J. Korean Soc. Appl. Biol. Chem. 54(6), 899-909 (2011)

Article

Influence of Chinese Milkvetch (*Astragalus sinicus* L.) with No-tillage on Soil Biotic Factors and Rice Yield

Min Keun Kim[1], Young Han Lee[1], Tae Ho Kang[2], and Han Dae Yun[2,3]*

[1]*Environment-friendly Research Division, Gyeongsangnam-do Agricultural Research and Extension Service, Chinju 660-360, Republic of Korea*

[2]*Division of Applied Life Science (BK21 Program), Gyeongsang National University, Chinju 660-701, Republic of Korea*

[3]*Research Institute of Agriculture and Life Science, Gyeongsang National University, Chinju 660-701, Republic of Korea*

Received July 19, 2011; Accepted October 26, 2011

The bacterial community, biotic factors, and rice yield of Chinese Milkvetch (*Astragalus sinicus* L.) with no-tillage and conventional chemical fertilizer treatment were evaluated. Analysis of bacterial isolates from each treatment plots with 16S rDNA sequences revealed 486 isolates from 18 different bacterial species. The frequency of spore-forming bacteria, including *Bacillus* sp. and some *Actinomycetes* appeared to be high under low temperature conditions, whereas *Proteobacteria* disappeared at low temperature. Most extracellular hydrolytic enzyme activity showing bacteria belong to low G + C Gram(+) bacteria (LGCGPB) compared to high G + C Gram(-) bacteria (HGCGPB) and *Proteobacteria*. In addition, the total soil carbon content in Chinese Milkvetch treated soil was higher than that of conventional tilled soil samples. Rice yield was slightly higher in the conventionaltilled field (4.13±0.08 Mg/ha) compared to the Chinese Milkvetch-treated field (3.94±0.15 Mg/ha). However, the field treated with Chinese Milkvetch followed by no-tillage was effective in sustaining LGCGP bacterial species that promote biogeochemical cycling and enrich microbial biomass on paddy soil (3.94±0.15 Mg/ha). These results showed that the Chinese Milkvetch treatment with no-tillage is effective in conserving the soil environment without significant yield loss.

Key words: biotic factors, chinese milkvetch, green manure, no-tillage, rice yield

Rice is a primary food source for nearly half of the world's population, and about 90% of the total area of rice fields is found in Asia [Lim *et al*., 2007]. Researchers have found that intensive agriculture with chemical fertilizer depresses soil enzyme activities. Soil microbial biomass, a living part of soil organic matter is an agent of transformation for added and native organic matter, and acts as a labile reservoir for plant-available nitrogen (N), phosphorus (P), and sulfur (S) [Jenkinson and Ladd, 1981]. The activity of microbial biomass is commonly used as a measure of the microbiological status of a soil [Nannipieri *et al.*, 1990], and to determine the effects of cultivation [Anderson and Domsch, 1978; Beyer *et al.*, 1991], field management [Perrott *et al.*, 1992] or contamination [Chander and Brookers, 1993] on soil microorganisms. Microbial activity-based indicators of soil quality may respond to disturbances over a shorter period of time than physical or chemical-based properties. As a consequence, some microbiological properties, such as bacterial extracellular enzyme activities, have been hypothesized to be potential indicators of soil quality due to their essential role in soil biology, easy of measurement, and rapid response to changes in soil management [Kandeler *et al.*, 1999]. The degradation of macromolecular carbon compounds is related to bacterial community composition, because the enzymatic capacity for the initial steps of degradation occurs in a comparatively limited number of microbial populations [Alvarez and Guerrero, 2000; Waldrop *et al.*, 2000].

Presently, one of the major environmental concerns is soil degradation and the release of CO_2 into the atmosphere.

*Corresponding author
Phone: +82-55-772-1962; Fax: +82-55-772-1969
E-mail: hdyun@nongae.gsnu.ac.kr

http://dx.doi.org/10.3839/jksabc.2011.136

Inefficient technologies have resulted in soil quality deterioration, organic matter losses and effects on air flow, and consequently on poor plant growth [Golchin *et al*., 1995]. However, the application of green manures to soil is considered a good management practice in most crop production systems in order to stimulate soil microbial growth and activity, with subsequent mineralization of plant nutrients, which should increase soil fertility and quality. Leguminous and non-leguminous plants are used as green manures. Leguminous green manures can fix large quantities of atmospheric nitrogen gas (N_2) and can provide useful amounts of organic matter in soil. Chinese Milkvetch (*Astragalus sinicus* L.), one of leguminous green manures, is mostly used in southern Korea due to overwintering. Chinese Milkvetch contains 2.25% nitrogen, which is available for rice plant assimilation immediately after the application of Chinese Milkvetch to the rice field. An agriculturally effective application rate of Chinese Milkvetch was observed to be 1.5 t/ha, which was equivalent to 110 kg/ha of N application.

On the other hand, no-tillage, conservation tillage, and/or proper field management prevent or minimize problems with soil erosion, chemical fertilizers, weed growth, degradation of soil fertility [Preston *et al.*, 2005]. Soils under no-tillage typically have greater mineralization potential due to more carbon or organic matter that accumulates with less decomposition in the field [Alvarez *et al.*, 1995]. In temperate coniferous regions, landscape organic matter availability is compromised by the accumulation of large amounts of litter in soil, so it is important that soil carbon is sequestered through productive tillage practices. Differences in carbon and N availability are likely to mediate microbial demand and activity, and to ultimately determine microbial processes, such as C and N cycling [Booth *et al.*, 2005]. Dissolved organic matter is mobile within the soil solution and has been proposed as important in the transport and supply of C and N to microbial populations. No-tillage has been proposed as an alternative to conventional seedbed preparations, because it leads to carbon increase in the topsoil [Kern and Johnson, 1993].

Bacterial communities are central to the functioning of terrestrial ecosystems, where they consist of a large number of different bacterial types [Keenedy and Gewin, 1997]. Paddy fields under rice cultivation, treated with chemical fertilizer and rice straw, showed a predominance of *Bacillus* sp., which included *Bacillus cereus* and *Bacillus licheniformis* across three stages of cultivation [Watanabe and Hayano, 1993].

We hypothesize that Chinese Milkvetch as an alternative fertilizer (organic farming), followed by no-tillage, will sustain beneficial bacteria for nutrient flow and enrich the soil with organic carbon by sequestering carbon. The objectives of the present study were to observe the effect of organic matter supplement with no-tillage to the paddy fields in a combined approach, and analyze the consequences of treatments on biotic factors such as soil C, microbial biomass, and rice yield. Furthermore, we strove to enumerate the numerically dominant species among the bacterial community in rice paddy soil as a function of seasonal changes.

Materials and Methods

Soil sample and rice cultivation. Soil samples were obtained from the experimental field (long. 35°12'17"N and lat. 128°07'13"E) at Gyeongnam Agriculture Research and Extension Services, Jinju, Gyeongnam, Korea. The soil was Ihyeon series, which is a member of the fine silty over coarse silty, mixed, mesic family of Dystric Fluventic Eutrudepts (Alluvial soils). These soils have moderately thick brown to dark brown silt loam A horizons and deep dark yellowish brown to brown silt loam cambic B horizons. C horizons are very deep brown weakly stratified silt loam. Chinese Milkvetch seed was sown at the rate of 66 kg ha^{-1} in 4 October, 2009 (early winter, previous year of rice cultivation). Two treatments were laid out in an randomized complete block design with three replicates, one sample treated with Chinese Milkvetch (CMV) 4.4 Mg/ha under no-tillage, no chemical fertilizer, and no pesticide, in conventional tillage field (T) with applied chemical fertilizer (N-K_2O=85.5−37.7 kg/ha) and herbicide such as benfuresate and enoxsulam. Soil samples were collected from each treated fields at different times (March, June, September, and December) were sieved through a 2-mm sieve and kept at 4°C prior to use. The climate is temperate coniferous with temperature range of 1 to 32°C.

Soil general properties and biotic factors analysis. Soil samples were collected from the field and were mixed, dried, and passed through a 2-mm diameter sieve before analysis. The chemical properties of the soil and organic matter were determined by soil analysis [Nelson and Sommers, 1982]. The total N was determined by micro-Kjeldahl digestion. Soil temperature measurements for two treatments were provided by the institute as mentioned above and were analyzed as the methods described by Moore-Kucera and Dick [2007]. Soil microbial biomass was estimated by using fumigation-extraction protocol [Bossio and Scow, 1998]. Upon maturation of the crop (usually in the early October), three 2-m long center rows were sampled from each plot for dry matter, grain yield, and the numbers were recorded at harvest.

Isolation of culture soil bacteria. One gram of each soil sample was added to 9 mL of sterile distilled water, mixed thoroughly to disperse the soil particles. Finally 1 mL of 10^{-4} dilution was plated on to Tryptic soy agar (TSA) medium and incubated at 30°C for 24 h. Pure isolates were obtained by four-way streaking on TSA plate. Total numbers of bacteria isolated were counted at each sample analysis. The bacterial colonies were initially screened and grouped by colony color and morphological characteristics.

Extracellular hydrolytic enzyme activity assay. Agar diffusion method was used for the detection of extracellular hydrolytic enzyme activity of the isolated soil bacteria. The isolates were grown on different enzyme activity indicator media such as cellulase activity indicator medium [Luria-Bertani (LB) medium containing, 0.5% (w/v) carboxymethylcellulose and 1.5% agar (w/v)], xylanase activity indicator medium [LB medium containing, 0.5% (w/v) oat spelt xylan and 1.5% agar (w/v)], mannanase activity indicator medium [LB medium containing, 0.5% (w/v) locust bean gum and 1.5% agar (w/v)], and lichenase activity indicator medium [LB medium containing, 0.5% (w/v) lichenan and 1.5% agar (w/v)] for the detection of cellulase, xylanase, mannose, and lichinase activity. To visualize the yellow halo zone surrounded by red background due to cellulase, xylanase, mannanase, and xylanase activities, the plates were flooded with 0.5% Congo Red solution for 30 min, rinsed with water, and then washed twice with 1 M NaCl [Cho *et al.*, 2007]. Moreover, the isolates were inoculated on pectinase (PGAase) activity indicator medium [LB medium containing, 1.0% (w/v) polygalacturonic acid and 1.5% agar (w/v)] and amylase activity indicator medium [LB medium containing, 1.0% (v/v) starch and 1.5% agar (w/v)]. To visualize the halo zone formations due to PGAase activity, the plates were flooded with 0.1% toluidine blue and potassium iodide and washed with distilled water [Cho *et al.*, 2007]. For determination of protease, lipase, esterase, and DNase activities, the isolates were also inoculated on protease activity indicator medium [LB medium containing, 1.0% (v/v) skim milk and 1.5% agar (w/v)], lipase activity indicator medium [LB medium containing 1.0% tricaprylin (v/v) and 1.5% agar (w/v)], esterase activity indicator medium [LB medium containing, 1.0% tributyrin (v/v) and 1.5% agar (w/v)], DNase activity indicator medium (Difco formular medium), and chitin activity indicator medium [LB medium containing, chitin 1 and 1.5% agar (w/v)].

Cloning and sequencing. Standard procedure for agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning related techniques were followed [Sambrook and Russell, 2001]. The 16S rDNA PCR products obtained from soil bacterial DNA were cloned into the pGEM-T Easy Vector as recommended by the manufacturer (Promega, Madison, WI). The preparation of obtained clones followed purification of recombinant DNA was carried out as described previously [Lin *et al.*, 2009; Ohshiro *et al.*, 2010; Rattanachomsri *et al.*, 2011]. *Escherichia* coli DH5á and recombinant *E. coli* were cultured in LB containing 50 μg/mL ampicillin at 37°C. Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Analysis of 16S rDNA Sequences. All reference sequences were obtained from the GeneBank and Ribosomal Database Project (RDP) databases. Our sequences were analyzed by the CHECK_CHIMERA program to exclude sequences from chimeric rDNA clones [Maidak *et al.*, 2000]. Similarity search against database entries was done using online BLAST search. Sequences were aligned using the multiple sequence alignment program CLUSTAL W version 1.6 [Tompson *et al.*, 1994]. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using the neighbour-joining methods [Saito and Nei, 1987]. Bootstrap analysis was performed with data resampled 1000 times using DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada).

Nucleotide sequence accession numbers. Nucleotide sequences are deposited in the GenBank database under the accession numbers FJ189757-FJ189798. For conventional tillage sample starts with FJ 189767-FJ189777 and FJ189795-FJ189796, and for Chinese Milkvetch treated soil sample starts with FJ189757-FJ189766 and FJ189792-FJ189794.

Statistical analysis. All data were statistically analyzed using the SAS software version 9.3 for Windows (SAS Institute, Cary, NC). Comparisons of grain yield and yielded components were performed using one-way analysis of variance (ANOVA). In addition, the Bonferroni t-test (Minimum Significant Difference) was used to detect and separate the mean treatment differences at 5.0% levels of significance (p <0.05).

Results

Analysis of culturable bacteria. The diversity of paddy field culturable soil bacterial species in two treatments was assessed at different intervals along with different stages of rice cultivation. A total of 18 bacterial species were observed among the 486 isolates obtained from two different soils samples. The sample from the

conventional tilled soil site revealed a total of 258 bacteria, among which 14 predominant bacteria represented 14 different species (Table 1). Bacterial community of the conventional tilled soil samples included Low G + C Gram-positive bacteria (LGCGPB), *B. licheniformis* (NSB09) predominant among *Bacillus* species, followed by *B. sporothermodurans* (NSB02), other *B.* species such as *B. subtilis* (NSB01), *B. licheniformis* (NSB13), *Bacillacea bacterium* (NSB04), *B. cereus* (NSB05), *Paenibacillus illinoisensis* (NSB08), *B. clausii* (NSB10), and *B. pumilus* (NSB11). Other predominant bacteria among high G + C Gram-positive bacteria (HGCGP) included *Staphylococcus pasteuri* (NSB07), *Microbacterium* sp. SSL14 (NSB03), *Micrococcus leutes* (NSB 12), *Streptomyces* sp. (NSB06), and *Proteobacteria*, *Rhizobium tropici* (NSB14). These sequences exhibited 97 to 100% identity to those found in databases. The sample from the Chinese Milkvetch-treated soil consisted of 228 bacteria, including 13 predominant bacteria, which represented 13 different species (Table 1). The Chinese Milkvetch-treated soil bacterial community mainly included LGCGPB such as *Bacillus* species along with few *Actinobacteria* and *Proteobacteria*; among the *Bacillus* species, *B. licheniformis* (MSB03) was significantly (n =47) dominant than in the conventional tilled soil samples followed by *B. sporothermodurans* (MSB12) and *B. subtilis* (MSB02). Among other species of *B. paenibacillus illinoisensis* (MSB08), *Paenibacillus pabuli* (MSB09), *B. clausii* (MSB10) *B. pumilus* (MSB11), *Bacillacea bacterium* (MSB05), *B. cereus* (MSB06) were observed. Other bacteria belong to HGCGP such as *Streptomyces colombiensis* (MSB05), *Lefisonia xyli* (MSB04), and *Proteobacteria R. tropici* (MSB13) were found. These sequences exhibited 97 to 100% identity to those found in NCBI databases. A search for the predominance of specific bacteria from each sample and 16S rDNA sequences revealed that one strain (MSB03 and NSB09) belonging to *B. licheniformis* was dominant in both samples, but numerically was higher in Chinese Milkvetch-treated soil sample (Table 1). The bacterial population at each stage of sampling varied significantly in both samples; the total number of bacteria (486 isolates) changed with seasonal variation along with growth stages of rice (Fig. 1). Among the two types of soil analyzed four times in a year, the samples cultured in the month of March showed 74 isolates from the tilled soil samples and 65 isolates from the Chinese Milkvetch-treated soil sample, and the highest bacterial population numbers were found in June, with 83 in conventional tilled soil samples and 79 in Chinese Milkvetch-treated soil samples. The gradual decrease in bacterial population was observed by September, with 56 observed in conventional tilled soil samples and 43 observed in Chinese Milkvetch-treated soil samples. Furthermore, populations were the lowest in December, 45 in conventional tilled soil samples and 41 in Chinese Milkvetch-treated soil samples. In December, spore-forming bacteria such as *Bacillus* sp. and some Actinomycetes, appeared high in number mainly due to extremely low temperatures (Fig. 2), whereas *Proteobacteria* disappeared at low temperature. Total population in field tillage soil samples was relatively higher than that in the Chinese Milkvetch-treated soil sample, which may be due to lower presence of *Actinobacteria* and *Proteobacteria*. The predominance of *Firmicutes* bacteria could be observed more clearly in both treated soil samples.

Analysis of 16S rDNA Sequences. Isolated 16S rDNA sequences of the bacterial strains from two treated soils (Tillage and Chinese Milkvetch) were analyzed with NCBI database entries; results of BLAST revealed that the sequences exhibited 97 to 100% identity to those found in databases. The nearest relatives of these strains are listed in Table 1. Our phylogenetic analyses placed 14 clones from conventional tilled soil sample sequences in the following three major groups of the bacterial phylum: (i) the phylum *Firmicutes*, which included mainly *Bacillus* sp., and *Staphylococcus* sp.; (ii) *Actinobacteria*, which included *Streptomyces* sp., *Microbacterium* sp., and *Micrococcus luteus*, and (iii) *Proteobacteria*, which included only *R. tropici* sp. In Chinese Milkvetch-treated soil sequences 13 clones were identified from three major bacterial groups (Fig. 3B).

Extracellular hydrolytic enzyme activity assay. Isolated soil bacterial strains were used for the extracellular hydrolytic enzyme assay (Table 2). Bacteria belonging to phylum *Firmicutes* exhibited greater degradation activity on many substrates compared to bacteria belong to phyla *Proteobacteria* and *Actinobacteria*. The mineralization ability of some soil bacteria was investigated, in addition to other specific enzyme activities, including: cellulase, xylanase, pectinase, and protease activities. Cellulase, xylanase, mannanase, amylase, and pectinase-producing isolates belonged to the *Bacillus* and *Paenibacillus* genera. All groups examined exhibited proteolytic activity, except *S. colombiensis* isolates. All isolates from the Chinese Milkvetch treated soil sampling site had possessed cellulase, amylase, xylanase, pectinase, mannanase, DNase, and esterase activities (Table 2). *B. licheniformis* (MSB03), *B. pumilus* (MSB09), and *B. sporothermodurans* (MSB12) exhibited all enzyme activities except for lipase and chitinase. All *Proteobacteria Leifsonia xyli* and *R.tropici* exhibited protease activity. Isolates from the conventional tilled soil sample displayed amylase, xylanase, pectinase, mannanase, DNase, esterase, and

Table 1. Bacterial isolates obtained from two different soil treatments samples

Bacterial isolates	Clone (Accession no.)	No. of isolates[a]				Phylum	Nearest relatives[b] (Accession no.[c])	Similarity (%)
		March	June	September	December			
Conventional tillage soil								
NSB01	FJ189767	4	5	4	5	LGCGPB[d]	*Bacillus subtilis* HDYM-23 (EF428247)	99
NSB02	FJ189768	6	7	3	4	LGCGPB	*Bacillus sporothermodurans* (AF329476)	99
NSB03	FJ189769	8	10	4	2	HGCGPB[e]	*Microbacterium* sp. SSL14 (EU373326)	99
NSB04	FJ189770	2	3	2	0	HGCGPB	*Bacillacea bacterium* KVD (DQ490420)	99
NSB05	FJ189771	6	4	2	2	LGCGPB	*Bacillus cereus* HNR10 (EU373359)	99
NSB06	FJ189772	7	6	4	5	HGCGPB	*Streptomyces colombiensis* (EF371424)	97
NSB07	FJ189773	6	9	7	4	LGCGPB	*Staphylococcus pasteuri* (EU373323)	97
NSB08	FJ189795	2	3	1	2	LGCGPB	*Paenibacillus illinoisensis* (AB073192)	99
NSB09	FJ189796	9	11	7	6	LGCGPB	*Bacillus licheniformis* ATCC 14580 (CP000002)	99
NSB10	FJ189774	2	1	1	2	LGCGPB	*Bacillus clausii* (AP006627)	100
NSB11	FJ189775	4	4	3	3	LGCGPB	*Bacillus pumilus* BPT (EU373327)	99
NSB12	FJ189776	9	8	7	4	HGCGPB	*Micrococcus luteus* CV39 (AJ717368)	100
NSB13	FJ189777	5	6	5	4	LGCGPB	*Bacillus licheniformis* BCRC 15413 (DQ993676)	99
NSB14	FJ189778	5	7	6	2	Proteobacteria	*Rhizobium tropici* (EF054889)	99
Chinese Milkvetch soil								
MSB01	FJ189757	5	4	2	3	LGCGPB	*Bacillus cereus* HNR10 (EU373359)	99
MSB02	FJ189758	6	5	2	3	LGCGPB	*Bacillus subtilis* HDYM-23 (EF428247)	99
MSB03	FJ189792	11	18	10	8	LGCGPB	*Bacillus licheniformis* ATCC 14580 (CP000002)	99
MSB04	FJ189759	5	6	4	1	HGCGPB	*Leifsonia xyli* (AJ717351)	98
MSB05	FJ189760	7	8	3	3	HGCGPB	*Streptomyces colombiensis* (EF371424)	97
MSB06	FJ189761	3	4	1	3	HGCGPB	*Bacillacea bacterium* KVD (DQ490420)	99
MSB07	FJ189793	3	2	3	2	LGCGPB	*Paenibacillus illinoisensis* (AB073192)	99
MSB08	FJ189762	4	5	3	2	LGCGPB	*Bacillus clausii* (AP006627)	100
MSB09	FJ189763	3	5	2	4	LGCGPB	*Bacillus pumilus* BPT (EU373327)	99
MSB10	FJ189764	4	4	2	3	LGCGPB	*Bacillus subtilis* 168 (Z99104)	99
MSB11	FJ189765	4	6	4	1	Proteobacteria	*Rhizobium tropici* (EF054889)	99
MSB12	FJ189766	8	10	6	6	LGCGPB	*Bacillus sporothermodurans* (AF329476)	99
MSB13	FJ189794	2	2	1	1	LGCGPB	*Paenibacillus pabuli* (AB073191)	99

Number of isolates was counted by the periodic sample analysis based on 16S rDNA sequence data.

[b]Closest relative species in the 16S rDNA sequence database. When more than one sequence had the same similarity, only the accession number of the first sequence is given.

[c]n parentheses inducate accession number of the nearest relatives.

[d]CGPB: low G + C Gram-positive bacteria.

[e]GCGPB: high G + C Gram-positive bacteria.

Table 2. Evaluation of extracellular hydrolytic enzyme activity isolated from the two treated soil samples

Isolates	Nearest relatives	Cellulase	Xylanase	Mannase	Pectinase	Amylase	Protease	Lipase	Esterase	DNase	Chitinase	Lichenase
MSB01[a]	*Bacillus cereus* HNR10 (EU373359)	-[c]	-	-	-	+++	+	-	-	-	-	-
MSB02	*Bacillus subtilis* HDYM-23 (EF428247)	-	+++	++	-	++	+++	++	++	+	+	+++
MSB03	*Bacillus licheniformis* ATCC 14580 (CP000002)	+++	++	+	+	++	+++	-	-	+	++	+++
MSB04	*Leifsonia xyli* (AJ717351)	-	-	-	-	-	+	-	-	-	-	-
MSB05	*Streptomyces colombiensis* (EF371424)	+	++	-	-	+	-	-	-	-	-	-
MSB06	*Bacillacea bacterium* KVD (DQ490420)	++	++	+	-	++	+++	-	++	+	-	++
MSB07	*Paenibacillus illinoisensis* (AB073192)	+	-	+	-	++	+	+		+	-	+
MSB08	*Bacillus clausii* (AP006627)	+	-	-	+	-	+	-	-	-	-	-
MSB09	*Bacillus pumilus* BPT (EU373327)	++	+	+++	-	+	++	W	++	++	-	-
MSB10	*Bacillus subtilis* 168 (Z99104)	++	-	-	+	++	+	-	-	++	-	-
MSB11	*Rhizobium tropici* (EF054889)	-	-	-	-	-	++	-	+	-	-	-
MSB12	*Bacillus sporothermodurans*(AF329476)	+++	+++	-	++	+++	+++	-	-	++	+	+++
MSB13	*Paenibacillus pabuli* (AB073191)	++	-	+++	-	+++	+	+	-	+	-	+
NSB01[b]	*Bacillus subtilis* HDYM-23 (EF428247)	-	+++	++	-	++	+++	++	++	+	+	+++
NSB02	*Bacillus sporothermodurans* (AF329476)	+++	+++	-	++	+++	+++	-	-	++	+	+++
NSB03	*Microbacterium* sp. SSL14 (EU373326)	-	-	-	W	-	+	+	-	W	-	-
NSB04	*Bacillacea bacterium* KVD (DQ490420)	++	++	+	-	++	+++	-	++	+	-	++
NSB05	*Bacillus cereus* HNR10 (EU373359)	-	-	-	-	+++	+	-	-	-	-	-
NSB06	*Streptomyces colombiensis* (EF371424)	+	++	-	-	+	-	-	-	-	-	-
NSB07	*Staphylococcus pasteuri* (EU373323)	-	-	-	-	-	++	-	+	-	-	-
NSB08	*Paenibacillus illinoisensis* (AB073192)	+	-	+	-	++	+	+	-	+	-	+
NSB09	*Bacillus licheniformis* ATCC 14580 (CP000002)	+++	++	+	+	++	+++	-	-	+	++	+++
NSB10	*Bacillus clausii* (AP006627)	-	-	-	-	-	+	-	-	-	-	-
NSB11	*Bacillus pumilus* BPT (EU373327)	++	+	+++	-	+	++	W	++	++	-	-
NSB12	*Micrococcus luteus* CV39 (AJ717368)	-	-	-	-	++	+	-	-	-	-	-
NSB13	*Bacillus licheniformis* BCRC 15413 (DQ993676)	++	++	-	-	+	+	-	-	+	+	+++
NSB14	*Rhizobium tropici* (EF054889)	-	-	-	-	-	++	-	+	-	-	+

[a,b]Total bacterial species isolates obtained from two treatments soil and sampled each soil at different intervals.

[c]Symbols indicate the degrees of enzyme activities formed around bacterial colonies: -, no enzyme activity; + (<2 mm), low enzyme activity; ++ (<4 mm), medium enzyme activity; +++ (<6 mm), high enzyme activity; W = Weak activity

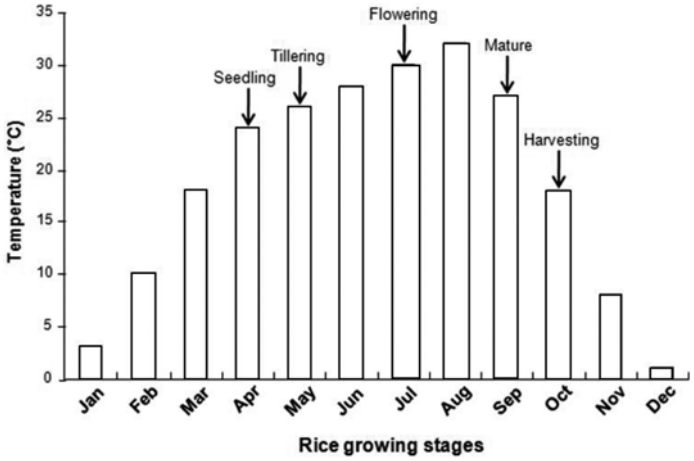

Fig. 1. The monthly mean temperature over one year including rice growing stages and soil sample collection periods.

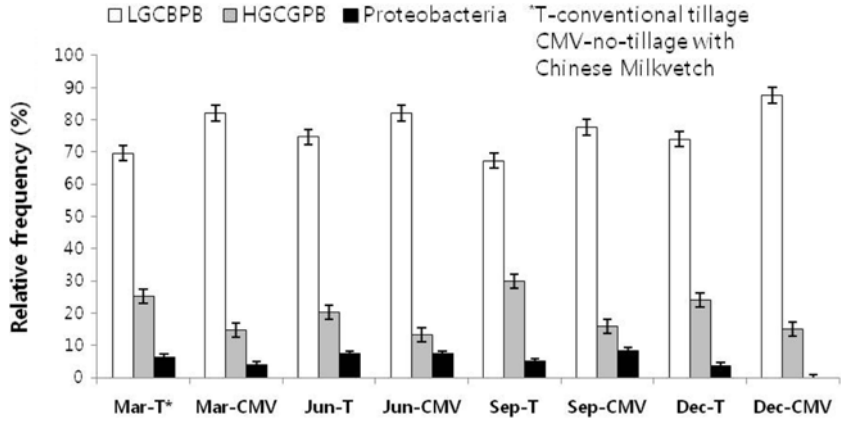

Fig. 2. Distribution of bacterial isolates on the analysis of 16s rDNA obtained from two treated soil samples at different time. The frequency of LGCGPB were high in both samples but was comparatively higher in Chinese Milkvetch. HGCGPB was higher in tillage soil sample. Distributions of clones were varied with seasonal and growth stages of rice.

protease activities. *B. licheniformis* (NSB09) and *B. sporothermodurans* (NSB02) possessed all enzyme activities tested. *S. colombiensis* (NSB06) exhibited cellulase, xylanase and amylase activities, whereas *Microbacterium* sp. (NSB03), *S. pasteuri* (NSB07), *M. luteus* (NSB12), and *R. tropici* (NSB14) exhibited only protease activity. Most of the isolates from both soil samples were belong to *Firmicutes*. *B. licheniformis* (MSB03) *B. sporothermodurans* (MSB12) and *B. subtilis* (MSB02) were involved in vigorous degradation of wide range of substrates. However, bacterial isolates from the Chinese Milkvetch-treated field displayed higher enzymatic activities than that of bacterial isolates collected from conventional tillage field soil sample.

Analysis of other biotic factors. Total organic carbon varied in the Chinese Milkvetch treated field from 37.6 to 31.8 g/kg, and from 33.8 to 28.7 g/kg in the conventional tilled soil samples (Fig. 4). In general, there was a statistically significant difference between Chinese Milkvetch treated and conventional tillage field. This could be attributed to the addition of Chinese Milkvetch as an alternative fertilizer and the NT practice, resulting in the accumulation of organic matter. However, there was an initial increase in organic carbon (March and June), which could be due to the supplementation of Chinese Milkvetch. Potentially, mineralizing carbon reflected microbial biomass pools in soil that accumulated in response to carbon inputs, and in response to a lack of decomposition in the field (Fig. 5). Our results showed similar patterns to those for total organic carbon and microbial biomass in Chinese Milkvetch treated soil samples compared to conventional tilled soil samples. Also we observed a decrease in total microbial biomass at the flowering stage of flooded rice.

Rice yield. Rice yield and yield contributing components were not significantly increased (Table 3). However, the total yield was higher in the conventional tilled plots (4.13±0.08 Mg/ha) than that of Chinese Milkvetch treated plots (3.94±0.15 Mg/ha), which was not significantly different.

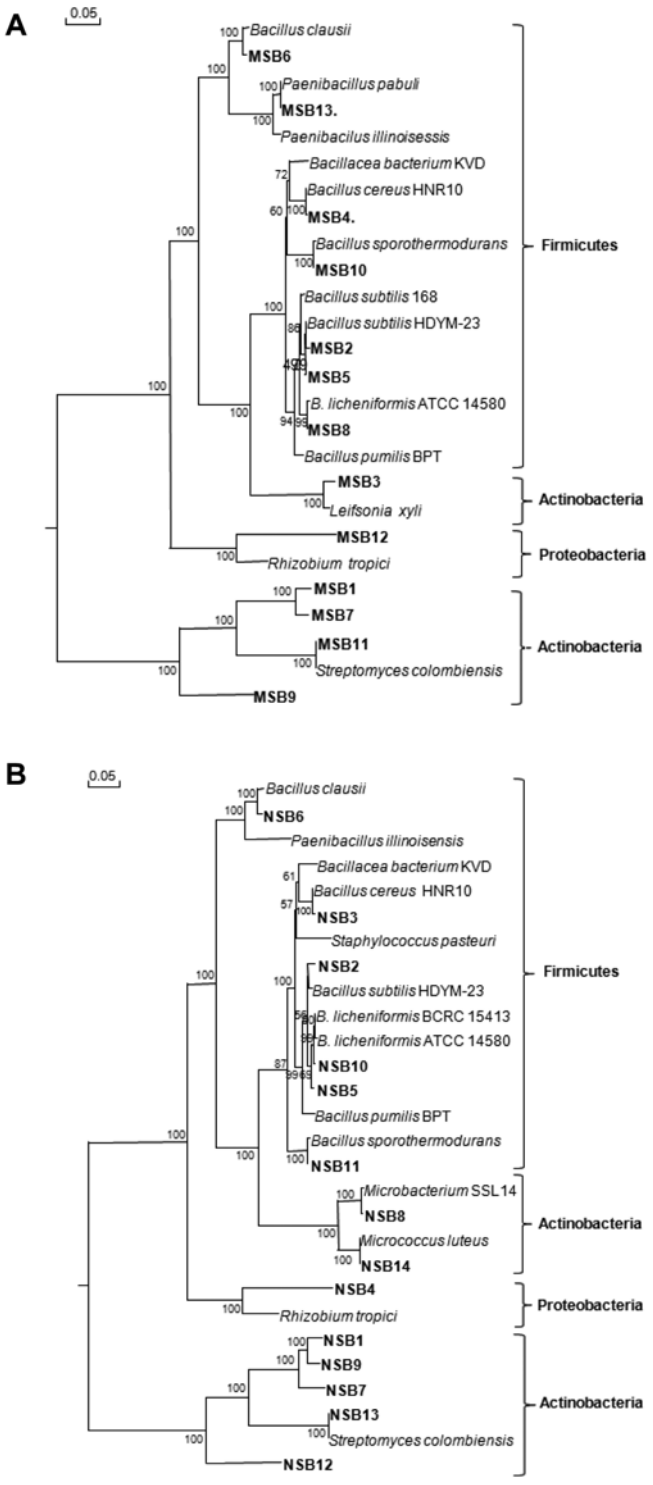

Fig. 3. Phylogenetic placement of bacterial isolates on the analysis 16S rDNA sequence from the treated soil of the paddy field. a, Chinese Milkvetch; b, conventional tillage. Numbers above each node are confidence levels (%) generated from 1000 boot strap trees. The scale bar is in fixed nucleotide substations per sequence position.

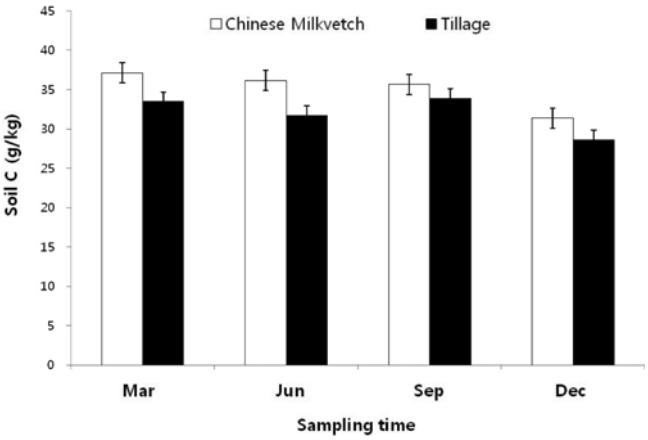

Fig. 4. Cumulative effect of Chinese Milkvetch on soil carbon content from samples collected at different time intervals. Soil carbon content altered with bacterial degradation and organic matter availability at different growth stages of rice. Error bar indicates the standard deviation on the three replication samples.

Discussion

Organic farming is responsible for material circulation in an agricultural ecosystem and enhanced crop production, with a minimal environmental load, thus promoting ecological balance. It is essential in an ecologically conscious production and management system. In this study, two experimental soil samples, a conventional tillage soil sample with applied chemical fertilizer and Chinese Milkvetch treated samples, were evaluated. The 16S rDNA sequence analysis of isolated bacteria showed a species distribution under three major phyla: *Firmicutes*, *Actinobacteria* and *Proteobacteria*. The *B. licheniformis* strain MSB03 was found dominant in Chinese Milkvetch treated samples, but no specific dominant bacterial species was observed in the conventional tilled soil. Bacterial frequency within phylum *Firmicutes* consisted mainly of *Bacillus* sp. was higher than that of strains belongs to *Actinobacteria* and *Proteobacteria*. Enzyme activity ~~as~~ evaluated by plate assay showed that most of *Bacillus* sp. degraded a wide range of synthetic substrates. There was no significant difference in bacterial species across treated soil samples, but specific population abundance varied across different treatments and with seasonal variation. Organic carbon and microbial biomass were both affected by treatments, resulting in high rice production yields.

The proportion of LGCGPB bacteria was greater in

Table 3. Comparison of grain yield and yield components of rice as affected by no-tillage (Chinese Milkvetch) and conventional tillage

Treatment	Tiller number No. plant^{-1}	No. of spikelets Thousand (m^{-2})	Ripened grain (%)	1,000-grain weight (g)	Grain yield (Mg/ha)
Tillage	13.1±1.24[a]	26.9±1.85	86.1±0.68	22.9±0.11	4.13±0.08
Chinese Milkvetch	12.1±0.75	25.5±1.07	88.0±0.45	22.3±0.08	3.94±0.15
MSD[b] (p <0.05)	NS[c]	NS	1.31	0.22	NS

[a]Mean ± standard deviation (n=3); [b]MSD, minimum significant difference; [c]NS, not significant at p <0.05.

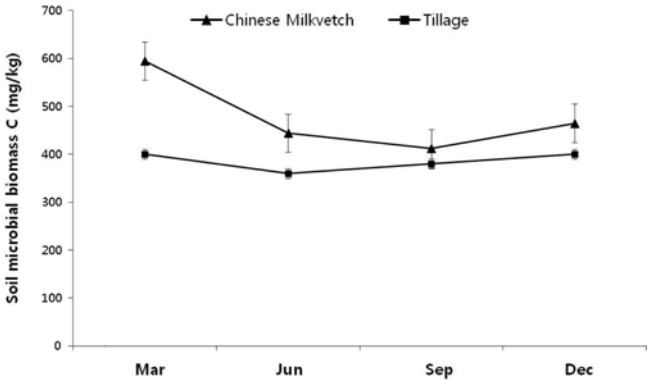

Fig. 5. Concurrent change in soil microbial biomass of the different soil samples collected at different time. Soil liable carbon varied with bacterial metabolism and Chinese Milkvetch matter content. Error bar indicates the standard deviation of three replication samples.

both samples tested, while HGCGPB and *Proteobacteria* were relatively greater in case of tillage field and lowest in Chinese Milkvetch samples (Fig. 2). Population were decreased in September (maturation stage), with further decreases in December (after harvesting). In December only spore-forming bacteria like *Bacillus* sp. can survive and restore nutrient flow in soils for plants, and for other microbes. Most of the *Proteobacteria* are known as plant pathogens [Preston *et al.*, 2005], and appear almost immediately when favorable climatic conditions (temperature and type of treatment) occur. In this study, we observed proteobacterial populations in two samples collected in March and June, but these populations were not observed when temperatures decreased. The effects of seasonal changes in the treated soil were more pronounced than in the conventional tilled soil samples, and are likely linked to the availability of nutrients throughout the year. Such changes could be attributed to an increase in the microbial biomass after Chinese Milkvetch treatment (Fig. 5). With the soil temperature varying between 5 and 32°C over the course of a year it is not surprising that climatic conditions have a large affect on microbial populations and community structure [Moore-Kucera and Dick, 2007]. Our results from total population analysis show maximum numbers in spring (June sample), with numbers from March following where the temperature was optimum for bacterial growth and the availability of nutrients.

Extracellular enzymes secreted from the bacteria play a major role in material recycling and in the continuation of nutrient cycling in nature. The stimulation of enzymes depends on the chemical composition of organic wastes in the soil. Many *Bacillus* sp. are known to secret a variety of extracellular enzymes [Rey *et al.*, 2004]. Soil hydrolases can provide an early indication of soil fertility status, since those enzymes are related to the mineralization of important nutrient elements, such as N, P and S [Preston *et al.*, 2005]. Bacterial isolates from soil samples showed degradation activity for many plant cell wall compounds. Our results show that most isolates exhibited maximal enzyme activity, indicating their role in biogeochemical cycles in nature. The general increase in biomass carbon observed can be attributed to a positive effect on organic materials and easily degradable materials [Tejada and Gonzalez, 2006]. Soil microbial biomass increases soil respiration and enzymatic activities. Soil respiration is the biological oxidation of organic matter to CO_2 by aerobic organisms, including bacteria. It is positively correlated with soil organic matter (SOM) content, and often with microbial biomass. The microbial biomass in-turn could be measured by the initial change in the soil respiration rate as a result of adding an easily decomposable substrate [Anderson and Domsch, 1978]. However, the correlation between total extracellular enzyme activity and community composition may be due to concurrent changes in the microbial biomass affected by organic matter with no-tillage.

We observed a higher frequency of *Bacillus* sp. in Chinese Milkvetch treated paddy soil compared to conventional tilled field soil, with the Chinese Milkvetch being the same as what was observed in an anoxic paddy soil sample [Watanabe and Hayano, 1993; Weber *et al.*, 2001]. Among the many *Bacillus* sp. identified, we could interpret an influential effect of the relative dominance of *B. licheniformis* in the Chinese Milkvetch treated soil samples, which is known to produce an assortment of extracellular enzymes that may contribute to nutrient cycling in nature [Rey *et al.*, 2004]. The results from the Chinese Milkvetch treated soil samples show dominance

of *B. licheniformis*, and the tests for secretion for extracellular enzyme activities revealed increased degradation of a wider range of synthetic substrates (Table 2). The variety of enzyme activities secreted from the bacteria help the crop to take the nutrients at the rhizosphere. These circustoms make it possible to keep the rice yield in common with conventional tilled soil (Table 3). The effect of Chinese Milkvetch treatment appeared to be distinct, with an increase in total soil carbon potentially translating into an effect on bacterial number and community. Meanwhile we could also see the effect of no-tillage on total soil carbon, which was lower in case of conventional tillage soil (Fig. 4). These results suggest that practicing no-tillage during rice cultivation has the potential to reduce additional degradation of organic matter and CO_2 increase into the atmosphere. It may be stated that soil management (no-tillage and soil carbon) directly influences soil carbon and carbon sequestration. The *Firmicutes* bacteria play a role in fixing the soil carbon and soil microbial biomass for keeping continuous flow of nutrients, which was observed more clearly in Chinese Milkvetch-treated soil samples than in conventional tilled soil samples. Furthermore, transformation of carbon in soil as influenced by land management and impact on global climatic change should not be overlooked [Huang *et al.*, 2004]. In conclusion, it was observed that use of Chinese Milkvetch with no-tillage may serve as an effective alternative to the use of chemical fertilizer.

Acknowledgments. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (03-2010-0250). T.H. Kang was supported by scholarships from the BK21 Program, Ministry of Education and Human Resources Development, Korea.

References

Alvarez R, Díaz R, Barbero N, Santanatoglia OJ, and Blotta L (1995) Soil organic carbon, microbial biomass and CO_2–C production from three tillage systems. *Soil Tillage Res* **33**, 17–28.

Alvarez S and Guerrero MC (2000) Enzymatic activities associated with decomposition of particulate organic matter in two shallow ponds. *Soil Biol Biochem* **32**, 1941–1951.

Anderson JPE and Domsch KH (1978) A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biol Biochem* **10**, 215–221.

Beyer L, Blume HP, and Peters M (1991) Biological activity and organic matter transformations in typical soils of Schleswig-Holstein. *Geoderma* **49**, 273–284.

Booth MS, Stark JM, and Rastetter E (2005) Controls on nitrogen cycling in terrestrial ecosystems: a synthetic analysis of literature data. *Ecol Monogr* **75**, 139–157.

Bossio DA and Scow KM (1998) Impact of carbon and flooding on soil microbial communities: Phospholipids fatty acid profiles and substrate utilization patterns. *Microb Ecol* **35**, 265–278.

Chander K and Brookes PC (1993) Residual effects of zinc, copper, and nickel in sewage sludge on microbial biomass in a sandy loam. *Soil Biol Biochem* **25**, 1231–1239.

Cho KM, Hong SY, Lee SM, Kim YH, Kahng GG, Lim YP, Kim H, and Yun HD (2007) Endophytic bacterial communities in ginseng and their antifungal activity against pathogens. *Microb Ecol* **54**, 341–351.

Golchin A, Oades JM, Skjemstad JO, and Clarke P (1995) Structural and dynamic properties of soil organic-matter as reflected by ^{13}C natural-abundance, pyrolysis mass-spectrometry and solid-state ^{13}C NMR-spectroscopy in density fractions of an oxisol under forest and pasture. *Australian J Soil Res* **33**, 59–76.

Huang PM, Wang MK, and Chiu CY (2004) Soil mineral-organic matter-microbe interactions: Impact on biogeochemical processes and biodiversity in soils. *Pedobiologia* **49**, 609–635.

Jenkinson DS and Ladd JN (1981) In *Soil Biochemistry*, (Vol. 5), Marcel Dekker, New York, NY.

Kandeler E, Tscherko D, and Spiegel H (1999) Long-term monitoring of microbial biomass, N mineralization and enzyme activities of a chernozen under different tillage management. *Biol Fertil Soils* **28**, 343–351.

Keenedy AC and Gewin VL (1997) Soil microbial diversity: Present and future considerations. *Soil Sci* **162**, 607–617.

Kern JS and Johnson MG (1993) Conservation tillage impacts on national soil and atmospheric carbon levels. *Soil Sci Soc Am J* **57**, 200–210.

Lim JM, Jeon CO, Lee GS, Park DJ, Kang UG, Park CY, and Kim CJ (2007) *Leeia oryzae gen*. nov., sp. isolated from a rice field in Korea. *Int J Syst Evol Microbiol* **56**, 39–44.

Lin L, Qiao YS, Ju ZY, Ma CW, Liu YH, Zhou YH, Zhou YJ, and Dong HS (2009) Isolation and characterization of endophytic *Bacillius subtilis* jaas ed1 antagonist of eggplant Verticillium Wilt. *Biosci Biotechnol Biochem* **73**, 1489–1493.

Maidak BL, Cole JR, Lilburn TG, Parker Jr, CT, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen, GJ, Pramanik S, Schmidt TM, and Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.

Moore-Kucera J and Dick RP (2007) PLFA profiling of microbial community structure and seasonal shift in soil of a Douglas-fir chronosequence. *Microb Ecol* **55**, 500–511.

Nannipieri P, Grego S, and Ceccanti B (1990) Ecological significance of the biological activity in soil. *Soil Biochem* **6**, 293–305.

Nelson DW and Sommers LE (1982) Total carbon, organic

carbon, and organic matter. In *Methods of Soil Analysis*, (Part 2). Soil Science Society of America, Inc. and American Society of Agronomy, Inc., Madison, WI.

Ohshiro T, Ohmoto Y, Ono Y, Ohkita R, Miki Y, Kawamoto H, and Izumi Y (2010) Isolation and Characterization of a Novel Fucoidan-Degrading Microorganism. *Biosci Biotechnol Biochem* **74**, 1729–1732.

Perrott KW, Sarathchandra SU, and Dow BW (1992) Seasonal and fertilizer effects on the organic cycle and microbial biomass in a hill country soil under pasture. *Australian J Soil Res* **30**, 383–394.

Preston GM, Studholme DJ, and Caldelari I (2005) Profiling the secretomes of plant pathogenic Proteobacteria. *FEMS Microbiol Rev* **29**, 331–360.

Rattanachomsri U, Kanokratana P, Eurwilaichitr L, Igarashi Y, and Champreda V (2011) Culture-independent phylogenetic analysis of the microbial community in industrial sugarcane bagasse feedstock piles. *Biosci Biotechnol Biochem* **75**, 232–239.

Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, Leon ALD, Xiang H, Gusti, V, Clausen IG, Olsen PB, Rasmussen MD, Andersen JT, Jorgensen PL, Larsen TS, Sorokin A, Bolotin A, Lapidus A, Galleron N, Ehrlich SD, and Berka RM (2004) Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. *Genome Biol* **5**, 1–12.

Saito N and Nei M (1987) The neighbor-joining method a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sambrook J and Russell DW (2001) Molecular Cloning: A Laboratory Manual, (3rd ed.), Cold Spring Harbor Laboratory Press, New York, NY.

Tejada M and Gonzalez JL (2006) Relationships between erodibility and erosion in a soil treated with two organic amendments. *Soil Till Res* **91**, 186–198.

Tompson, JD, Higgins DG, and Gibson TJ (1994) CLUSTAL W improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Waldrop MP, Balser TC, and Firestone MK (2000) Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem **32***, 1837–1846.

Watanabe K and Hayano K (1993) Distribution and identification of proteolytic *Bacillus* spp. In paddy field soil under rice cultivation. *Can J Microbiol* **39**, 674–680.

Weber S, Stubner S, and Conrad R (2001) Bacterial population colonizing and degradation rice straw in anoxic paddy soil. *Appl Environ Microbiol* **67**, 1318–1327.