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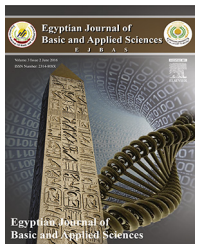

Full Length Article

Effect of soil disinfection with chemical and biological methods on bacterial communities

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ABSTRACT

Little is known about the effect of soil disinfection on bacterial communities. Soils were treated with an effective chemical fumigant chloropicrin and biofumigant mustard greens (*Brassica juncea*). While mustard greens did not affect the soil bacterial community structures very much, chloropicrin greatly reduced soil biomass and bacterial species richness. Chloropicrin also influenced the bacterial community structure, making the phylum *Firmicutes* dominant by occupying about 75%. In more than two months, the proportion of *Firmicutes* was reduced to the basal level, and the phyla *Bacteroidetes* and *Proteobacteria* became dominant. Since mustard greens worked as carbon sources for soil reduction, soils were treated with wheat bran and a low concentration of ethanol. Soil reduction with wheat bran and ethanol did not influence the soil bacterial community structures. Beta diversity analyzed by Principal Coordinate Analysis showed that bacterial communities in the soils except chloropicrin-applied soils formed a cluster. All together, biofumigant mustard greens, a probable substitute for chloropicrin, were demonstrated to cause much less damage on soil bacterial community than chemical chloropicrin.

1. Introduction

Soil bacteria play important roles for the maintenance of the soil ecosystem by regulating several significant soil processes, such as decomposition of organic materials, nutrient recycling and mineralization, and inducing pollutant degradation. Many studies have already revealed that agronomic and crop protection practices significantly influence both function and structure of soil microbial communities [1–3].

Soil disinfection with chemical methods, such as pesticides, herbicides and fumigants, has been applied to control weeds, plant diseases and soil borne toxic pathogens all over the world [4]. Some of these chemicals are known to damage

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the environment, be toxic to human, and have some negative effects on soil microorganisms [5–7]. Methyl bromide, one of the highly effective fumigants, is banned in several developed countries because it damages the ozone layer [8]. Chloropicrin, which is widely used in green houses, is also banned by EU as a pesticide for agricultural purposes due to its carcinogenic effects [9]. Chloropicrin is generally regarded as effective on fungal pests, but less effective on nematodes and weeds than Methyl bromide [10].

For the promotion of environment friendly agriculture, the use of organic compounds and green materials such as rice bran [11], oil cake [12], charcoal and ashes are now increasingly attempted in controlling weeds and soil borne pathogens. For the search of alternatives of chemical fumigants, studies are initiated on various aspects to find out the suitable biofumigants. Biofumigation is the agronomic practice of using volatile chemicals (allelochemicals) released from decomposing plant tissues to suppress pests [13,14]. Most of the studies have been done to search for biofumigants with *Brassica* families [15] plants containing isothiocyanate, which has the biocidal effects to nematodes, bacteria, fungi, insects and germinating seeds of weeds. However, very few information is available for the effects of biofumigants on whole soil bacterial communities. On the basis of these consequences, the main objective of this study was to figure out the effects of biofumigants and chemical fumigant chloropicrin application on the bacterial community structures along with soil reduction treatments. *Brassica* family plants for biofumigation are used as carbon sources for soil reduction. Wheat bran and a low concentration of ethanol were used for soil reduction.

Next-generation DNA sequencing technology, in particular pyrosequencing using the Roche/454 platform, has been applied to studies in microbial ecology [16–18]. In this study, we surveyed the bacterial community composition by using barcoded 16S rRNA gene 454-pyrosequencing technology. We found that the chloropicrin fumigants greatly affect the natural soil bacterial composition, whereas the application of biofumigant and reductive treatment did not affect the natural soil bacterial communities too much.

2. Materials and methods

2.1. Soil sampling, preparation and physicochemical properties

2.1.1. Soil preparation and soil sample collection

Plastic containers containing approximately 45 kg of soil were used for the experiment. The soil was sampled from the farm of Education and Research Center for Subtropical Field Science, Kochi University. Each treatment was conducted in five replicates. 1.4 kg of small pieces of shoots and 100 g of roots from two-month-old mustard greens (*Brassica juncea*) were mixed with soil (mustard greens). Soils were mixed with 0.24 kg of wheat bran (wheat bran) and 2% ethanol (ethanol). Containers were covered by plastic sheets and submerged in water for about one month for soil reduction disinfection. Soils were treated with 4 ml of chloropicrin in two holes and covered by plastic sheets for 10 days (chloropicrin). Two tomato seedlings were planted in each container after the first treatment. Soil samples were periodically collected at 5 cm depth from the surface. Time schedule of soil treatment and soil sampling was summarized (Fig. 1).

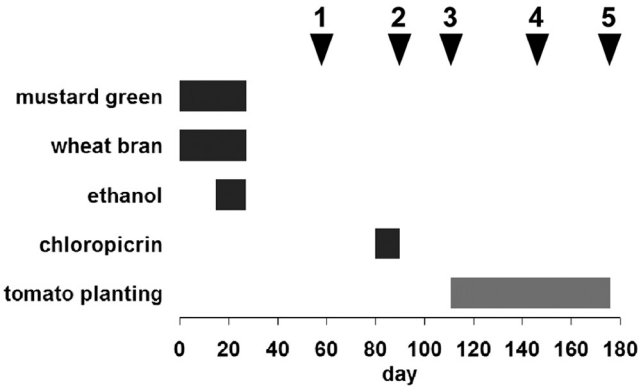

Fig. 1 – Time schedule of soil treatments and soil sampling. Soil was treated with materials shown on the left throughout time indicated by black bars. Tomato plants were grown in the soil during time shown by a gray bar. Soils were collected on the day shown by arrowheads.

2.1.2. Analysis of soil physicochemical properties

Physicochemical properties of soil were analyzed with the following methods. Soil samples were air-dried and passed through a sieve with 2 mm mesh. Soil particle size was determined by the pipette method with sodium hexametaphosphate as dispersing agent. Soil pH was determined in water in a soil solution ratio of 1:5 using the glass electrode. Total carbon and nitrogen contents were analyzed using an NC analyzer (JM1000CN, J-Science). After fresh soil samples were passed through a sieve with 4 mm mesh, soil organic carbon was extracted with a 0.5 M K_2SO_4 solution in a soil to solution ratio of 1:5 and the C concentration was determined by a TOC meter (TOC-VCPH, Shimadzu) [19].

2.2. 454 pyrosequencing and data analysis

DNA was extracted from soil using ISOIL for Beads Beating (Nippon Gene). 0.5 g of soil was disrupted at 5500 rpm for 45 seconds using a Micro Smash MS-100 (Tomy Seiko). The extracted DNA was diluted, sonicated for 5 min, and used as PCR template. The hyper variable V4- and V5-region of 16S rRNA gene was PCR-amplified. The forward primer F563-LXA contained a sequence (CCATCTCATCCCTGCGTGTCTCCGAC) in its 5′ end and a key sequence (TCAG), followed by titanium adaptor (MID1 to MID6, Roche) and specific sequence (AYTGGGYDTAAAGNG). The reverse primer was BSR926-LB (5′-CCTATCCCCTGTGTGCC TTGGCAGTCTCAGCCGTCAATTYYTTTRAGTTT-3′). The PCR product (about 450-bp in size) was purified by Agencourt AMPure XP using sizing buffer (7% PEG6000 and 1 M NaCl). Emulsion PCR was done with Lib-L kit (Roche) and amplicons were analyzed on GS Junior 454 system (Roche).

Raw sequence data were processed and analyzed using QIIME 1.8 [20] through OTUMAMi 3.13 [21]. RDP was used in classifying the sequences into phylum/class and clustering the sequences into operational taxonomic units (OTUs). Representative sequences were selected from each OTU and used

Table 1 – Physical characteristics of the soil samples.[a]

Soil treatment	Date[b]	pH	Organic C	Total C	Total N	C/N ratio	Particle size (%)		
			(g kg^{-1})				Sand	Silt	Clay
Before treatment		5.95	0.85	16.4	1.37	11.9	58.2	22.0	19.8
None	2	5.94	0.64	16.7	1.39	12.0	60.5	20.8	18.6
	3	6.22	0.58	15.6	1.41	11.1			
	4	5.88	0.58	16.1	1.45	11.1			
	5	5.85	0.59	16.1	1.44	11.2			
Mustard green	1	5.98	0.68	17.2	1.54	11.2	61.0	21.0	18.0
	2	6.03	0.77	17.1	1.40	12.2	59.7	21.1	19.2
	3	6.29	0.64	16.5	1.48	11.2			
	4	5.89	0.66	16.6	1.52	10.9			
	5	6.02	0.71	17.0	1.53	11.1			
Wheat bran	1	5.90	0.74	17.5	1.52	11.5	59.5	22.1	18.4
	2	5.95	0.85	17.8	1.42	12.5	59.6	21.6	18.8
	3	6.13	0.60	16.5	1.50	11.0			
	4	5.75	0.63	16.5	1.52	10.9			
	5	5.85	0.68	16.2	1.50	10.8			
Ethanol	1	5.84	0.69	16.6	1.43	11.6	60.5	21.4	18.1
	2	5.81	0.50	17.2	1.43	12.1	59.7	21.8	18.4
	3	6.09	0.37	15.9	1.42	11.2			
	4	5.69	0.43	15.7	1.44	10.9			
	5	5.70	0.47	15.9	1.45	10.9			
Chloropicrin	3	5.90	0.07	15.5	1.41	11.0			
	4	6.17	0.11	15.3	1.44	10.7			
	5	5.83	0.09	15.7	1.42	11.0			

[a] Average of triplicate measurements.
[b] Date corresponds to the sampling time in Fig. 1.

for calculation of Chao I richness estimates [22] by using RDP pipeline [23]. The observed species data were picked form QIIME pipeline used in generating rarefaction graph by using Microsoft Excel program. The hierarchical heatmap was drawn by using OTUMAMi [21]. The bar plot for relative abundance of major phylum was performed by R command generated in OTUMAMi and visualized by statistical package R 3.0.2. The Jackknifed Principal Coordinate (PCoA) analysis plot was generated by using the UniFrac distance [24] in QIIME pipeline and visualized by incorporated EMPEROR software. The sequenced read data have been deposited in DDBJ Sequence Read Archive (DRA) under accession numbers DRA003990 and DRA003993.

3. Results

3.1. *Soil physical properties*

The physical properties of the studied soils were determined and no remarkable difference was observed among different treatments (Table 1). The study soil was classified as sandy clay loam soil and weakly acidic.

3.2. *Soil organic carbon content*

The organic carbon in the soil could be derived from the microbial biomass [19]. The organic carbon slightly increased during initial steps of soil reduction with wheat bran and mustard greens (Fig. 2), indicating that during these steps, the microbial biomasses increased. In the chloropicrin-treated soil, the soil biomass was drastically lower and remained low throughout the study (Fig. 2 and Table 1). Tomato plants grew well in all the soils. No significant difference was observed in growth rates (data not shown).

3.3. *Bacterial richness and diversity in soils with different treatments*

Large-scale pyrosequencing of 16S rRNA genes provides more sequence information in terms of profiling of phylogenetic

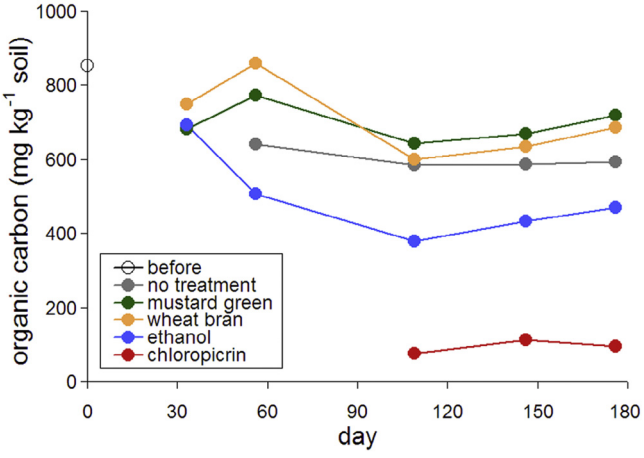

Fig. 2 – Organic carbon contents in the soil with different treatments. Soils with different treatments were collected at days indicated by circles. Soil organic carbon was extracted with a 0.5 M K_2SO_4 solution in a soil to solution ratio of 1:5, and the carbon concentration was determined by a TOC meter.

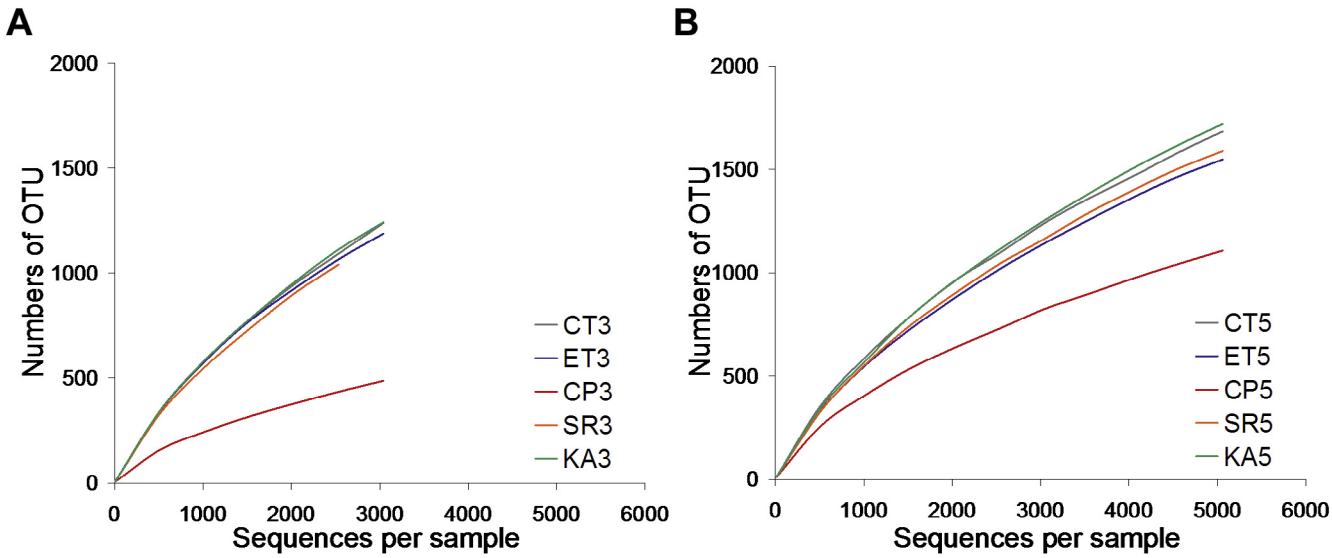

Fig. 3 – Rarefaction analysis of soils treated with different materials. Rarefaction for observed and estimated species richness was calculated, and rarefaction curves were drawn with QIIME. (A) CT3, KA3, SR3, ET3, and CP3 used soils treated with none, mustard greens, wheat bran, ethanol, and chloropicrin, respectively, at sampling time 3. (B) CT5, KA5, SR5, ET5, and CP5 used soils treated with none, mustard greens, wheat bran, ethanol, and chloropicrin, respectively, at sampling time 5.

diversity and community composition compared to the traditional Sanger-based sequencing of clone libraries [16]. The pyrosequencing-based analyses of the V3–V4 regions of 16S rRNA genes were conducted at two stages (at sampling times 3 and 5). Species richness was estimated with both rarefaction curves (Fig. 3) and Chao 1 indices (Table 2). Bacterial species richness among soils with different treatments was almost the same, except chloropicrin treatment. Species richness in the chloropicrin-applied soils was about half of those in the other soils at sampling time 3, and slightly increased at sampling time 5 (Fig. 3). Bacterial diversity in the chloropicrin-applied soils was much lower than those in the other soils estimated by Shannon diversity index (Table 2). These observations are consistent with less contents of organic carbon (Fig. 2) in the chloropicrin-applied soils.

Table 2 – Richness and diversity of the microbial communities.

Sample	No. of sequences	No. of OTUs[a]	Chao 1[b]	H′[c]
CT3	2,021	656	1,229	5.73
KA3	1,514	621	117	5.87
SR3	1,434	542	1,253	5.62
ET3	1,449	547	1,092	5.75
CP3	1,219	225	548	4.01
CT5	8,050	2,335	3,806	7.05
KA5	10,639	2,787	4,551	7.09
SR5	14,047	2,986	4,664	6.96
ET5	11,582	2,596	4,050	6.90
CP5	4,473	1,075	2,073	5.86

[a] Number of OTUs at a maximum distance of 0.03.
[b] Chao 1 index at a maximum distance of 0.03.
[c] Shannon diversity index at a maximum distance of 0.03.

3.4. *Alpha diversity at phylum level*

Major phyla detected in the soils were *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, *Firmicutes*, *Acidobacteria*, *Gemmatimonadetes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*, and *Nitrospirae* (Fig. 4). *Acidobacteria* and *Proteobacteria* were the most abundant phyla in the control soil without any treatment at both sampling times. These two phyla were also dominant in the other soils except the chloropicrin-applied soils. The dominance of *Acidobacteria* was slightly reduced after planting tomato. This might be due to the incorporation of some bacteria in the tomato rhizosphere. When chloropicrin was applied to the soil, *Firmicutes* became predominant, occupying about 75% of the soil bacterial community. At sampling time 5, the abundance of *Firmicutes* was reduced and *Bacteroidetes* appeared. Although *Proteobacteria* became most abundant, the bacterial community composition in the chloropicrin-applied soils was still different from those in the other soils (Fig. 4).

3.5. *Alpha diversity at OTU level*

Bacterial community compositions of the soils were examined at descending levels of biological classification. The heatmap was constructed with OTUs, which were relatively abundant at more than 1% in any of the soils. Most of the abundant OTUs belonged to *Firmicutes* in the chloropicrin-applied soils just three weeks after treatment (Fig. 5A), which is in good agreement with α diversity at phylum level. The most dominant OTU #3024 belonged to genus *Alicyclobacillus*. The member of genus *Flavisolibacter* (OTU #904) was observed as major OTU. In two months after chloropicrin treatment, most of the *Firmicutes* disappeared or became less abundant. OTUs belonging to phylum *Bacteroidetes* became dominant (Fig. 5A). The

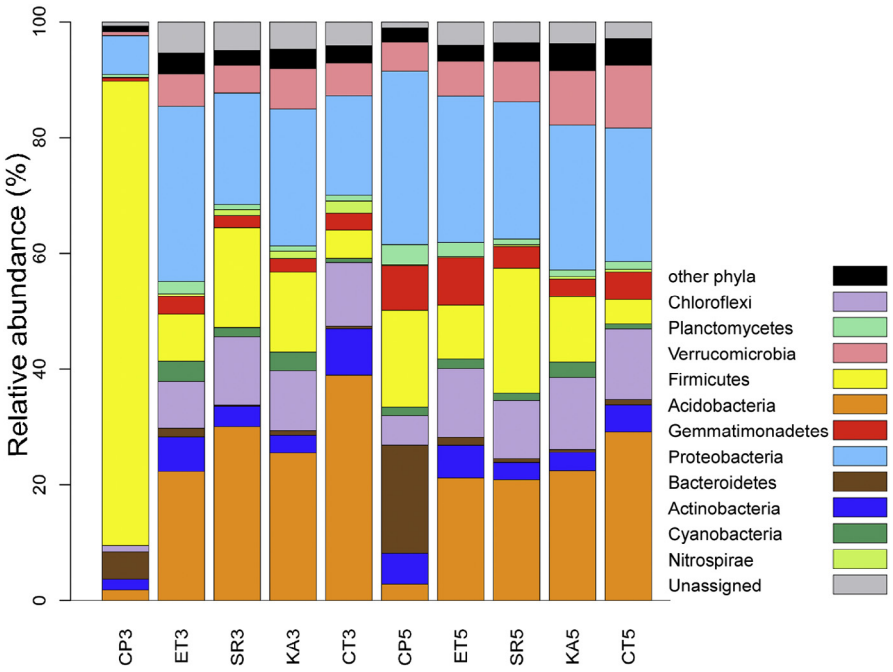

Fig. 4 – Relative abundance of major phylum groups in the soils treated with different materials. Soils were treated with materials shown on the bottom. The abbreviations are the same as Fig. 3.

dominant OTU was genus *Flavihumibacter* (OTU #2988). Several OTUs belonging to *Proteobacteria* also became abundant.

OTU #3551 belonging to genus *Bradyrhizobium* was one of the dominant bacteria in the other soils (Fig. 5B). Since this OTU dominantly existed in the control soil and even in the chloropicrin-applied soils (Fig. 5A), this bacterium appeared to be dominated in the soils used in this experiment. The other dominant OTU #2871 belonged to genus *Candidatus Solibacter*. *Candidatus Solibacter* is a member of *Acidobacteria*, which agrees with α diversity in phylum level (data not shown). OTU #1467 belonging to genus *Clostridium* was dominant in both soils treated with wheat bran and mustard greens. Compositions of dominant bacteria were stable for two months between sampling times 3 and 5 (Fig. 5B).

3.6. *Beta diversity*

β Diversity was calculated with the Jackknifed PCoA. As indicated with α diversity analysis, bacterial community compositions in the soils treated with ethanol, wheat bran, and mustard greens formed one cluster with those in the untreated soils (Fig. 6). This phenomenon could be explained as follows: the application of chloropicrin killed most of the dominant bacteria species, and the bacterial community structure was reorganized in two months. However, the community structure did not move back to the original soil bacterial composition.

4. Discussion

This study investigated the effects of several materials for soil disinfection on the structure of the soil bacterial community. Two biomaterials, wheat bran and mustard greens, and one chemical, ethanol at low concentration, were used for soil reduction. One chemical, chloropicrin, was used for fumigation. DGGE is a fast and relatively easy way to observe the overall bacterial community structure and composition [25]. DGGE results were almost compatible with the results of another pyrosequencing method (data not shown), indicating that the combination of these two methods could be an effective way to analyze bacterial community structure. We demonstrated that the chloropicrin-applied soil contained a very different bacterial composition from the other soils. *Firmicutes* became dominant with about 75% of bacterial species in the chloropicrin-applied soils. Drastic reduction of organic carbon corresponding to biomass was also observed in the chloropicrin-applied soils. Despite these much differences of the bacterial community structures, planted tomato plants grew and formed pericarps in the chloropicrin-treated soils as normal as in other soils (data not shown). We are going to elucidate the relationship between bacterial diversity in the soil and impacts on the agriculture, such as plant growth and productivity, in the next step.

Chloropicrin is a toxic gas, which directly kills the soil living organisms and decreases the biomass [26]. Chemical fumigant disrupts the cell walls of microorganisms [5]. Since *Firmicutes* forms spores, the spore form could be resistant to chloropicrin and survive even after chloropicrin treatment. Although we did not test the effectiveness of chloropicrin on the pests, no weeds grew on the soils. Chloropicrin is demonstrated to be effective against several weeds in a commercial strawberry field [27]. Even three months later after chloropicrin treatment, the bacterial community structure did not go back to the original state, although *Firmicutes* was no longer dominant. All together, we conclude that chloropicrin completely disturbs the bacterial community structure.

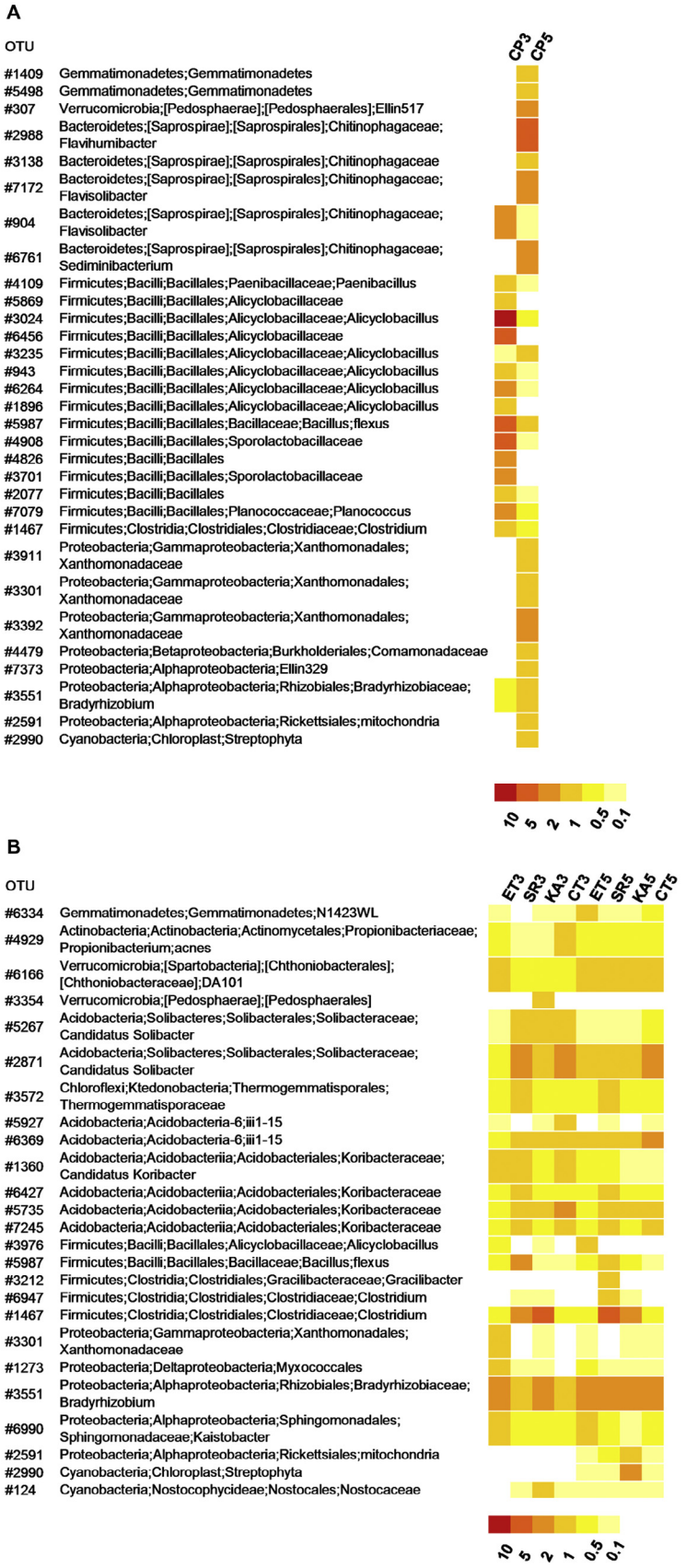

Fig. 5 – Relative abundance of major OTUs in the soils. OTUs, which existed more than 1% in the selected soil, were picked and the heatmap was constructed. OTUs in (A) chloropicrin-applied soils and (B) other soils were indicated.

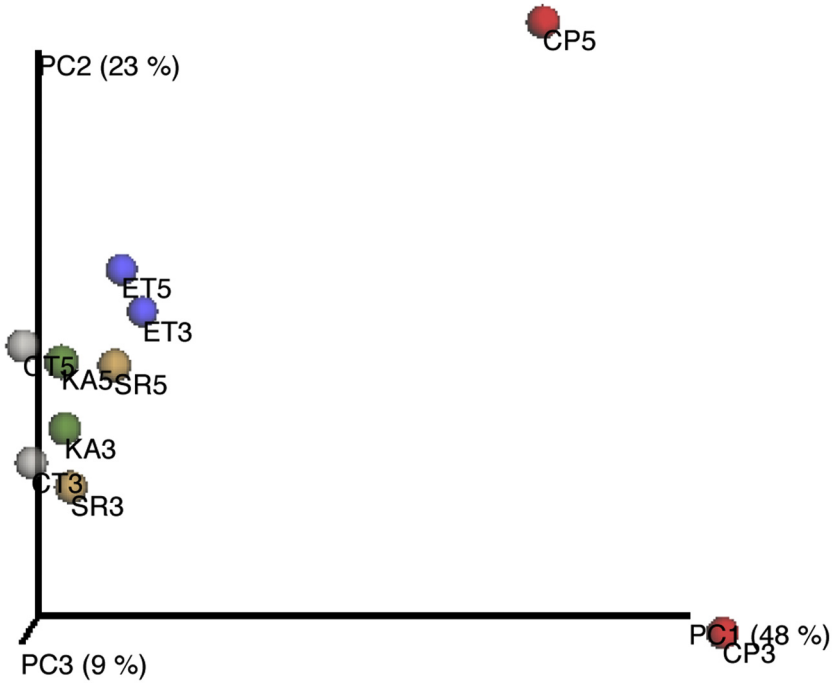

Fig. 6 – Weighted Jackknifed PCoA. PCoA visualized the difference of bacterial compositions. The Jackknifed plot was illustrated based on the calculated weighted UniFrac distance metrics.

The bacterial community structures of the soils were not affected by soil reduction with several carbon sources, wheat bran, ethanol, and mustard greens. Soil reduction consumes oxygen in the soil, so the eukaryotes such as fungi cannot survive in such reduced atmosphere, although we did not measure the number of eukaryotic microorganisms. Many of the soil bacteria are facultative anaerobic and could survive even after soil reduction with carbon sources. This is why the bacterial compositions in the soils treated with wheat bran, ethanol, and mustard greens were almost similar to that in the control soil. Weeds grew in the wheat bran-applied and mustard greens-applied soils (data not shown), indicating that wheat bran and mustard greens are not as strong as chloropicrin, which completely inhibited weed growth, at least for weed control.

Mustard greens work as biofumigant as long as carbon sources for soil reduction. A biotoxic element isothiocyanate is formed from the *Brassica* plants decomposition [28,29]. Volatiles released from chopped leaf materials of *Brassica* plants inhibit growth of a variety of plant pathogenic fungus of potato, including *Rhizoctonia solani*, *Phytophthora erythroseptica*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, and *Fusarium sambucinum* [30]. *B. juncea* and *Brassica rapa* cause over 91–95% mortality of encysted eggs of nematode *Globodera pallida* [31,32]. *B. juncea* and *B. rapa* contain 2-propenylglucosinolate and 3-butenylglucosinolate as main glucosinolates, respectively. The biocidal activity of these glucosinolates is comparable with the efficacy of synthetic pesticides and antibiotics gentamicin [31,32]. In this sense, synthetic pesticides, such as methyl bromide and chloropicrin, could be replaced by *Brassica* plants. *Brassica* plants are attractive materials for both soil reduction and biofumigation.

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