Suppression of spinach wilt disease by biological soil disinfestation incorporated with *Brassica juncea* plants in association with changes in soil bacterial communities

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Highlights

- Effects of biological soil disinfestation (BSD) on spinach wilt were investigated.
- BSD-treatment using *Brassica juncea* plants reduced the wilt disease incidence.
- Soil bacterial compositions in BSD soil were examined by clone library analysis.
- Species in the *Clostridia* class dominated in the bacterial communities in BSD soil.
- BSD should be applied before every cropping to suppress the disease effectively.

Abstract

Biological soil disinfestation (BSD) is a method of controlling soil-borne pests and diseases through anaerobic decomposition of plant biomass incorporated in field soil with temporary irrigation and covering with sheets. In this study, effects of BSD on suppression of spinach wilt disease were investigated by two different field experiments using mainly *Brassica juncea* plants as plant biomass. Soil bacterial community compositions were analyzed with clone library analysis based on 16S rRNA gene sequences to determine the relationship between the bacterial composition in the treated soil and suppression of the disease. For the BSD-treated soils, oxidation-reduction potential dropped, and acetate was usually detected at high concentrations. Although the control treatment (irrigation and polythene covering without biomass) decreased the wilt disease incidence in spinach plants cultivated in the treated plot as

compared with those for the non-treated plot, BSD-treatments suppressed the disease more effectively. The clone library results showed that both non-treated and control soils contained diversified bacterial communities of various phylogenetic groups, while members of the *Firmicutes* mainly from the class *Clostridia* were dominated for the BSD-treated soils. The clostridial groups detected were diverse and the major clone groups were closely related to strictly anaerobic fermentative bacteria such as *Clostridium saccharobutylicum*, *C. cylindrosporum*, *C. sufflavum*, *C. xylanovorans*, etc. These clostridial groups were almost eliminated from the soil bacterial community when the BSD-treated soil was treated again with irrigation and covering without biomass before the next cropping, in which the wilt disease was hardly suppressed.

Keywords

Anaerobic bacteria; Biological soil disinfestation (BSD); *Brassica juncea*; Clone library; Clostridial group; *Fusarium oxysporum*, Wilt disease

1. Introduction

Soil-borne diseases are recognized as important limiting factors in the production of vegetable crops. The outbreak of soil-borne diseases inflicts major economic damage on crop

worldwide. Fusarium wilt of spinach (*Spinacia oleracea* L.), caused by *Fusarium oxysporum* f. sp. *spinaciae*, has been reported as the most serious disease of spinach cropping (Correll et al., 1994; Horinouchi et al., 2010). It causes damping-off, wilting, root rot, and discoloration of the vascular system of seedlings and mature plants. To combat the disease, preplant soil disinfestation is essential. Although soil fumigation with methyl bromide, chloropicrin or other chemicals has been used successfully to control the disease, their use has been associated with potential severe environmental problems or damages for human health (Kuniyasu et al., 1993; Gina et al., 2008).

Biological soil disinfestation (BSD) is a method for controlling soil-borne pests and diseases through anaerobic decomposition of plant biomass that was mainly developed in the Netherlands (Blok et al., 2000; Messiha et al., 2007) and Japan (Shinmura, 2004; Momma, 2008). Recently, BSD has become popular in the world, especially in organic agriculture as an alternative of chemical fumigation. For BSD, plant biomass is incorporated into soil followed by application of irrigation water and covering the soil surface with transparent plastic film for about three weeks to induce reduced soil conditions and to maintain suitable soil temperature (Shinmura, 2000, 2004). Thereafter, crops can be cultivated after removing the plastic film and plowing the field. Plant biomass sources such as *Brassicaceae* plants, wheat bran, rice straw, rice bran, *Avena* spp., grasses, or other organic substances have been reported to be used

successfully for BSD against soil-borne pests and diseases (Mojtahedi et al., 1991; Sarwar and Kirkegaard, 1998; Shinmura, 2004; Goud et al., 2004).

Brassicaceae plants are known to contain bioactive substances and have been widely used for biofumigation in soil (Kirkegaard et al., 1996; Larkin and Griffin, 2007). Biofumigation was originally designated to include the particular use of Brassicaceous cover crops and the plants have become associated with the practice of BSD as promising biomass for incorporation (Stapleton et al., 2000). The decomposition of glucosinolates in the *Brassicaceae* plant tissues may cause release of isothiocyanates (ITCs), in addition to thiocyanates, nitriles, and oxazolidinethiones, which are toxic to many soil pathogens (Sawar and Kirkegaard, 1998; Fahey et al., 2001). Thus, incorporation of the *Brassicaceae* plants in soil for BSD should be advantageous for suppression of plant pathogens over other organic substances. *Brassica* species such as *Brassica juncea*, *B. napus*, *B. nigra*, *B. oleracea*, and *B. campestris* used as BSD material or biofumigant were reported to control various soil-borne diseases and crop mortality caused by *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., *Verticillium* spp. *Alternaria alternata*, *Colletotrichum dematium*, and plant parasitic nematodes (Tsror et al., 2007; Mattner et al., 2008; Ramirez et al., 2009). A number of brassicas are available in the world market, of which mustard greens or Indian mustard (*B. juncea* var. *cernua*) is widely grown at both subsistence and commercial levels. The potentiality of use of *B. juncea* plants in BSD is attributed to the

availability of seeds, easy and quick growth, and year round production in most of the areas in the world.

In our previous studies, BSD treatments with mustard greens (*B. juncea* var. *cernua*) and *Avena* plants, as well as wheat bran, successfully controlled the population of the pathogens (*F. oxysporum* f. sp. *lycopersici*, wilt pathogen of tomato and *F. oxysporum* f. sp. *spinacea*, wilt pathogen of spinach) in two pot experiments using soil from different districts of Japan (Mowlick et al., 2012, 2013). Based on the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and clone library analyses, we found that bacterial communities in the BSD-treated soils were greatly changed and strictly anaerobic groups, especially fermentative members in the class *Clostridia*, became major bacterial groups in the communities together with some other aerobic or facultatively anaerobic bacteria from the classes including *Bacilli* and *Gammaproteobacteria*. The *Clostridia* population increased in soil was suggested to play an important role in the control of pathogens through their activities or products.

Considering the large scale production or repeated cultivation of crop, we should give importance to the field experiments to increase the reliability of the findings from the model experiments. In this study, we intended to confirm the effects of BSD in field conditions to suppress the spinach wilt disease using *B. juncea* plants and the changes in the soil bacterial

community compositions by the treatments. Moreover, the BSD-treated fields were treated again with irrigation and covering without biomass incorporation before the next cropping, and changes or recovery of bacterial community in the soil and duration of BSD-effects on diseases suppression were also investigated. Molecular clone library analysis (Maidak et al., 1999) based on 16S rRNA gene sequences was mainly carried out to determine the bacterial community compositions in the soil samples.

2. Materials and methods

The experiments were carried out in greenhouses at two different fields located in two districts in Japan at a distance of more than 500 km from each other. Spinach was grown based on the organic farming method (without applying chemical fertilizers or pesticides) throughout the cultivation period for both experiments.

2.1. Soil samples for Experiment 1

Soil samples for Exp. 1 were collected from a field experiment of BSD in a greenhouse (8 × 21 m^2) of Agricultural Research Center, Nara (34.49 ^oN, 135.96 ^oE), Japan during June 2010. A total of three treatments were carried out in this experiment using a Randomized Complete Block Design with three replications. The unit plot size for each treatment was 2.5 × 4 m^2. Soil

(brown forest loamy soil, pH 6.5-7.0) was treated with two plant biomass sources, *B. juncea* var. *cernua* (Mustard greens) and *Avena sativa* plants, as BSD-treatments in a greenhouse field. Spinach had been continuously cultivated since 2009 and natural infection of wilt disease of spinach had occurred in the field. The *Brassica* and *Avena* plants were cultivated beforehand in the same greenhouse for two months (15 April to 15 June) and used for the BSD-treatments. The plants were cut to pieces by a hammer knife mower and immediately incorporated into the soil by a rotary tractor at the rate of 10.4 kg/m^2 (= 1.1 kg in dry weight/m^2) and 3.29 kg/m^2 (= 0.44 kg in dry weight/m^2), respectively. For the control-treatment, none of plant material or any other substances was incorporated into the soil. All the plots including the control were irrigated sufficiently to exceed the field capacity of moisture content (56.4%) and covered with agricultural transparent polyethylene film tightly to provide reducing conditions in soil for three weeks (15 June-06 July). The plots were plowed well when the sheets were removed after three weeks. Spinach was seeded in every plot about a week later (15 July). The natural wilt disease incidence (%) was recorded during the cultivation by visual observation of 100 spinach plants (hill) per plot.

Temperatures in soil (5 and 10 cm depth) and air inside the greenhouse were recorded every 10 minutes by data loggers (TR-724, T & D Co. Ltd.). Oxidation-reduction potential (ORP) of soil (at 15 cm depth) were measured at intervals of two or three days at four points of every

treatment by electrodes (Ag/AgCl) inserting into the soil directly.

Soil samples for the measurement of volatile fatty acids (VFAs) in soil and clone library analysis of bacterial communities were collected at every week during the treatment. Each soil sample (100 g) was obtained from the upper 10 cm of soil depth and mixed well in sterile polyethylene bags. Similarly, an original field soil sample without any treatment was also collected. Soil samples collected were kept in a freezer (-20^{o}C) immediately after the sampling and preserved there until use. Soil samples collected at two weeks of the treatments were used for the clone library analysis based on the data for various soil conditions examined. The names of clone libraries were designated considering the name of the place (Nara), the sampling date (two weeks), and control or the type of biomass as NCO (non-treated soil), N2C (control/irrigated without biomass), N2B (*Brassica* /Mustard-treated soil), and N2A (*Avena*-treated soil).

2.2. Experiment 2

Soil samples for Exp. 2 were collected from a field experiment of BSD in greenhouses (5.5 × 17.5 m^2) of Agricultural Research Center, Yamaguchi, Japan (34.9 ^{o}N, 131.3^{o}E) during June 2010. The soil was gray lowland soil (sandy loam, pH 6.5) and the plot size for each treatment

was 1.5 × 5.5 m^2. The number of treatments used in this experiment was six distributed in a Randomized Complete Block Design with three replications. Spinach had been also continuously cultivated and natural infection of wilt disease of spinach had occurred in the field. The soil was treated with plant biomass of two different varieties of *B. juncea*, that is, *B. juncea* var. *cernua* (Mustard greens) and *B. juncea* var. *crispifolia* (Azamina, one of green vegetables commonly cultivated in this district). The name of the treatments was designated considering the name of the place (Yamaguchi), control or types of biomass (C, control; B, *Brassica*/Mustard; Ba, *Brassica*/Azamina).

Both *B. juncea* varieties were cultivated (30 Nov. 2009-8 Apr. 2010) to use as the BSD-materials for the BSD plots (YB and YBa) in the same plots. The plant material was incorporated into soil by the rotary tractor at the similar way as described for incorporation of plants in Exp. 1 together with applying irrigation water and covering with a double layer of agricultural transparent sheet with low-permeability for gas (Barrier Star film, TOKANKOUSAN Co. LTD; Sky Coat film, C.I. KASEI Co. LTD). For the control-treatment (YC), soil was covered with the same film after irrigation without plant biomass. Soil samples were collected from all the plots in the similar way as described above for Exp.1 after three weeks of the treatments (8-30 Apr. 2010). Spinach was then seeded and cultivated (10 May-17 June) in the treated soil. Natural wilt disease incidence was recorded based on the observation of

plants for 10 different places of 0.1 × 1 m^2 of each plot during the cultivation and fresh marketable yields (g/m^2) were determined by the weights of plants harvested at the end of cultivation for the same area.

As the next treatments after spinach cultivation in the YC, YB, and YBa fields, the field soil was treated in the same way as described above for the control treatment (covering after irrigation without biomass incorporation) for each soil (designated as YCC, YBC, and YBaC, respectively). Soil samples were also collected from the plots after three weeks of the treatments (25 June-16 July). For all plots, spinach was cultivated (22 July-9 Sep.), and both natural wilt disease incidence and yields of spinach were also recorded for each field. Temperature in soil with sheets (10 cm depth) and air inside the greenhouse during the soil treatments was also recorded by data loggers.

2.3. Measuring volatile fatty acids concentrations of soil samples

The concentrations of volatile fatty acids (VFAs) for the soil samples were measured by gas chromatography (Hitachi G-3900) as described previously (Mowlick et al., 2012; Ueki et al., 1986) and the concentrations are shown in the text as those determined in the supernatant of slurry samples.

2.4. DNA extraction, PCR amplification, clone library, and sequencing

A composite sample (3 g) was prepared by taking 1 g soil of each triplicate soil sample. One g of soil from the composite sample was used for DNA extraction. According to the instructions of 'Ultra Clean™ Soil DNA Isolation kit' (MO BIO Laboratories, Inc., Carlsbad, CA, USA), DNA was extracted from the soil and used for PCR amplification. Bacterial 16S rRNA genes were PCR-amplified using a primer set B27f (5'-AGA GTT TGA TYM TGG CTC AG-3') and U1492r (5'-GGY TAC CTT GTT ACG ACT T -3'). The composition of PCR mixture (50 µl) and PCR amplification conditions were followed as described in the previous study (Mowlick et al., 2012). Amplified DNA fragments were confirmed after agarose gel electrophoresis and ethidium bromide staining.

Clone library analyses were conducted to determine the bacterial community composition of the soil samples collected as described previously. The PCR products of DNA from these soil samples were purified according to the instructions of QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, and USA) and cloned into *Escherichia coli* JM109 competent cells following the protocol of pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). The vector-harboring clones containing an insert of appropriate sizes (about 1500 bp) were obtained in the

Luria-Bertani (LB) plates by the standard method (Kaku et al., 2005). Nucleotide sequencing (about 600 bp) was performed for a total of 96 clones unless otherwise stated for each soil sample using a sequence primer U515f (5' GTG YCA GCM GCC GCG GTAA-3') according to the Dye Terminator method using a capillary sequencer at Takara Co. Ltd.

2.5. Database search, construction of trees, and statistical analysis

The database searches for related 16S rRNA gene sequences were carried out with BLAST program and GenBank database (Altschul et al., 1997). The ClustalW program of DDBJ was used to align the nucleotide sequences of the clone libraries. The phylogenetic trees were made using the neighbor-joining method (Saitou and Nei, 1987) with Njplot program in ClustalW package (Thompson et al., 1994). Determination of OTUs (operational taxonomic unit), bootstrap resampling analysis, chimera checking, rarefaction analysis, calculation of coverage (C), Shimpson's- (D) and Shannon-Wiener-diversity index (H'), etc. were conducted as described in our previous study (Mowlick et al., 2013).

2.6. Accession numbers of the nucleotide sequences

The nucleotide sequences retrieved from the clone library analyses have been deposited in DDBJ/GenBank and assigned under the accession numbers AB746034-AB746159 (125 entries for Exp. 1) and AB744235-AB744467 (232 entries for Exp. 2).

3. Results

3.1. Experiment 1

3.1.1. Soil status and disease incidence of spinach

The soil temperature under the sheets (10 cm depth) was much higher (24.7 to 35.5ºC based on the average daily values) as compared with that without the sheets (22.6 to 26.2ºC) throughout the period of soil treatments (Fig. 1a). The temperature at 5 cm in depth was usually higher (about 0.5-1.0ºC) than that at 10 cm irrespective of with or without sheets (data not shown). The air temperature inside the greenhouse ranged from 21.2 to 29.0ºC and was comparable to the soil temperature without the sheets.

The ORP value for non-treated soil was fairly constant at around +350 to +400 mV. The values for Mustard- and *Avena*-treated soil dropped very rapidly to below -100 mV within three

days, and then decreased to -200 or -250 mV until 13-15 days (Fig. 1b), indicating the development of strongly reduced conditions in these treated soils. The ORP value for the control soil (irrigation and covering without plant biomass) also decreased early in the treatment, while it never decreased under -100 mV.

Concentrations of VFAs in each soil were determined once a week (Table 1). No VFAs was detected from the control as well as non-treated soil samples throughout the period of treatment. For the Mustard-treated soil, acetate was detected at rather high (15-17 mmol/l) concentrations for the first two weeks, and decreased (<4 mmol/l) by the third week. Lower concentrations (1-2 mmol/l) of propionate and butyrate were detected throughout the treatment. For *Avena*-treated soil, acetate was detected only from the first week sample and a small amount of propionate was detected from all samples.

Non-treated plots resulted in a high incidence of spinach wilt (41.5%), but the control treatment considerably reduced disease incidence (66% reduction) relative to the non-treated plot (Table 2). However, both BSD-treatments, the *Brassica* and *Avena* plant biomass treatments, reduced the incidence more effectively (by 88-90%) than the control treatment.

3.1.2. Clone library analysis for soil bacterial community structure

To know and compare the changes in the soil bacterial community structures after the treatments, clone library analysis was carried out for the four soil samples including the non-treated (original) soil (48 clones for each soil sample). The phylogenetic affiliations of the clones designated by phylum or class levels of the domain *Bacteria* were determined for all clones (Table 3), and the phylogenetic trees were constructed for all libraries (data not shown).

The clone library for the non-treated soil (NCO) showed much diversified populations of different phylogenetic groups. The most dominant taxonomic group from the soil sample was allocated to the phylum *Proteobacteria* (32.5% of the total number of clones) and the majority in the phylum was placed in the *Alphaproteobacteria* class. Other sequences were placed in the phyla *Bacteroidetes*, *Actinobacteria*, TM7, *Acidobacteria*, etc. For the control library N2C, diversified bacterial populations were also detected and the clones in the *Proteobacteria* phylum were the most dominant (37.6%), including the clones in the class *Deltaproteobacteria* (12.5%), which were not found in the library NCO. *Clostridium straminisolvens* and *Clostridium xylanovorans* appeared as the closest relatives of some clones in the *Clostridia* groups (a spore-forming, strictly anaerobic bacterial group).

Clone libraries for both BSD-treated soil samples showed substantial differences as compared with those of the libraries NCO and N2C. In case of the Mustard-treated library N2B, 27.8% clones were assigned to the class *Clostridia* in the phylum *Firmicutes*. For the

Avena-treated library N2A, the number of members of the class *Clostridia* also increased as compared with those of the libraries NCO and N2C. The closely related clostridial species of the dominant clone groups in these two BSD-treated libraries were *Clostridium saccharobutyricum*, *Thermincola carboxydiphila*, *Clostridium tetanomorphum*, and *Clostridium orbiscindens*.

3.2. Experiment 2

3.2.1. Effects of treatments on soil status and crop

The average air and soil (10 cm depth) temperatures during the first treatment (in April) in the greenhouse were 18.1 and 21.7ºC with great daily fluctuations. For the second treatment (June to July), the average air temperature was 30.7 and that of soil temperature was 35.7ºC (data not shown). Considerable amounts of VFAs were detected from both BSD-treated soils with *B. juncea* plants incorporation (YB and YBa) (Table 4). Acetate was the major VFA with butyrate and propionate as the minors. No VFAs was detected where the soil was irrigated without plant biomass incorporation (YC, YCC, and YBC), with an exception of YBaC where traces of acetate and propionate were detected.

BSD treatment with both *Brassica* plants (Mustard and Azamina) effectively reduced the

incidence of spinach wilt by 87% and 75% relative to the control treatment, respectively, and almost the same and high amounts of fresh marketable yields were obtained from both BSD plots (Table 5). When both BSD plots were treated again with irrigation and covering without biomass incorporation (YBC or YBaC), the treatments hardly suppressed the outbreak of disease of spinach cultivated after the treatment, especially for the YBaC plot. Furthermore, the high disease incidence level for the YCC plot showed that repetition of irrigation without biomass incorporation had no effect on disease suppression.

3.2.2. Clone library analysis for soil bacterial community structure

Out of the soil samples obtained from the plots, five samples except from the YBaC plot were used for the analysis of the bacterial community compositions (Table 6). The clone library YC showed much diversified populations of different phylogenetic groups at almost similar ratios as described for the control library (N2C) of Exp. 1. For the Mustard-treated clone library YB, the members of the *Firmicutes* were exceedingly dominated (64.2%), of which the majority (38.8%) was clostridial clones. The clostridial groups were also dominant (44.4%) in the Azamina-treated library YBa. The clone groups affiliated with the class *Bacilli* (a spore-forming, aerobic or facultatively anaerobic group) were the second most dominant for both libraries (25.4

and 12.5%, respectively).

Of all the phylogenetic trees constructed, the trees for both BSD-treated libraries (YB and YBa) are shown in Fig. 2. Clones related to *Clostridium cylindrosporum* (8 clones, 93-95%), *C. saccharobutylicum* (4 clones, 91-100%), and *C. sufflavum* (4 clones, 90-93%) were dominant clostridial groups in the library YB, whereas those related to *C. cylindrosporum* (12 clones, 92-93%), *C. saccharobutylicum* (6 clones, 90-92%), *C. xylanovorans* (3 clones, 94-95%), and *Pelotomaculum schinkii* (3 clones, 88-89%) were dominant for the library YBa. The clone groups related to *Bacillus pycnus* (5 clones, 100%), *Bacillus circulans* (4 clones, 99%), and *Bacillus niacini* (4 clones, 96-97%) were also dominated in these libraries.

3.2.3. Effects of irrigation-repeats on the bacterial communities

In case of the clone library YCC, diversified bacterial populations were also detected, but clones related to the *"Symbiobacterium*" clade in the *Firmicutes* were detected as the most dominant group, which was not found in the library YC (Table 6). The ratio of clones from the *Bacilli* class increased and those from the phylum *Acidobacteria* decreased as compared with the library YC. Besides, the bacterial groups belonging to the class *Gammaproteobacteria* and the phylum *Bacteroidetes*, which emerged in the library YC, seemed to be eliminated in the

library YCC. The major dominant clone clusters for this library were related with *Symbiobacterium thermophilum* (16 clones, 90-95%) and *B. niacini* (7 clones, 98-99%) in the *Firmicutes*.

For the clone library YBC, the typical clostridial members became minor in the community, while the members in the *Bacilli* and the *"Symbiobacterium*" clade occupied the dominant places. Other major groups belonged to the phyla *Proteobacteria*, *Chloroflexi*, and *Acidobacteria*. The major clone groups from the YBC clone library were related to *S. thermophilum* (10 clones, 90-95%), *Longilinea arvoryzae* (10 clones, 87-89%) in the *Chloroflexi*, and *Pseudolabrys taiwanensis* (6 clones, 92-97%) in the *Alphaproteobacteria*, etc. Among the clostridial groups, two clones related to *C. saccharobutylicum* (99%) were found.

3.2.4. Bacterial diversity in the soil bacterial communities

The numbers of OTUs (at 97% similarity) recognized for the clone libraries showed almost a similar pattern according to the number of clones. Rarefaction analysis based on the OTU clustering showed that the curves for all the treated soils were far from the saturation with lower coverage values and bacterial richness in soil was not recovered for all soil samples (data not shown). The estimates of diversity in the communities (Table 7) showed almost the same and

high levels of bacterial diversities for most of the soil samples.

4.3. Phylogenetic diversity of clostridial groups from both experiments

Since members in the *Clostridia* were the specific group increased in the BSD-treated soils and suggested to play important roles to suppress the pathogens, the compositions of clostridial communities for all BSD soils were compared by constructing a phylogenetic tree consisting of all OTUs assigned to the class *Clostridia* from all (both Exp. 1 and 2) BSD-treated samples (Fig. 3). Most of the OTUs in the class consisted of more than two clones as shown above, and some of OTUs contained only one clone. All the clostridial clones are classified into four clusters (the clusters I, III, IV, and XIVa) (Collins et al., 1994) together with various groups such as *Caloramator*, *Oxobacter*, and *Pelotomaculum*. The result indicates that the clostridial communities were diverse irrespective of the BSD-treatments.

It was revealed that OTUs related to *C. saccharobutylicum*, *C. cylindrosporum*, *C. tetanomorphum*, *C. sufflavum*, *C. xylanovorans*, and *P. schinkii* were recognized in most of the BSD-treated clone libraries as the dominant groups and clones related to *O. pfennigii* were also detected commonly as a relatively minor group.

4. Discussion

In this study, we investigated the potential of *B. juncea* plants as BSD material to suppress the spinach wilt disease. Spinach plants cultivated in all BSD-treated soils with *B. juncea* incorporation showed much lower disease incidences as compared with the non-treated or control plots for both experiments. The results were similar to our previous studies of model experiments (Mowlick et al., 2012, 2013), indicating the effectiveness of BSD using *B. juncea* plants for actual cultivation of spinach in the field conditions.

The exact mechanism for the suppression of soil pathogens in the BSD-treated fields is not yet clearly known. For the *Brassica*-treated fields, ITCs from the tissue damage of *Brassica* leaves have been mentioned as the major causal factors to kill the pathogens (Matthiessen and Kirkegaard, 2006; Mattner et al., 2008). Apart from the function of glucosinolates, studies have demonstrated that the pesticidal activity of *Brassica* spp. is likely due to other factors such as releasing of aldehydes, acids and other sulfur- and nitrogen-containing compounds during plant growth or biomass decomposition in soil (Gamliel and Stapleton, 1993; Bending and Lincoln, 1999). In our preliminary experiments using closed pots containing soil incorporated with *B. juncea* plants, it was shown that volatile bioactive substances were released from soil only at the early period (3-4 days) of the treatment, that is, before the start of active decomposition of

plants by microbes in soil (data not shown). Thus, it was strongly suggested that microbial decomposition of plants during the BSD-treatment for three weeks should be greatly associated with soil disinfestation in addition to the bioactive substances directly released from the plants.

In Exp. 1, covering with sheets (control) reduced the disease incidence as compared with non-treated soil. A moderately reduced condition was developed in the soil during the treatment and it appeared that some anaerobic bacterial groups in the classes *Deltaproteobacteria* and *Clostridia* increased in the soil bacterial community. These changes in the soil condition might affect the population of soil pathogens and bring about the suppression of disease incidence. It is generally thought that BSD-treatment effectively suppresses soil pathogens at lower temperature as compared with solarization. Since the soil temperature under the sheets for Exp. 1 was not so high throughout the treatment (usually lower than 40^{o}C at the highest during daytime), it is not likely that increase in temperature or solarization was the major cause of the suppression of pathogen.

In this study, we investigated various states of BSD-treated soils such as ORP, VFAs accumulation or changes of bacterial community composition, etc. The decrease in ORP (Blok et al., 2000) and accumulation of VFAs such as acetate and butyrate in soil (Momma et al., 2006; Katase et al., 2009) have been pointed out as important aspects of BSD, that are associated with the proliferation of anaerobic fermentative bacteria in soil. We noticed that ORP

values (Exp.1) decreased considerably for the BSD-treated samples as compared with the control and non-treated soils, thereby might stimulate growth and multiplication of anaerobic bacteria. Actually, a large number of anaerobic bacteria including clostridial groups were detected as the closest relatives for the clones from the BSD-treated soil samples of Exp. 1 (N2A and N2B) as well as Exp. 2 (YB and YBa). Although the ratios of the clostridial group populations were not so high as in our previous studies of the model experiments (Mowlick et al., 2012, 2013) especially for Exp. 1, they were also dominating in the clone libraries obtained in this study. Besides, high concentrations of VFAs were also detected from most of the BSD-treated soil samples. Since anaerobic bacteria such as clostridial groups are known to produce VFAs or other compounds during fermentation of substrates for their growth, the detection of VFAs from the BSD-treated soils coincided well with the presence of a large number of clostridial groups in the clone libraries for the BSD-treated soils. Thus, it was shown that incorporation of *Brassica* plants also strongly stimulated growth of the strictly anaerobic clostridial groups in field soil. Of the two *Brassica* plants used as BSD materials in this study, Mustard plants showed a slightly higher disease suppressing effect than Azamina, however, differences in the effects of both plants should be confirmed by further experiments.

Avena sativa, a member of the grass family (*Gramineae*), has been also known to produce bioactive chemical compounds, including phenolics, glycosides, benzoxazinones, and amino

acids, which have mainly allelopathic effects on other plants or soil-borne pests and diseases (Putnam and DeFrank, 1983; Stapleton, 2009). Thus, the *Avena* plants also have the advantage to suppress pathogens in a similar manner as that for the *Brassica* plants, and actually showed effectiveness to suppress the disease in this study. We did not include *Brassica* or *Avena* plants incorporation without polythene covering to see whether the biofumigation of these bioactive plants alone could produce disease reduction comparable to BSD. These should be studied in future as remaining subjects to discriminate between effects of bioactive substances of plants and microbial activities during BSD treatments.

The clone libraries for the non-treated soils NCO (Exp. 1) contained diversified bacterial groups of different phyla and classes as noticed in case of non-treated soils of the previous studies (Mowlick et al., 2012, 2013). Since we obtained almost similar community profiles for the non-treated and control (without biomass) soils in the previous studies as well as Exp. 1 of this study, we did not analyze the communities for the non-treated soil of Exp. 2. However, the bacterial compositions of the control soils N2C (Exp. 1) and YC (Exp. 2) indicated that the soil upon irrigation without plant biomass incorporation kept diverse groups in the bacterial communities in soil with only slight changes, which also coincided with our previous studies of the model experiments.

Most of the closely related clostridial species found in the BSD soil samples such as *C.*

saccharobutylicum, *C. cylindrosporum*, *C. sufflavum*, *C. tetanomorphum*, *C. xylanovorans*, *O. pfennigii*, and *P. schinkii* were also placed as the dominant clone groups for our model experiments (Mowlick et al., 2012, 2013). These diverse clostridial species are known to form various products including VFAs, alcohols or other compounds such as indole or skatole during decomposition of biomass (Macfarlane and Macfarlane, 1995; Rainey et al., 2009; Wiegel, 2009), suggesting the possible role of pathogenic suppression by the fermentation products of the related species in BSD-treated soil in practice. Besides the *Clostridia*, clones closely related to *B. niacini*, *B. circulans*, *B. pycnus*, etc. of the *Bacilli* class were also major groups in the BSD-treated libraries. Since the species in the *Bacillus* have been well known to participate in various enzymatic activities (Wang et al., 2002; Hariprasad et al., 2011), the group may play some roles in the disease control. In the further studies, isolation of bacteria relating to these dominant groups from the BSD-treated fields and their physiological and other metabolic characteristics should be studied to determine the functions in pathogenic suppression.

The YCC and YBC clone libraries showed the increased ratios of clones from the *Firmicutes* phylum, especially from the class *Bacilli*, as compared with that for the library YC, but the *Clostridia* became a minor group even in the library YBC. The results indicated that irrigation without biomass incorporation (YBC) did not maintain the clostridial dominancy even the field soil had harbored diverse clostridial species at large ratios just after the previous BSD-treatment.

A unique group relating to *S. thermophilum* (a new clade within the *Firmicutes* or, possibly, a novel phylum, Beppu and Ueda, 2009) was found to dominate in the libraries YCC and YBC. Similar results were obtained from the analysis of bacterial community for the control soil (without biomass) of our model experiment (Mowlick et al., 2013).

The estimates of diversity indicated a high diversity in the soil bacterial communities after the BSD-treatment even though the percentages of the clones in the *Firmicutes*, especially in the class *Clostridia*, remarkably increased as compared with the non-treated or control soils. Various species from these newly proliferated groups in the BSD-treated soil apparently supported the high bacterial diversity at the species (OTU) level, indicating the abundance of bacterial species in soil for changing the community structures according to circumstances.

In this study, effectiveness of BSD with *B. juncea* plants incorporation in fields can be observed clearly by the suppression of spinach wilt disease, accumulation of VFAs, and increased ratios of the clostridial groups for the BSD-treated libraries from both experiments. Moreover, it was shown that the control treatment (irrigated and polythene covered without plant biomass incorporation) could not fully reduce the plant disease incidence as compared with BSD-treatments. Thus, BSD should be a suitable option to control the soil-borne disease and *B. juncea* as a plant biomass can suppress the spinach wilt disease effectively. Moreover, plant biomass is always necessary to be incorporated for successful functioning of BSD before

cultivation of crops. We isolated many clostridial strains from the BSD-treated soil samples during the study. We are now investigating to clarify the effects of anaerobic bacteria on growth of soil pathogenic fungi using the bacterial isolates relating to the major clostridial clones found in the BSD-treated soils.

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FIGURE LEGENDS

Fig. 1. Time courses of changes during the treatments of Exp. 1 in (a) temperatures of soil (10 cm depth) (Δ, without polythene wrapping; ▲, under polythene wrapping) and air in the greenhouse (◇) and (b) oxidation-reduction potential (ORP) (◆, non-treated; □, control; ●, Mustard-treated; ○, *Avena*-treated).

Fig. 2. Neighbor-joining trees showing the phylogenetic relationships of all OTUs derived from YB (Mustard-treated soil) (a) and YBa (Azamina-treated soil) (b) based on 16S rRNA gene sequences from Exp. 2. Bootstrap values (n = 1,000) above 70% are indicated at branch nodes. The scale bar represents 2% estimated difference in nucleotide sequence position. The name of each clone starts with the clone library designation of both YB and YBa. As the outgroup, *Sulfolobus acidocaldarius* (D14053) (the domain *Archaea*) 16S rRNA gene sequence was used. Accession numbers of the species are shown in the parentheses. Numbers in the parentheses aside each clone name denote the number of clones assigned to the OTU. Each clone name without parenthesis represents one OTU with one clone. The abbreviation *C.* indicates the genus *Clostridium*. Other abbreviations: α-, β-, γ and δ-Prot, *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, respectively; Actino, *Actinobacteria*; Acido, *Acidobacteria*; Gemma,

Gemmatimonadetes, Symbio, *Symbiobacterium*; Bacter, *Bacteroidetes*; Plancto, *Planctomycetes*; Verruco, *Verrucomicrobia*; Chlo, *Chloroflexi*. Designation of clone names with YB and YBa correspond to the YCB and YCBa libraries, respectively, deposited in the DDBJ/Genbank.

Fig. 3. Neighbor-joining tree showing the phylogenetic relationships of clostridial clones (all OTUs from BSD-treated samples of Exp. 1 and 2) based on 16S rRNA gene sequences (according to the clostridial cluster analysis by Collins et al., 1994). As the outgroup, *Bacillus subtilis* DSM10 16S rRNA gene sequence was used. The abbreviation *C*. indicates the genus *Clostridium*. The name of each clone starts with the clone libraries with BSD treatments. N2B and N2A, Mustard- and *Avena*-treated soil, respectively from Exp. 1, whereas YB, Mustard-treated; YBa, Azamina-treated; YBC, fields irrigated and covered again without biomass for YB field from Exp. 2. Tree construction and other notifications are similar as described in Fig. 2.

Fig. 1

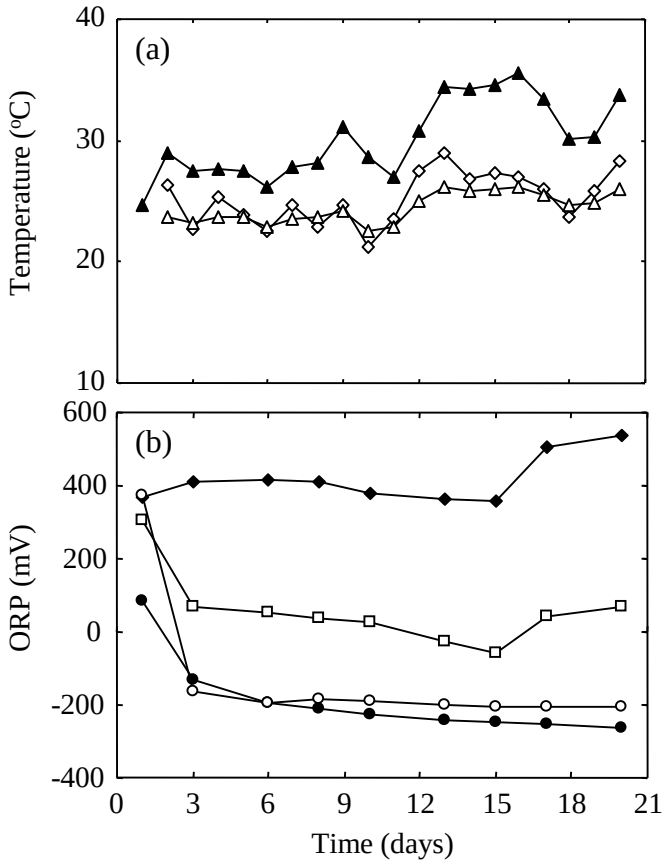
(a)
Temperature (ºC)
40
30
20
10
(b)
ORP (mV)
600
400
200
0
-200
-400
0
3
6
9
12
15
18
21
Time (days)

Fig. 2

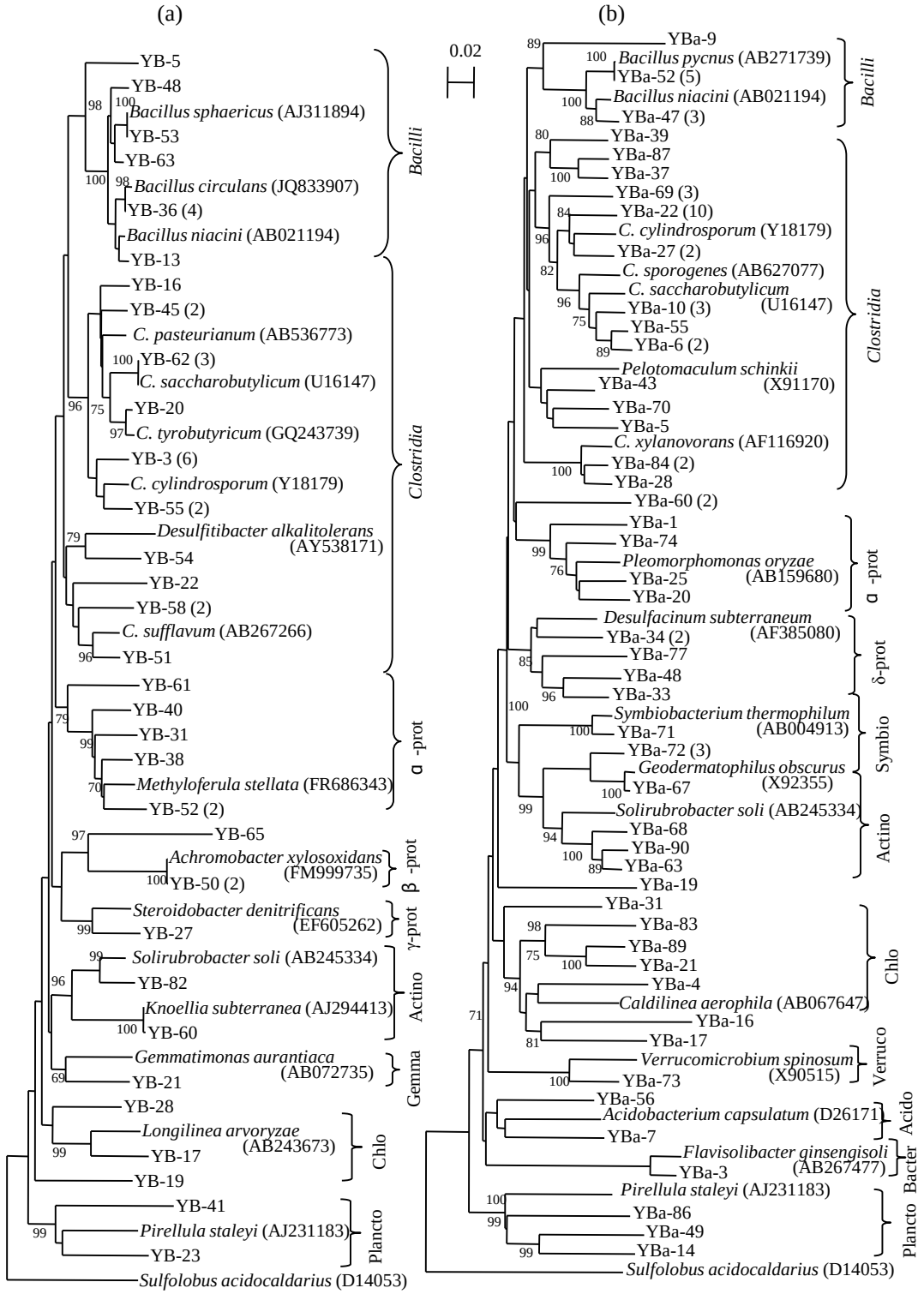
(a)
YB-5
YB-48
Bacillus sphaericus (AJ311894)
YB-53
YB-63
Bacillus circulans (JQ833907)
YB-36 (4)
Bacillus niacini (AB021194)
YB-13
YB-16
YB-45 (2)
C. pasteurianum (AB536773)
YB-62 (3)
C. saccharobutylicum (U16147)
YB-20
C. tyrobutyricum (GQ243739)
YB-3 (6)
C. cylindrosporum (Y18179)
YB-55 (2)
Desulfitibacter alkalitolerans (AY538171)
YB-54
YB-22
YB-58 (2)
C. sufflavum (AB267266)
YB-51
YB-61
YB-40
YB-31
YB-38
Methyloferula stellata (FR686343)
YB-52 (2)
YB-65
Achromobacter xylosoxidans (FM999735)
YB-50 (2)
Steroidobacter denitrificans (EF605262)
YB-27
Solirubrobacter soli (AB245334)
YB-82
Knoellia subterranea (AJ294413)
YB-60
Gemmatimonas aurantiaca (AB072735)
YB-21
YB-28
Longilinea arvoryzae (AB243673)
YB-17
YB-19
YB-41
Pirellula staleyi (AJ231183)
YB-23
Sulfolobus acidocaldarius (D14053)
Bacilli
Clostridia
α -prot
β -prot
γ-prot
Actino
Gemma
Chlo
Plancto
0.02
(b)
YBa-9
Bacillus pycnus (AB271739)
YBa-52 (5)
Bacillus niacini (AB021194)
YBa-47 (3)
YBa-39
YBa-87
YBa-37
YBa-69 (3)
YBa-22 (10)
C. cylindrosporum (Y18179)
YBa-27 (2)
C. sporogenes (AB627077)
C. saccharobutylicum (U16147)
YBa-10 (3)
YBa-55
YBa-6 (2)
Pelotomaculum schinkii (X91170)
YBa-43
YBa-70
YBa-5
C. xylanovorans (AF116920)
YBa-84 (2)
YBa-28
YBa-60 (2)
YBa-1
YBa-74
Pleomorphomonas oryzae (AB159680)
YBa-25
YBa-20
Desulfacinum subterraneum (AF385080)
YBa-34 (2)
YBa-77
YBa-48
YBa-33
Symbiobacterium thermophilum (AB004913)
YBa-71
YBa-72 (3)
Geodermatophilus obscurus (X92355)
YBa-67
Solirubrobacter soli (AB245334)
YBa-68
YBa-90
YBa-63
YBa-19
YBa-31
YBa-83
YBa-89
YBa-21
YBa-4
Caldilinea aerophila (AB067647)
YBa-16
YBa-17
Verrucomicrobium spinosum (X90515)
YBa-73
YBa-56
Acidobacterium capsulatum (D26171)
YBa-7
Flavisolibacter ginsengisoli (AB267477)
YBa-3
Pirellula staleyi (AJ231183)
YBa-86
YBa-49
YBa-14
Sulfolobus acidocaldarius (D14053)
Bacilli
Clostridia
α -prot
δ-prot
Symbio
Actino
Chlo
Verruco
Acido
Bacter
Plancto

Fig. 3

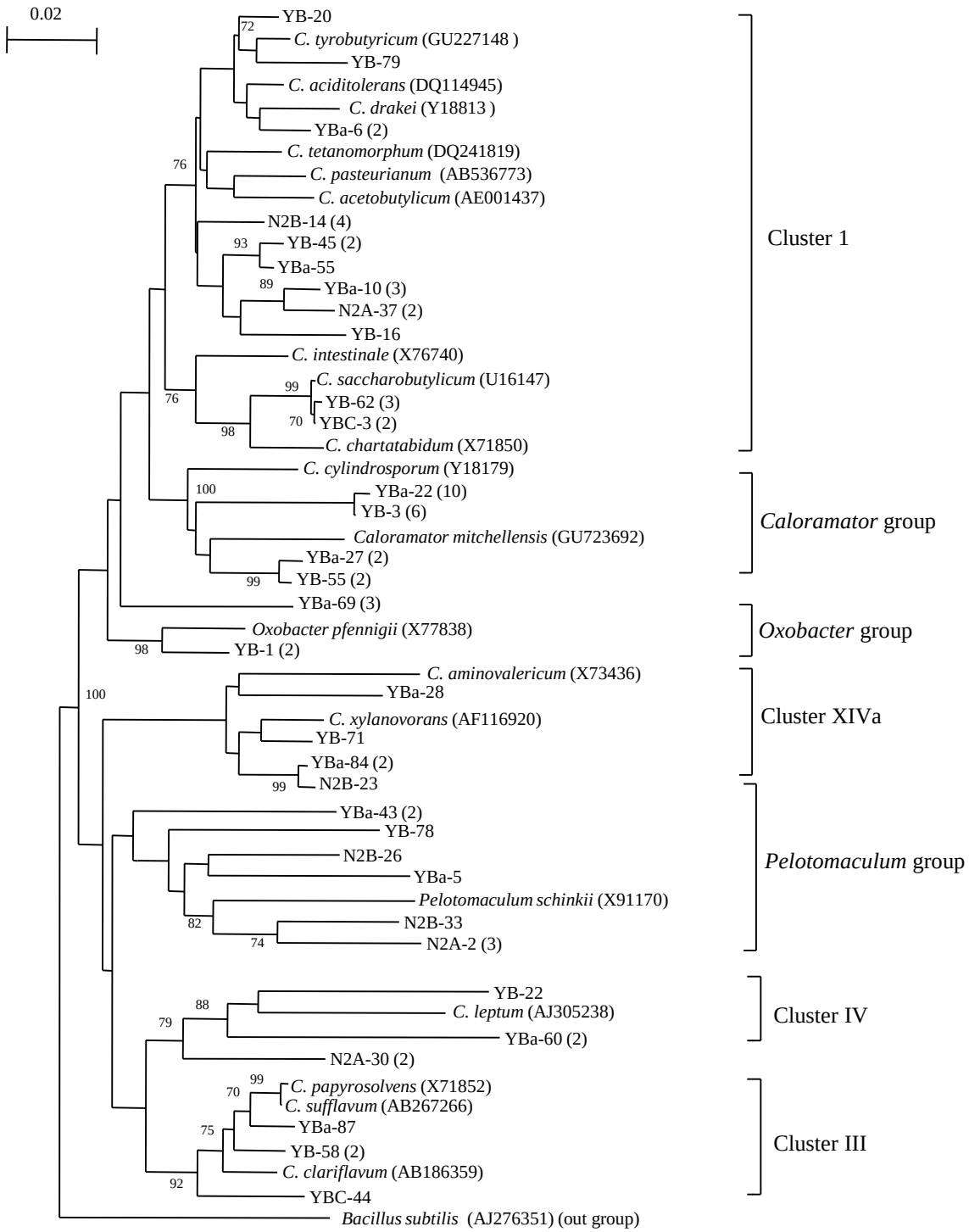
0.02
YB-20
C. tyrobutyricum (GU227148)
YB-79
C. aciditolerans (DQ114945)
C. drakei (Y18813)
YBa-6 (2)
C. tetanomorphum (DQ241819)
C. pasteurianum (AB536773)
C. acetobutylicum (AE001437)
N2B-14 (4)
YB-45 (2)
YBa-55
YBa-10 (3)
N2A-37 (2)
YB-16
C. intestinale (X76740)
C. saccharobutylicum (U16147)
YB-62 (3)
YBC-3 (2)
C. chartatabidum (X71850)
C. cylindrosporum (Y18179)
YBa-22 (10)
YB-3 (6)
Caloramator mitchellensis (GU723692)
YBa-27 (2)
YB-55 (2)
YBa-69 (3)
Oxobacter pfennigii (X77838)
YB-1 (2)
C. aminovalericum (X73436)
YBa-28
C. xylanovorans (AF116920)
YB-71
YBa-84 (2)
N2B-23
YBa-43 (2)
YB-78
N2B-26
YBa-5
Pelotomaculum schinkii (X91170)
N2B-33
N2A-2 (3)
YB-22
C. leptum (AJ305238)
YBa-60 (2)
N2A-30 (2)
C. papyrosolvens (X71852)
C. sufflavum (AB267266)
YBa-87
YB-58 (2)
C. clariflavum (AB186359)
YBC-44
Bacillus subtilis (AJ276351) (out group)
Cluster 1
Caloramator group
Oxobacter group
Cluster XIVa
Pelotomaculum group
Cluster IV
Cluster III

Table 1. VFAs concentrations detected from differently treated samples[a]

Plant biomass incorporated	Weeks of treatment	VFAs (mmol/l)[b]		
		Acetate	Propionate	Butyrate
Brassica (Mustard)	1	15.5	1.0	1.3
	2	17.0	1.6	1.0
	3	3.8	1.7	0.3
Avena	1	4.9	0.4	-[c]
	2	-	0.2	-
	3	-	1.7	-

[a] No VFAs was detected from Control (irrigated and polythene covered witl biomass) and non-treated soil. [b] Means of triplicate samples. [c] Not detected.

Table 2. Wilt disease incidence of spinach cultivated in differently treated soil for Exp. 1

Treatment	Wilt disease incidence (%)[a]
Non-treated	41.5 ± 7.7
Control	14.0 ± 1.5
Brassica (Mustard)	5.2 ± 1.5
Avena	3.9 ± 0.6

[a] Mean ± SD.

Table 3. Composition profiles of phylogenetic groups of bacteria based on 16S rRNA gene sequences from clone libraries of Exp. 1.

Phylum or Class	Clone library[a] (% of relative abundance)			
	NCO	N2C	N2B	N2A
Alphaproteobacteria	24.4	15.3	8.3	8.3
Betaproteobacteria	5.4	5.6	-[b]	5.6
Gammaproteobacteria	2.7	4.2	13.9	8.3
Deltaproteobacteria	-	12.5	2.8	2.8
Acidobacteria	5.4	5.6	8.3	2.8
Verrucomicrobia	5.4	5.6	2.8	16.7
Bacteroidetes	13.5	4.2	8.3	11.1
Planctomycetes	2.7	2.8	2.8	-
Firmicutes (*Clostridia*)	2.7	8.4	22.2	19.4
Firmicutes (*Bacilli*)	2.7	4.2	5.6	2.8
Gemmatimonadetes	2.7	1.4	-	2.8
TM7	8.1	2.8	-	2.8
Actinobacteria	10.8	15.3	13.9	2.8
Chloroflexi	2.7	1.4	5.6	8.3
Others	10.8	8.4	5.6	5.6

[a] NCO, non-treated soil; N2C, control soil (control (irrigated and polythene covered without biomass); N2B, *Brassica* (Mustard)-treated soil; N2A, *Avena* -treated soil. [b] Not detected.

Table 4. VFAs accumualtion for the soil samples of Exp. 2

Soil sample[a]	VFAs (mmol/l)		
	Acetate	Propionate	Butyrate
YC	-[b]	-	-
YB	4.5	1.9	2.7
YBa	9.0	2.6	2.3
YCC	-	-	-
YBC	-	-	-
YBaC	0.3	0.2	-

[a] YC, control (irrigated and polythene covered without biomass); YB, BSD (Mustard)-treated; YBa, BSD (Azamina)-treated; YCC, YBC, and YBaC, fields irrigated and covered again without biomass for YC, YB, and YBa fields, respectively. [b] Not detected.

Table 5. Wilt disease incidence and yields of spinach cultivatede in the treated fields for Exp. 2

Soil smaple[a]	Wilt disease incidence (%)[b]	Yield of spinach (g/m^2)[b]
YC	58.8 ± 37.3	858 ± 601
YB	7.5 ± 8.5	4,556 ± 717
YBa	14.7 ± 12.1	4,425 ± 599
YCC	55.0 ± 20.1	1,484 ± 859
YBC	31.0 ± 25.6	2,265 ± 820
YBaC	65.0 ± 20.1	416 ± 188

[a] YC, control (irrigated and polythene covered without biomass); YB, BSD (Mustard)-treated; YBa, BSD (Azamina)-treated; YCC, YBC, and YBaC, fields irrigated and covered again without biomass for YC, YB, and YBa fields, respectively. [b] Mean ± SD.

Table 6. Composition profiles of phylogenetic groups of bacteria based on 16S rRNA gene sequences from clone libraries of Exp. 2.

Phylum or class	Clone library[a] (% of abundance)				
	YC	YB	YBa	YCC	YBC
Alphaproteobacteria	9.1	10.4	5.6	10.0	17.7
Betaproteobacteria	9.1	4.5	-[b]	6.7	3.8
Gammaproteobacteria	7.6	2.9	-	-	1.3
Deltaproteobacteria	9.1	-	6.9	2.2	2.5
Acidobacteria	13.6	-	2.8	8.9	6.3
Verrucomicrobia	3.0	1.5	1.4	3.3	1.3
Bacteroidetes	6.1	-	1.4	-	3.8
Planctomycetes	4.5	4.5	4.2	4.4	2.5
Firmicutes (*Clostridia*)	3.0	38.8	44.4	1.1	7.6
Firmicutes (*Bacilli*)	6.1	25.4	12.5	16.7	14.0
Firmicutes (*Symbiobacterium*)	-	-	1.4	17.8	12.7
Gemmatimonadetes	6.1	1.5	-	7.8	-
Actinobacteria	12.1	4.5	9.7	6.7	2.5
Chloroflexi	9.1	1.5	8.3	3.3	21.5
Others	1.5	4.5	1.4	11.1	2.5

[a] YC, control (irrigated and polythene covered without biomass); YB, BSD (Mustard)-treated; YBa, BSD (Azamina)-treated; YCC and YBC, fields irrigated and covered again without biomass for YC and YB fields, respectively. [b] Not detected.

Table 7. Estimates of bacterial diversity from the field samples of Exp. 2

Soil sample[a]	YC	YB	YBa	YCC	YBC
No. of total clones	66	67	72	89	79
No. of total OTUs	45	43	46	43	46
Coverage (%)	57.5	56.7	51.4	73	63.3
Shimpson's diversity index	0.98	0.98	0.97	0.96	0.98
Shannon-Wiener diversity index	5.32	5.17	5.14	5.05	5.25

[a] YC, control (irrigated and polythene covered without biomass); YB, BSD (Mustard)-treated; YBa, BSD (Azamina)-treated; YCC and YBC, fields irrigated and covered again without biomass for YC and YB fields, respectively.