weaver diversity index (H_f') was obtained with values between 2.77 for CT and 3.01 for GM. Statistical analysis (ANOVA) performed using substrate richness (S) from potential substrate utilization patterns as the input data, showed that the GM treatment increased the microbial diversity.

Keywords: Microbial diversity, organic farming, paddy soil, CLPP, PCR-DGGE

INTRODUCTION

Rice production is commonly associated with conventional farming practices including the use of chemical fertilizers and pesticides. Therefore, conventional farming practices, if not managed properly, can have adverse environmental consequences (Schionning et al., 2004). To ensure human survival it is necessary to maximize rice production while minimizing negative effects on

the environment (e.g. soil quality and microbial diversity). Alternative farming systems such as organic farming are required. Organic farming avoids or minimizes the use of synthetic fertilizers, pesticides and antibiotics. It has a positive effect on the build up of soil organic matter (SOM), mainly because of high organic matter inputs to the soil (green manure or rice straw), which is beneficial for soil microbial biomass and activity. Soil micro-organisms such as bacteria, fungi, protozoa, and nematode, play an important role in maintaining soil quality in agricultural management systems. Sustaining soil fertility is governed largely by the decomposition activity of the microorganisms. Agricultural management practices have been reported to influence soil microbial community structures (Sun et al., 2004). Microbial communities in soil mediate key processes that control ecosystem carbon (C) and nitrogen (N) cycling as well as other beneficial plant nutrients. The essence of sustainable agriculture is the maintenance of viable, diverse populations and functioning microbial communities in the soil. Microbial diversity relates to environmental impact and soil functioning, both of which seem inadequate for use as indicators (Anderson, 2003). Therefore, analyses of soil microbial diversity and community structures are essential when monitoring environmental influences on soil quality. Bacteria occurring in the soil are excellent indicators of soil health because they are involved in many soil processes and they respond quickly to changes in the soil ecosystem (Winding, 2004). Bacterial diversity is critical to ecosystem functioning because of the diversity of processes for which bacteria are responsible, including decomposition and nutrient cycling (Kennedy, 1999).

Recently a number of different methods and approaches have been used to investigate the microbial community. Previously investigation of microbial populations was determined by total plate count, which underestimates the actual soil microbial population. This is because less than 0.1% of agricultural soil micro-organisms are culturable. The BIOLOG™ EcoPlate assay is sensitive to changes in the short term from management practices. To investigate microbial diversity in the soil more data are required for PCR-amplified (16S rDNA) and analysed by DGGE to study genetic diversity of bacterial in the paddy soil. To accomplish this, a careful and thorough investigation of factors affecting bacterial diversity is required. A full appreciation of the genetic diversity of soil bacteria has not yet been accomplished compared to functional diversity. The aim of this paper is to investigate microbial diversity to serve as a biological indicator which can be further developed for use in an organic rice field.

METHODOLOGY

Study area, experimental design and soil sampling procedures

The study area was used to grow rice under organic and conventional farming at the Surin Rice Research Center, located in Korat Plateau, Muang District, Surin Province, Thailand (latitude 14^{o} 55'N and longitude 103^{o} 25'E). The studied soil belongs to the Roi Et series, which is widely distributed and used for rice production in northeastern Thailand. This soil has properties of an infertile acid loam and sandy loam to a depth of 10 cm, which is the active rhizosphere for rice plants and good microbiological activity. The experimental design consisted of four plots, each with an area of 0.16 ha. All four plots included (1) CF, using a chemical fertilizer (16-16-8, N-P_2O_5-K_2O), (2, 3) two organic treatments; one received Sesbania rostrata as GM, and the other treatment/plot with RS, and (4) control plot (CT), which has no external source of plant nutrients. Rice (Oriza sativa, KDML 105 variety) was grown on all plots under rainfed lowland conditions. Representative top soil samples were taken from the plots which have been cultivated with rice for eleven years. After sampling, all samples were kept at 4^{o}C for total plate count and BIOLOG™ EcoPlate analysis, and -20^{o}C for PCR-DGGE analysis. Each individual treatment was sampled three times in 2007; before growing rice in May, when maximum tillering occurred, and immediately after rice harvest. A topsoil layer (0-10 cm) was collected using a soil core device with diameter of 2.5 cm. Based on random sampling, six points were chosen from each plot and

when the sampling was completed, topsoil from the six points was thoroughly mixed into one sample.

Total plate count analysis

The effect of agricultural management practice on the total number of culturable bacteria was determined by the plate count technique (pour plate) of colony-forming units (CFU). Five grams of fresh soil were prepared in 45 ml sterile ¼ Ringer solution (Merk, Germany), and homogenized in a centrifuge tube (50 ml) using vortex for 3 min. at maximum speed. Then the samples were centrifuged for 5 min at 129 x g (or 1,074 rpm) (Calbrix et al., 2007) and the supernatant liquids were serially diluted (10-fold steps) with sterile ¼ Ringer solution and 0.1 ml aliquots of each dilution were pipetted into a sterile Petri plate in triplicate, then melted tryptic soy agar (TSA) was poured in and mixed with the sample. All plates were incubated at 37^{o}C for 48 hr.

Community levels physiological profile (CLPP) analysis

CLPP was undertaken using the Biolog^{TM} Ecoplate and each plate containing 96 microtiter wells was divided into three replicated sets. Thirty-two wells in each of the sets contained different substrates and a blank control well contained water. Soil extracts were prepared with 5 g of fresh soil in 45 ml sterile ¼ Ringer solution (Merk, Germany), and homogenized in a centrifuge tube (50 ml) using vortex for 3 min. at maximum speed. Then the samples were centrifuged for 5 min. at 129 x g (or 1,074 rpm) (Calbrix et al., 2007) and the supernatant liquid was diluted (10^{-3} dilution) with sterile ¼ Ringer solution and inoculated onto BIOLOG^{TM} EcoPlate system (Biolog Inc., CA, USA). The aliquots of 130 µl from a 10^{-3} dilution were inoculated into the microplates. The plates were incubated at 37^{o}C in dark. Color development in each well was examined optical density (OD) at 405 nm every 12 h for 7 d (Zak et al., 1994) using a microplate reader (DNM-9602G, Beijing Prolong New Technology Co., Ltd).

Polymerase chain reaction-denature gradient gel electrophoresis (PCR-DGGE) analysis

For the analysis, variations of diversity of microbial genes in paddy soil were studied using directly extracting total DNA, and amplifying 16S rDNA by PCR-DGGE. Community DNA was directly isolated from the paddy soil samples without duplicate using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument as described by the manufactures (MP Biomedicals, Solon, Ohio, USA). Extracted DNA was then purified by Sephadex G-50 Mini Column® according to the manufacture (Geneaid, Taiwan) to remove humic acid and fulvic acid contamination. Total bacterial communities were analysed with specific primer sets; 357f-GC and 518r (Kurisu et al., 2002; Muzer et al., 1993). Extracted DNA was amplified by PCR amplification using these specific primer sets (357f-GC and 518r) for 35 cycles in a 24 µl reaction volume. PCRs were performed using a thermal cycler (BioRad Laboratories, lnc). PCR mixtures were amplified as follow: preincubated at 94°C for 9 min, 35 cycles of denaturing (30 sec at 94°C), annealing (30 sec at 53°C), and extension (30 sec at 72°C) and a final elongation was at 72°C for 10 min (Kurisu et al., 2002). Analysis of PCR products by DGGE was performed according to Muzer et al. (1993) with modifications using a DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, USA). A 20 µl volume of amplified products was loaded onto 8% (wt/vol) polyacrylamide gel in 1x TAE buffer reservoir (7 L). The 8% (wt/vol) polyacrylamide gel (bisacrylaminde gel stock solution, 37.55:1 (Bio-Rad Laboratories, lnc) were prepare with denaturing gradients of urea and formamide ranging from 20 to 70%. The electrophoresis was run at 60°C, first for 300 min at a constant voltage of 130 V. After electrophoresis, the gel was incubated for 20 min in 15 mL of Milli-Q water containing two drops of ethidium bromide and photographed with a Polaroid camera. The gels were analyed using Quantity One software (Bio-Rad Laboratories lnc, USA).

Data analysis and statistical analysis

Plate count data were normalized by log transformation and significant differences between group means were determined by analysis of variance (ANOVA) and calculation of the minimum significant difference by the Least-Significant Different (LSD) test at P = 0.05. The net optical diversity (OD) at 405 nm for each substrate well for BIOLOG^{TM} EcoPlate was calculated by subtracting the control well OD from the substrate well OD. If this subtraction gave a negative number, 0 was used in the subsequent analyses. Microbial activity in each microplate was expressed as average well-color development (AWCD). The AWCD was determined by the following equation; AWCD = $\sum OD_i/31$, where OD_i is the optical density value from each of the 31 wells by water blank substraction (Garland and Mills, 1991). The absorbance values at 72 h were used to calculate diversity indices. Shannon-Weaver index (H_f') was calculated as follows; $H_f' = -\sum p_i (\ln p_i)$, where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates ($\sum OD_i$). Substrate richness (S_f) is the number of different substrates that were used by the bacterial community, i.e. equivalent to species richness in the soil, and was calculated by counting all positive OD reading (Zak et al., 1994). The AWCD, S_f, and H_f' were analyzed by ANOVA and comparisons of means by the LSD test at P = 0.05. The genetic diversity indices were calculated from DGGE profiles. Scanned gels analyzed the intensity of individual bands (peak height of bands) in all lanes with Quantity One software package. Genetic diversity was evaluated by calculating Shannon-Weaver index (H_g') as follows; $H_g' = -\sum (n_i/N)\ln(n_i/N)$, where n_i is the intensity of band i as judged by its peak height, i is the number of bands in DGGE gel profile and N is the sum of all peak heights in a given DGGE profile.

RESULTS AND DISCUSSION

Total plate count

Lower numbers of culturable heterotrophic bacteria (CFU) were isolated from the CT because this plot had low SOM (5.85±3.32 g kg^{-1}) and also low in biological activity such as basal respiration (3.80±1.95 mg CO_2-C kg^{-1} d^{-1}), potentially mineralizable nitrogen (8.71±3.55 mg NH_4-N kg^{-1}, microbial biomass carbon and nitrogen (0.331±0.355 g kg^{-1} and 0.186±0.158 g kg^{-1}) (Thuithaisong et al., 2010). Two-way ANOVA of the results revealed that total plate counts were significantly ($P<0.05$) affected by the four different management practices. However, a GM plot ($2.29x10^6$ CFU g^{-1} of dry soil) and CF plot ($1.80x10^6$ CFU g^{-1} of dry soil) were not significantly different ($P>0.05$) (Table 1). This implied that without plant nutrients provide to the soil, growth of culturable bacteria was restricted. Both GM and CF at the rate applied are equally effective in supporting a better growth and perhaps survival of the cultivable bacteria, because they both contain an array of organic compounds such as carbohydrates, fatty acids and peptide that are substrates for growth of culturable bacteria.

Community level physiological profiles

The overall color development in the BIOLOG^{TM} Ecoplates was calculated as the mean of all 31 wells. The temporal changes obtained from AWCD were different between soil samples, with the CT plot being the lowest, suggesting a lower level of microbial community in this plot. The number of carbon sources that gave positive reactions for each soil treatments again highlighted the lower values for CT compared to the other plots (Table 1). The GM plot had a high AWCD value and a high number of carbon sources. All indices of bacterial diversity (functional diversity) in GM plot increased the microbial diversity, especially the Shannon-Weaver index (H_f') and substrate richness (S_f) as indicated by their highest values at 3.01 and 14.78 respectively.

Polymerase chain reaction-denaturing gradient gel electrophoresis

Bacterial community structure determined by DGGE banding patterns are showed in Fig.1. Numerous distinct DGGE bands, resulting from differences between the 16S rDNA gene sequences of different bacterial species, were obvious. Each band represents at least one unique ribotype, as reflected by the relative band intensity on a DGGE gel. The higher the intensity, the more dominant is the bacterial ribotype corresponding to that band. The DGGE patterns from the paddy soils of different agricultural management practices (CT, RS, GM and CF) had distinctly different features of bacterial community, suggesting that different soil environmental conditions influence bacterial community structure. Based on the DGGE image, the expression level of 16S rDNA gene sequences was strongly expressed in samples collected in May but they were weak in October and December. SOM, which is beneficial for soil microbial growth, was highest in the samples collected in May (those soils were under aerobic condition). Therefore the intensity of DGGE bands in May was expressed strongly due to the aerobic bacteria. In contrast bacterial populations from rice soils of the four plots decreased in all the samples collected in October. Because such samples were obtained under anaerobic condition the intensity of DGGE bands has lowest. The degradation of SOM by aerobic bacteria was strongly inhibited because oxygen levels were reduced by flooding. However, the intensity of DGGE bands in soil samples collected in December was higher than in October because the soils were again under aerobic conditions. The Shannon-Weaver Index (H_g') was quite similar between RS and GM plots and their H_g' was higher than from CF and CT soil treatments.

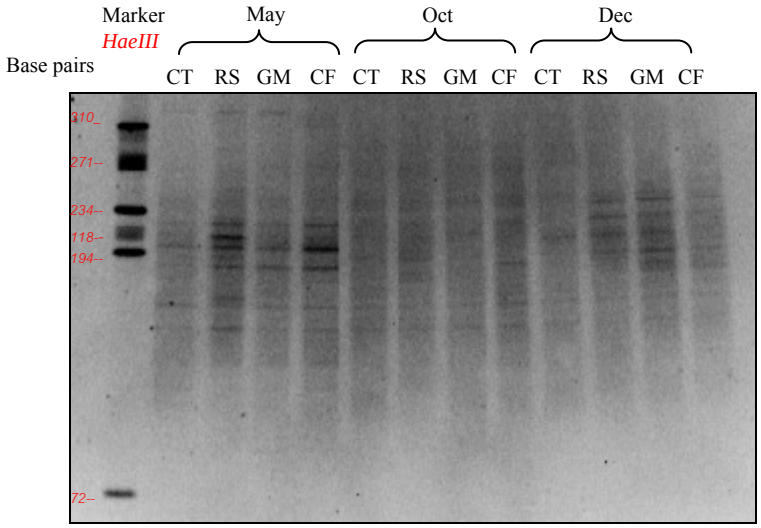

Fig. 1 DGGE images of PCR-amplified products from different agricultural practices (CT, RS, GM and CF) during different sampling time (May, October, and December)

Table 1 Effect of different farming management practices on soil microbial communities

Plot	Total plate count (CFU g^{-1} of dry soil)	AWCD	No. of C sources utilized	Functional diversity		Genetically diversity	
				(H_f')	(S_f)	(H_g')	(S_g)
CT	$8.42\pm5.49\times10^{4\,a*}$	$0.19\pm0.10^{\,a}$	$6.0\pm3.87^{\,a}$	$2.77\pm0.15^{\,a}$	$5.3\pm2.96^{\,a}$	$1.62\pm0.32^{\,a}$	$6.0\pm2.00^{\,a}$
RS	$3.23\pm4.04\times10^{5\,b}$	$0.28\pm0.16^{\,b}$	$9.7\pm5.95^{\,b}$	$2.83\pm0.15^{\,a}$	$9.0\pm1.73^{\,b}$	$2.08\pm0.23^{\,a}$	$9.0\pm3.00^{\,a}$
GM	$2.29\pm1.61\times10^{6\,c}$	$0.46\pm0.30^{\,c}$	$13.9\pm7.93^{\,c}$	$3.01\pm0.82^{\,b}$	$14.8\pm4.12^{\,c}$	$1.96\pm0.43^{\,a}$	$8.3\pm3.05^{\,a}$
CF	$1.80\pm1.33\times10^{6\,c}$	$0.30\pm0.17^{\,d}$	$8.8\pm4.57^{\,b}$	$2.86\pm0.12^{\,a}$	$10.0\pm3.04^{\,b}$	$1.89\pm0.38^{\,a}$	$8.3\pm2.52^{\,a}$

** Values are expressed as means and standard deviations followed the different regular letters (a, b, c) in row are significantly different (P<0.05) by Least Significant Different (LSD). AWCD=average well color development, H_f' =Shannon-Weaver diversity index of functional diversity, H_g' =Shannon-Weaver diversity index of genetic diversity S_f =substrate richness, and S_g=species richness.*

This indicates that the influence of management practice on soil microbial communities did occur. Based on the finding organic farming (RS, GM) seemed to have an edge over the conventional management (CF). However, the statistical analysis (ANOVA) performed for the Shannon-Weaver index (H_g') and species richness (S_g) from DGGE profiles data showed no significant differences between the four plots (CT, RS, GM and CF) (Table 1).

CONCLUSION

Based upon the results obtained from this study soil management practices have influenced microbial diversity, both in functional and genetic diversity, in the rice plots studied. Further a narrow range of Shannon-Weaver diversity index (H_f') was obtained with the values between 2.77 for CT to 3.01 for GM. In the statistical analysis performed using substrate richness (S_f) from potential substrate utilization patterns data, the GM treatment increased microbial diversity. Even within the constraints of organic farming practices, it is possible for farmers to adopt and continue this application to improve soil bio-physiochemical properties, provided that the rate and source of organic materials used is site specific.

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