Usefulness of Japanese radish residue in biological soil disinfestation to suppress spinach wilt disease accompanying with proliferation of soil bacteria in the *Firmicutes*

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Abstract

Biological soil disinfestation (BSD) is an effective method to suppress soil-borne plant diseases by incorporation of plant biomass into soil under reduced, anoxic condition. Usefulness of Japanese radish (daikon) residue as plant biomass for BSD was investigated by both model and field experiments in comparison with the effects of *Brassica juncea* plants or wheat bran. Considerable amounts of acetate together with minor amounts of propionate and butyrate were detected from the radish-treated soils at similar levels with those in soils treated with *B. juncea* plants or wheat bran. BSD treatments with radish residue reduced spinach wilt disease incidence in both model and field experiments. When the BSD-treated soil was treated again with irrigation and covering without biomass before next cropping, however, wilt disease was hardly suppressed. Clone library analysis based on 16S rRNA gene sequences was carried out to determine the changes in the bacterial community compositions in the treated soil samples. The analyses showed that the bacterial communities in the radish-treated soils were dominated by members of the classes *Clostridia* and *Bacilli* of the phylum *Firmicutes* in both experiments. The clostridial groups detected were diverse and the major operational taxonomic units (OTUs) were closely related to *Clostridium saccharobutylicum*, *Clostridium sufflavum*, *Clostridium xylanovorans*, and *Oxobacter pfennigii*, which had been commonly detected as the dominant groups in BSD-soils treated with *B. juncea* plants or wheat bran in our previous studies. The

dominant clone groups belonging to the *Bacilli* class were closely related to several species such as *Bacillus niacini*, *Bacillus circulans*, and *Bacillus pycnus*. Dominancy of the *Bacilli* groups seemed to increase when radish residue was repeatedly applied as BSD material.

Keywords

Anaerobic bacteria; Biological soil disinfestation (BSD); Japanese radish (Daikon); *Firmicutes* group; *Fusarium oxysporum*, Spinach wilt

Abbreviations

BSD, biological soil disinfestation; ITC, isothiocyanate; OTU, operational taxonomic unit; PCR-DGGE, polymerase chain reaction-denaturing gradient gel electrophoresis; VFAs, volatile fatty acids.

1. Introduction

Public demands to protect the global environment have stimulated research to reduce the use of pesticides and to find environmentally friendly and cost effective alternatives for controlling plant diseases. In this context, biological methods without using chemicals offer attractive ways

to suppress plant pathogens. Biological soil disinfestation (BSD) is a method developed mainly in the Netherlands (Blok et al., 2000; Messiha et al., 2007) and Japan (Shinmura, 2004; Momma, 2008) as an ecological alternative to chemical fumigation. BSD requires incorporation of plant biomass into field soil together with excess irrigation water before the start of cropping. The soil surface is then tightly covered with polythene sheets to maintain anoxic soil condition for about three weeks (Shinmura, 2000, 2004). Crops can be cultivated after this period upon removal of the sheets and plowing of soil. It has generally been thought that bioactive substances released from plant biomass incorporated into soil as well as various compounds produced by microbes in soil during decomposition of plants synergistically suppress soilborne plant pathogens. Among plant biomass sources, the *Brassicaceae* plants have been used as promising BSD materials or biofumigants (Sarwar and Kirkegaard, 1998; Goud et al., 2004). Most of the *Brassicaceae* plants may release isothiocyanates (ITCs), substances that are toxic to soilborne pests and pathogens, by the myrosinase activity to hydrolyse glucosinolates commonly present in the *Brassicaceae* plant tissues (Sawar and Kirkegaard, 1998; Fahey et al., 2001).

Spinach (*Spinacia oleracea* L.) is an important vegetable crop in many countries that is mainly cultivated from seed sowing. In case of direct seed sowing, BSD seems to be more advantageous as seeds sown in the field may often be prone to attack by a number of soilborne diseases during their establishment. Spinach cultivation is greatly hampered by many fungal

soilborne diseases. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *spinaciae* has been reported as the most serious disease of spinach in Japan (Muslim et al., 2003; Horinouchi et al., 2010). The disease causes damping-off, wilting, root rot, vascular discoloration, and death of seedlings as well as mature spinach plants (Hungerford, 1923; Larsson and Gerhardson, 1992).

In our previous studies with BSD treatments using model experiments (Mowlick et al., 2012; 2013a), *Brassica juncea* plants, a species of the *Brassicaceae*, as well as wheat bran and *Avena sativa* plants were incorporated into soil to determine the effects on pathogenic suppression and soil bacterial community development. The BSD treatments successfully controlled populations of the pathogens *F. oxysporum* f. sp. *lycopersici* (wilt pathogen of tomato) and *F. oxysporum* f. sp. *spinaciae* (wilt pathogen of spinach). Furthermore, *B. juncea* plants were effectively used in field experiments as BSD material to combat spinach wilt (Mowlick et al., 2013c). Using molecular analyses such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and clone library methods based on 16S rRNA gene sequences, we observed that bacterial communities in these BSD-treated soil samples were greatly changed and anaerobic groups, especially in the class *Clostridia* of the phylum *Firmicutes*, became major bacterial groups in the communities together with some other aerobic or facultatively anaerobic bacteria in the classes *Bacilli* and *Gammaproteobacteria*. It

seemed that the activities of these bacterial groups that proliferated in BSD-treated soil strongly affected the survival and growth of pathogens in the soil.

In this study, we intended to determine the efficacy of plant biomass from the family *Brassicaceae* when incorporated into soil as BSD material. Radish (*Raphanus sativus* L.), belonging to the family *Brassicaceae,* is an important vegetable crop in the world that is consumed in China, India, Japan, Korea, the European countries, and America as raw (salad), cooked, brined and fermented (pickled), and dried forms. In Japan, many varieties of *R. sativus* have been widely cultivated. Especially, Japanese radish (*R. sativus* var. *longipinnatus*), commonly known as "daikon" in Japanese (that is, "large root") is cultivated all over Japan as one of the most popular vegetables with high production. A daikon plant develops a white thick root (composed of a hypocotyl and a main root, 20-35 cm long and about 7-8 cm in diameter) and radical leaves (about 20 pinnate compound leaves with more than 50 cm long rachis). Young leaves of daikon are also cooked as a brightly-colored, nourishing vegetable however, the roots are mainly used in various popular foods (Morgan and Midmore, 2003). As a Brassicaceous crop, daikon plants or their products also contain glucosinolates or ITCs (Diana et al., 1985; Okano et al., 1990). In fact, grated raw roots of daikon, called "hot daikon" in Japan, have a very hot or pungent taste. Due to larger size of the roots and leaves, a vast amount of radish products can be obtained every year in Japan. Thus, a lot of radish residue is wasted after

collecting marketable plants from the fields. Furthermore, most of the leaves of grown daikon are usually discarded during harvesting and transportation. Hence, the radish plants seem to be very promising as material for BSD treatments and may have greater possibilities to be used in controlling soilborne diseases. Besides, the availability of radish seeds in the market, the ease of growing radish, and the rapid growth of radish plants may increase the potential of using radish biomass as BSD material.

The objective of this research was to determine the usefulness of Japanese-radish (daikon) residues as BSD treatments in suppressing spinach wilt disease in model and field experiments. Changes in soil bacterial community composition due to radish incorporation were also investigated by clone library analysis. Furthermore, repetition of BSD treatment in the same fields was also investigated to evaluate the persistence of BSD effects on spinach wilt and soil bacterial community structures.

2. Materials and methods

2.1. A model experiment of BSD using pots and plastic bags

A model experiment of BSD using both pots and closed plastic bags was conducted in

Agricultural Research Centre, Yamaguchi, Japan (34.9°N, 131.3°E) during 2010. A total of five treatments were assigned in this experiment using a completely randomized design with four replications. Soil group was gray lowland (sandy loam). Roots of daikon (cv. *Yakushakoiki*), as well as *B. juncea* plants and wheat bran were used as BSD material, and the effects on suppression of wilt disease of spinach were compared. Fresh daikon (radish) roots were broken into pieces (about 1 cm^3) with a food processor and *B. juncea* plants harvested before the flowering stage were cut into pieces (about 1 cm) with a cutter before application. Radish roots, *B. juncea* plants, and wheat bran were applied to soil at the rates of 10, 5, and 2 kg/m^2, respectively. For control treatment, no plant biomass was incorporated into soil. *F. oxysporum* f. sp *spinaciae* YSF-1 (wilt pathogen of spinach) cultivated in Komada's *Fusarium*-selective medium (Komada, 1975) was incorporated into soil at about 10^4 CFU/g of dry soil.

Pots (16.0-17.5 cm in diameter or 0.02 m^2 with 19.8 cm in height, four pots for each treatment) were filled with each treated soil (working volume 3.3 L) prepared as described above and the soil was irrigated. The amount of water for irrigation (the ratio of soil and water, 4.3:1.2) was determined based on our previous BSD field experiments (Mowlick et al., 2013c). Each pot was kept airtight throughout the treatment by covering with a double layer of transparent sheets with low gas permeability (Barrier Star film, TOKANKOUSAN Co. LTD; Sky Coat film, C.I. KASEI Co. LTD). The pots were placed in a greenhouse (2 June) without

disturbance during the treatment in ambient temperature. The sheets were removed after three weeks and the pots were kept open to dry the soil. Thereafter, the pots were prepared for cropping and spinach (cv. *Summer Top*) was seeded (9 July) in all pots (15 seeds/pot) placed in the same greenhouse. Spinach was also seeded in pots filled with original soil, without any treatment but inoculated with the pathogen. After seedlings emergence, the pots were inspected regularly and intercultural operations such as watering, weeding and insect control were done when necessary following common agricultural practices in the area. Wilt disease incidence was recorded in all pots after a month (6 August). Temperature in pot soil was recorded by a data logger (Ondotori, T&D Corp.) during the soil treatment.

Plastic bags (17 cm × 11 cm × 3.3 cm) were used for sampling of soil during the BSD treatments under the same conditions. Each bag was filled with soil (430 g), treated in the same way as for pot soil as described above, watered (120 mL), and sealed tightly to create anoxic conditions in the bag. The treated bags were placed in the same greenhouse at the same time as the pots (2 June). The bags were opened (one for each condition) and soil was sampled at weekly intervals for four weeks in all treatments. Soil samples collected were kept in a freezer (-20^{o}C) immediately after the sampling and preserved there until use. The bags once opened and used for soil sampling were discarded and other bags incubated under the same treatment conditions without opening were used for the next sampling.

2.2. A BSD experiment in a field greenhouse using radish residue

A BSD experiment using radish residue was carried out in a greenhouse located in a research field at the Agricultural Research Centre, Yamaguchi, Japan. The soil was gray lowland soil (sandy loam) and the size of each treatment plot was 1.5 × 5.5 m^2 distributed in a randomized complete block design with three replications. Spinach had been continuously cultivated in the greenhouse and natural infection of spinach by Fusarium wilt disease had occurred. Nonmarketable roots of daikon (cv. *Natsutsukasa*) were collected from a vegetable sorting house before shipping. Most of the above ground parts (leaves) were usually removed and discarded before collection of radish roots from fields. But the bottom (about 20 cm) of rachises of compound leaves was left on each root. Therefore, the above ground parts were also used as BSD material. The radish residue collected was broken into pieces by a hammer knife mower and incorporated into soil (about 15-20 cm depth) by a rotary tractor at a rate of 20 kg/m^2. The soil in the greenhouse was treated three times by BSD and spinach was cultivated after each treatment during 2009-2010 as described below. The plot names were designated according to the name of the place (Yamaguchi), control or types of biomass (C, Control; R, Radish; B, *Brassica*/Mustard; Ba, *Brassica*/Azamina).

1) The first treatment: For the first BSD treatment and cropping of spinach, radish residue was applied as described above together with enough irrigation water (more than 80-100 L/m^2) and the soil surface was covered with sheets (16 September 2009) (YR plot). As the control treatment (Control 1), soil was irrigated with the same amount of water and covered with sheets without biomass incorporation. The greenhouse was closed during the treatment to maintain ambient temperature and humidity. After three weeks (7 October 2009), the sheets were removed and the treated plots were dried and plowed. Spinach (cv. *Trad*) was seeded using a seeding machine (Gonbe, Mukai Kogyo Inc.) with an inter-row spacing of 10 cm and intra-row spacing of 16 cm resulting in 63 plants/m^2 (14 October 2009). Plants were watered as needed (about 10 minutes in the morning). Weeds were pulled by hand until two weeks after seeding and insect pests were mainly controlled using insect screens (4-mm mesh size) set at all openings of the greenhouse. Insecticides were used when need arose. Occurrence of wilt disease was monitored during growth of the plants and yields were recorded after about a month (17 November).

2) The second treatment: The YR plot in the first treatment was divided into four plots for the second treatment. Since production of daikon in this district is generally decreased from January to May, it was difficult to obtain enough amounts of radish residue for the next BSD treatments. Therefore, two different varieties of *B. juncea*, that is, *B. juncea* var. *cernua*

(Mustard greens) and *B. juncea* var. *crispifolia* (Azamina, one of the green vegetables commonly cultivated in this district) were cultivated after the first cropping of spinach (from 30 November 2009). After about four months of cultivation (8 April 2010), the *Brassica* plants were used as BSD material in the second treatment. Both *Brassica* plants harvested were cut into pieces by a hammer knife mower and immediately incorporated into the plots (YRB for Mustard greens and YRBa for Azamina, respectively) with a rotary tractor at the rate of 9.6 kg/m^2. After irrigation, the soil surface was covered with sheets as described above. For YRC plots, both cultivated *Brassica* plants were removed after harvest without incorporation into soil (YRC-B for Mustard greens cultivated plot and YRC-Ba for Azamina cultivated plot). Control 1 plot of the first treatment was used for the second control treatment (Control 2) and *Brassica* plants were also cultivated after spinach cultivation, but the plants were removed in the same way as those for YRC plots. Soil samples were collected from all plots after 3 weeks (30 April 2010). For each soil sample, 100 g of soil was obtained from the upper 10 cm soil depth in triplicate and preserved in a similar way as described for the model experiment. Spinach (cv. *Active*) was seeded and cultivated (10 May-17 June) in the treated plots in a similar way as mentioned above.

3) The third treatment: After spinach cultivation in the YRB and YRBa plots, soil was again treated with radish residue and sheet-covering as the third treatment (YRBR and YRBaR),

respectively (25 June). In case of other treatments (Control 3, YRCC-B, and YRCC-Ba), the soil was irrigated without biomass incorporation and covered. Soil samples were also collected from the plots after three weeks of treatment (16 July). Spinach (cv. *Summer Top*) was cultivated (22 July-9 September) in these treated plots. The second and third treatments as well as cropping of spinach were carried out in parallel with the field experiment (Exp. 2) described previously (Mowlick et al., 2013c).

Pellet-type organic fertilizer containing fish meal (Kumiai Ube Yuki 100: N, 70 g/kg; P_2O_5, 40 g/kg; K_2O, 10 g/kg) (MC FERTICOM, Co., LTD) was used as preplanting fertilizer to supply nitrogen (20 g/m^2) in each plot. The amounts of fertilizer applied were determined based on the nitrogen content measured by the microdiffusion method using samples extracted from each plot with 2 M KCl. Additional fertilizer was not applied throughout the cultivation. The soil and air temperatures inside the greenhouse during the treatments were recorded by data loggers. For all plots, natural wilt disease incidence was recorded based on the observation of plants in 10 different locations (each measuring 0.1 × 1 m^2) in 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.

2.3. Determination of volatile fatty acids (VFAs) concentrations in soil

The concentrations of volatile fatty acids (VFAs) in the soil samples collected were measured by gas chromatography (Hitachi G-3900) as described previously (Mowlick et al., 2012; 2013c).

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

All procedures were conducted as described previously (Mowlick et al., 2013c). For soil DNA extraction, each composite sample (3 g) from triplicate soil samples was used following the instruction of 'Ultra Clean™ Soil DNA Isolation Kit'. PCR amplification of bacterial 16S rRNA genes was done 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').

Molecular clone library analyses (Maidak et al., 1999) based on 16S rRNA gene sequences were carried out to determine the bacterial community compositions in the soil samples as described previously (Mowlick et al., 2013a). Nucleotide sequencing (about 600 bp) was carried out for a total of 96 clones using a sequence primer U515f (5' GTG YCA GCM GCC GCG GTAA-3') according to the Dye Terminator method (capillary sequencer at Takara Co. Ltd.).

2.5. Data analysis

Disease incidence and yield data were analyzed using MSTAT-C statistical software (Nissen, 1983) and mean separation was conducted according to Duncan's multiple range test (DMRT). Database searches for related 16S rRNA gene sequences were carried out with the 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. Phylogenetic trees were made using the neighbor-joining method (Saitou and Nei, 1987) with the Njplot program in the ClustalW package (Thompson et al., 1994). Construction of OTUs (operational taxonomic unit at 97% similarity level), bootstrap resampling analysis, chimera checking, and rarefaction analysis were carried out as described previously (Mowlick et al., 2013c). Coverage of the clone libraries including the diversity indexes was calculated using an online biodiversity calculator (http://www.alyoung.com/labs/biodiversity_calculator.html).

2.6. Accession numbers of nucleotide sequence

The nucleotide sequences obtained from the clone library analyses have been deposited in DDBJ/GenBank under the accession numbers AB745762-AB746033 (271 entries) for the model

experiment and AB744468-AB744648 (180 entries) for the greenhouse experiment.

3. Results

3.1. The model experiment

3.1.1. Soil status during treatment

Soil temperature in the pots changed with large diurnal changes, and the average, maximum, and minimum temperatures during the treatments were 27.3ºC, 39.4ºC (in the daytime), and 19.7ºC (in the night), respectively. The cumulative soil temperature was 573ºC. Changes in concentrations of VFAs in the treated soil were determined by using the soil samples collected from the closed bags (Fig. 1). No VFAs were detected from the original soil as well as the control soil throughout the treatment. Considerable amounts of VFAs were detected from all BSD-treated soil samples. Acetate was the major component followed by butyrate and propionate for most of the treated soils, and the amounts detected were comparatively higher during the three weeks of treatments than other incubation periods. The changes in the concentrations of VFAs in the radish-treated soil showed a similar pattern as those in the

Brassica-treated soil with the highest concentrations of acetate at about 13-14 $mmolL^{-1}$. Butyrate was detected at a higher concentration in case of wheat bran-treated soil (up to 22 $mmolL^{-1}$).

3.1.2. Effects on spinach wilt disease of BSD using radish in pot soil

Spinach was cultivated in the treated pot soil and wilt disease incidence was determined. All spinach plants were affected by the disease (100% incidence) in both original and control pots, whereas all BSD-treatments reduced the incidence to 15-32% (Table 1). Radish biomass was determined to be as effective BSD material for suppression of spinach wilt as *Brassica*- and wheat bran-treatments.

3.1.3. Bacterial community structures based on clone library analysis

The bacterial community compositions in soil during the treatments were subjected to clone library analysis using the soil samples collected from the closed bags at one week of the treatments. Table 2 shows the phylogenetic affiliations of the clones from these soil samples based on percentages of abundance. The clone library for the control soil (without biomass

incorporation) consisted of diverse bacterial groups allocated mainly to the phyla *Acidobacteria* (about 34% of the total number of clones) and *Proteobacteria* (30%). On the other hand, the library of radish-treated soil sample showed substantial differences as compared with the control library and contained members of the *Firmicutes* phylum (at similar ratios of those in the *Clostridia* and *Bacilli*) as the dominant and major groups in the community. The most dominant clone groups belonging to the *Firmicutes* phylum from this BSD library were closely related to *Clostridium saccharobutylicum* (8 clones, 95-99% sequence similarity with the closest one) and *Bacillus niacini* (10 clones, 99-100%). Other phylogenetic groups of this library detected were from the phyla *Proteobacteria*, *Actinobacteria*, and *Chloroflexi*. For other two BSD libraries (*Brassica*- and wheat bran-treated soil), members of the *Firmicutes* phylum including both the classes *Clostridia* and *Bacilli* were also dominant groups in the communities in consistence with our previous studies using these BSD materials (Mowlick et al., 2012, 2013a,b,c). Thus, it was shown that radish residue brought about similar changes in the soil bacterial community as those brought about by *Brassica* plants or wheat bran biomass.

3.2. The field experiment

3.2.1. Temperature and soil status

For the first treatment, air temperature in the greenhouse was monitored. The average, minimum, and maximum temperatures were 33.1, 21.4, and 44.8ºC, respectively. The average air (in the greenhouse) and soil (10 cm depth) temperatures during the second treatment were 18.1 and 21.7ºC, respectively, with great daily fluctuations (the cumulative soil temperature, 442ºC). For the third treatment, the average air temperature was 30.7 and the soil temperature (10 cm depth) was 35.7ºC (the cumulative soil temperature, 750ºC). VFAs concentrations were determined for the soil samples obtained at the ends of the second and the third treatments (Table 3). No VFAs were detected in Control 2 and 3 plots. All BSD-treated soils contained considerable amounts of VFAs (acetate as the major VFA followed by butyrate and propionate as minor components). Almost the same concentrations of VFAs were detected from both soil samples of YRB and YRBa as well as YRBR and YRBaR. The amounts of acetate and butyrate were almost more than double in the third BSD treatments by radish incorporation as compared with those in the second treatments using *Brassica* plants. When the nitrogen contents in field soil immediately after the radish-treatments were measured to determine the application amount of fertilizer, it appeared that the soil contained more than 15 g/m^2 of nitrogen (mainly ammonium nitrogen) (data not shown), which was enough for the next spinach cropping without application of the fertilizer.

3.2.2. Wilt disease incidence and yield of spinach

For the first treatment, severe spinach wilt disease did not occur even in the control plot, but radish-BSD resulted in complete suppression of the disease (Table 4). For the second cropping, the incidence of wilt in the control plot increased significantly (44.5%) and it was also rather high for YRC plots (YRC-B and YRC-Ba), although the levels were lower than that of Control 2 plot. BSD treatments with incorporation of *B. juncea* plants (YRB and YRBa) remarkably reduced spinach wilt (3-6% incidence) and large amounts of marketable products (4000-4800 g/m^2 yield) were obtained from these plots. As for the third cropping, severe wilt disease broke out in the three plots without biomass incorporation (Control 3, YRCC-B, and YRCC-Ba). In the case of BSD-treated soil using radish residue, wilt incidence was rather low, especially for the YRBR plot. Spinach yields were higher in both BSD plots (YRBR and YRBaR) as compared with those of the three plots without biomass incorporation. Thus, the BSD plots without biomass incorporation hardly suppressed the outbreak of spinach wilt in the next cropping. It was also shown that repetition of irrigation without biomass incorporation (Control 2 and Control 3) had no effect on disease suppression.

3.2.3. Clone library analysis for the radish biomass-treated soil samples

Out of the soil samples obtained, six samples were used for analysis of bacterial community compositions (Table 5). The clone library Control 2 showed much diversified populations of various phylogenetic groups from the phyla *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, and others. For the BSD clone library YRB, members of the phylum *Firmicutes* especially from the class *Bacilli* (44%) were exceedingly dominant with 20% from the *Clostridia*. Besides, the clone library YRBa showed the clones belonging to the *Bacilli* (48%) and *Clostridia* (26%) almost at a similar ratio as detected in the library YRB. The phylogenetic trees for the YRB and YRBa clone libraries (Fig. 2A, B) showed a similar type of closely related species of the dominant clone groups from the *Clostridia* and *Bacilli* with some exceptions. The dominant clostridial groups were closely related to *C. saccharobutylicum*, *Clostridium xylanovorans*, and *Oxobacter pfennigii*, whereas those related to *B. niacini*, *Bacillus circulans*, and *Bacillus pycnus* were from the class *Bacilli*.

In the clone library Control 3, diversified bacterial populations were also detected, but some changes were observed in the *"Symbiobacterium*" clade and the *Bacilli* class in the *Firmicutes* as well as in the phyla *Acidobacteria* or *Bacteroidetes* in comparison with those in Control 2. When BSD was repeated by radish incorporation (the clone library YRBR), the ratio of clones

from the *Bacilli* was much higher (70%) as compared with that of the *Clostridia* (19%). The phylogenetic tree for the clone library YRBR (Fig. 3A) demonstrated that dominant clone clusters were related with *B. pycnus*, *B. niacini*, and *Paenibacillus ruminocola* from the class *Bacilli* as well as *C. saccharobutylicum*, *C. xylanovorans*, and *Clostridium sufflavum* from the *Clostridia*. For the clone library YRBaR, almost all members in the library were from the classes *Clostridia* (63%) and *Bacilli* (33%). The phylogenetic tree for the YRBaR clone library (Fig. 3B) showed an exceeding proliferation of clone groups related to *C. saccharobutylicum*, *C. xylanovorans*, and *Thermoanaerobacterium saccharolyticum* from the *Clostidia* as well as *B. niacini*, *B. circulans*, and *B. pycnus* from the *Bacilli*.

3.2.4. Bacterial diversity of the clone libraries

Rarefaction analysis based on OTU clustering (Fig. 4) showed that the curves for the control soils (Control 2 and 3) were far from saturation with lower coverage values. The curves for YRB and YRBa overlapped, whereas those for the YRBR and YRBaR libraries seemed to reach near the plateau. The number of OTUs (at 97% similarity) declined greatly for the clone libraries YRBR and YRBaR as compared with those of the YRB and YRBa libraries (Table 6). The estimates of diversity in the communities demonstrated that radish biomass incorporation in

the repeated BSD decreased bacterial diversity in soil.

4. Discussion

Addition of various radish biomass in soil has been reported to reduce soilborne diseases of onion, potato, celery, and other solanaceous vegetables together with increase in crop yields (Larkin and Griffin, 2007; Justo et al., 2008, Anita, 2012). In this study radish biomass incorporation greatly suppressed spinach wilt in both model and greenhouse experiments indicating that Japanese radish was an effective biomass in suppressing the disease. Harmful effects of radish incorporation on growth of spinach were not observed throughout the experiments from every possible aspect. Furthermore, it was shown that incorporation of radish into soil also input nutrients necessary for growth of plants at least in the next cropping.

In addition to the experiments presented here, we have carried out many BSD experiments in greenhouses. The results obtained suggested that if the cumulative time of soil temperature (10 cm depth) at higher than 30^{o}C reached more than 250-280 hours during the treatments, it was very promising to succeed in suppression of soil pathogens. It was also indicated that soil temperature higher than 40^{o}C (200 hours of cumulative time) by solarization effects should enhance suppression of pathogens. Concerning the field experiments in this study, soil

temperature for the third cropping kept higher than 30°C for most of the period of treatment (cumulative time of more than 350 hours). For the first cropping, soil temperature might be changed similarly judging from the air temperature. Meanwhile, for the second cropping, although the soil temperature was lower than 30°C for most of the period and the cumulative soil temperature was much lower than that of the third cropping, the BSD treatment suppressed wilt disease effectively. The results indicate that although it is desirable to keep high soil temperature to succeed in BSD, the method is also applicable even during colder periods with appropriate treatments to establish sufficient reduced condition in soil.

Goud et al. (2004) found that the suppressive effect of BSD was maintained for several years to control verticillium wilt disease of perennial crops. However, most plants may be more sensitive to soilborne pathogens at the seedling stage. When radish-treated field was again irrigated without biomass incorporation, the plots showed higher disease incidence in a similar way as observed in the control plots indicating that radish biomass should be incorporated before every spinach cropping for effective suppression of wilt disease. Our study showed that yield of spinach was also increased by radish incorporation in soil probably due to effective suppression of wilt disease in consistence with our previous research (Mowlick et al., 2013c) where yields of spinach were increased markedly by BSD treatments. High temperature during the third cropping season might increase the disease incidence as a whole.

Radish residue incorporation resulted in accumulation of considerable amounts of VFAs in soil, especially of acetate in all conditions. Production of VFAs such as acetate and butyrate in soil are reported as an important aspect of BSD (Momma et al., 2006; Katase et al., 2009), which is associated with proliferation of anaerobic fermentative bacteria in soil. Actually, a number of anaerobic bacterial species including clostridial groups were detected as the closest relatives for the clones from the radish-treated soils in both experiments. These results agreed well with our previous reports mentioned above where high concentrations of VFAs in various BSD-treated soils were detected in both model and field experiments.

All control libraries so far examined by us were shown to harbor diversified bacterial communities of various phylogenetic taxa, whereas the radish-treated as well as other BSD libraries showed an extraordinary development of clones belonging to the phylum *Firmicutes*. Bacterial groups under the class *Bacilli* of the phylum *Firmicutes* were detected as the dominant groups for all clone libraries of BSD with radish incorporation. As compared with the ratios of the *Bacilli* groups in the *Brassica* plants-treated soil (Mowlick et al., 2013c), it was revealed that these groups were proliferated by the repeated incorporation of radish biomass. Especially for YRBR, repetition of radish incorporation to the YRB soils seemed to enhance remarkable proliferation of the *Bacilli* groups, and closely related to *B. niacini*, *B. circulans*, *B. pycnus*, and *P. ruminocola*. *Bacillus* species are recognized as biocontrol agents that kill soilborne pathogens

by synthesizing different kinds of antibiotics and enzymes, as well as by their longer survival due to their ability to form endospores (Emmert et al., 1999; Wang et al., 2002; Lee et al., 2006; Hariprasad et al., 2011). *Bacillus* species have also been reported to reduce plant disease incidence by competition, growth promotion and induction of resistance (Cawoy et al., 2011). Therefore, although they are basically aerobic or facultatively anaerobic, proliferation of *Bacilli* groups might play some roles in the suppression of spinach wilt during BSD treatments.

Although clostridial groups were relatively lower in percentages in radish-treated soils (except YRBaR), similar types of clostridial species were also found as the closest relatives in radish-treated soil, especially for the library YRBaR, as those detected in the *Brassica*- or wheat bran-treated soils in the present study as well as in our previous research (Mowlick et al., 2012, 2013a,c). Clone groups related to *C. saccharobutylicum*, *C. sufflavum*, and *C. xylanovorans* recognized in most of the BSD-treated soils in our previous work were also detected as major groups in the radish-treated clone libraries. These 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). The high concentrations of VFAs detected in these soils also indicated proliferation of clostridial groups in these field soils, suggesting their important roles in disease suppression in radish-treated soil in practice.

The estimates of diversity indicated a high diversity in the soil bacterial communities after BSD with *Brassica* incorporation in our previous study (Mowlick et al., 2013c), whereas radish incorporation decreased the diversity in this study probably due to proliferation of less diverse *Bacilli* groups. Especially for YRBR, bacterial diversity decreased remarkably as the ratios of the *Bacilli* increased. The results indicated that the *Bacilli* rather than the *Clostridia* reduced the overall diversity in radish-treated soil.

In this study, usefulness of radish residue for BSD treatment was observed clearly as spinach wilt was suppressed both in the pots and field greenhouse. Thus, radish residue can be used as BSD material for the suppression of plant pathogens in a similar manner as for other plant biomass sources like *B. juncea* or wheat bran. The exact mechanism of suppression of soil pathogens by BSD has not been elucidated. As a further study, it may be necessary to compare the type and content of glucosinolates in radish residue used in this study with those of *B. juncea* plants. The abundance of populations in the *Firmicutes*, especially in the *Bacilli*, was greatly increased by radish biomass incorporation. Bacterial groups from both the *Bacilli* and *Clostridia* might have important roles in suppressing spinach wilt. We have isolated many strains in the *Clostridia* and the *Bacilli* from BSD-treated soil. We are now examining their physiological characteristics to clarify the effects of these bacteria on survival or growth of spinach wilt pathogen and to determine their functions in suppression of soilborne plant diseases.

Furthermore, changes in fungal communities in soil after BSD treatments should be examined in future to determine the comprehensive effects of BSD on soil microbial communities.

Acknowledgments

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FIGURE CAPTIONS

Fig. 1. Changes in concentrations of volatile fatty acids in (A) *Brassica*- (B) Wheat bran-, and (C) radish-treated soil. Symbols: Δ, acetate; ■, propionate; ▲, butyrate.

Fig. 2. Neighbor-joining trees showing the phylogenetic relationships of all operational taxonomic units (OTUs) derived from the libraries YRB (BSD-treated soil with radish and Mustard greens, subsequently) (A) and YRBa (BSD-treated soil with radish and Azamina, subsequently) (B) based on 16S rRNA gene sequences. The name of each clone starts with the clone library designation of both YRB and YRBa. Abbreviations: *C.*, *Clostridium*; α-, β-, and γ-Prot, *Alpha-*, *Beta-*, and *Gammaproteobacteria*, respectively; Actino, *Actinobacteria*; Acido, *Acidobacteria*; Gemma, *Gemmatimonadetes*, Symbio, *Symbiobacterium*; Bacter, *Bacteroidetes*; Plancto, *Planctomycetes*; Verruco, *Verrucomicrobia*, Chlo, Chloroflexi. Bootstrap values (n = 1,000) above 70% are indicated at branch nodes. The scale bar represents 2% estimated difference in nucleotide sequence position. As the outgroup, *Sulfolobus acidocaldarius* (D14053) (the domain *Archaea*) 16S rRNA gene sequence was used. Accession numbers of the species are shown in parentheses. Numbers in 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.

Fig. 3. Neighbor-joining trees showing the phylogenetic relationships of all operational taxonomic units (OTUs) derived from the libraries YRBR (BSD-treated soil with radish, Mustard greens, and radish, subsequently) (A) and YRBaR (BSD-treated soil with radish, Azamina, and radish, subsequently) (B). The name of each clone starts with the clone library designation of both YRBR and YRBaR. Abbreviations: *C.*, *Clostridium*; α- and δ-Prot, *Alpha-* and *Deltaproteobacteria*, respectively; Gemma, *Gemmatimonadetes*, Symbio, *Symbiobacterium.* Tree construction and other notifications are similar as described in Fig. 2.

Fig. 4. Rarefaction curves for the 16S rRNA gene sequences from all clone libraries. Refer to Tables 3 and 4 for the clone library names.

Fig. 1

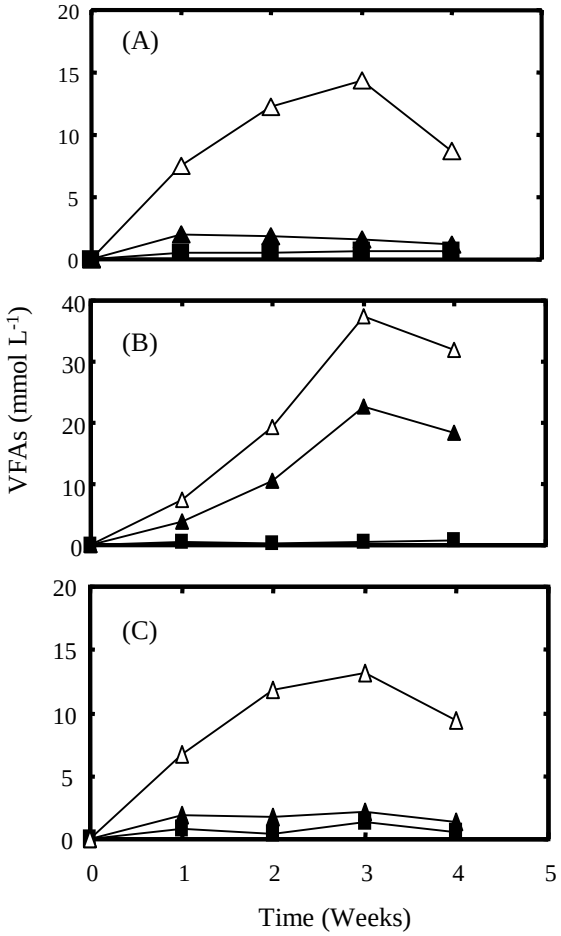
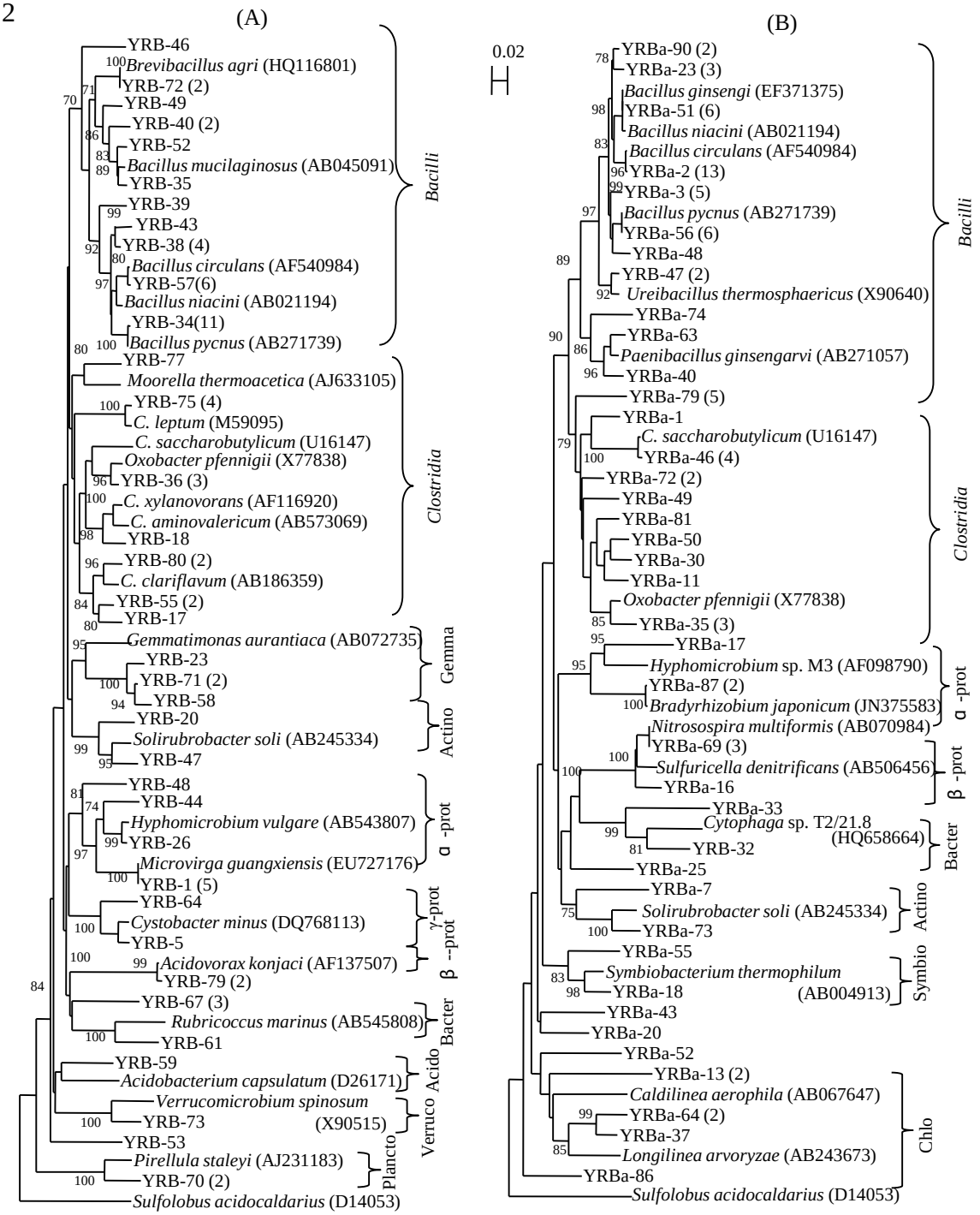


Fig. 2


(A)
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Brevibacillus agri (HQ116801)
YRB-72 (2)
YRB-49
YRB-40 (2)
YRB-52
Bacillus mucilaginosus (AB045091)
YRB-35
YRB-39
YRB-43
YRB-38 (4)
Bacillus circulans (AF540984)
YRB-57(6)
Bacillus niacini (AB021194)
YRB-34(11)
Bacillus pycnus (AB271739)
Bacilli
YRB-77
Moorella thermoacetica (AJ633105)
YRB-75 (4)
C. leptum (M59095)
C. saccharobutylicum (U16147)
Oxobacter pfennigii (X77838)
YRB-36 (3)
C. xylanovorans (AF116920)
C. aminovalericum (AB573069)
YRB-18
YRB-80 (2)
C. clariflavum (AB186359)
YRB-55 (2)
YRB-17
Clostridia
Gemmatimonas aurantiaca (AB072735)
YRB-23
YRB-71 (2)
YRB-58
Gemma
YRB-20
Solirubrobacter soli (AB245334)
YRB-47
Actino
YRB-48
YRB-44
Hyphomicrobium vulgare (AB543807)
YRB-26
Microvirga guangxiensis (EU727176)
YRB-1 (5)
α -prot
YRB-64
Cystobacter minus (DQ768113)
YRB-5
γ-prot
β --prot
Acidovorax konjaci (AF137507)
YRB-79 (2)
YRB-67 (3)
Rubricoccus marinus (AB545808)
YRB-61
Bacter
YRB-59
Acidobacterium capsulatum (D26171)
Acido
Verrucomicrobium spinosum
YRB-73
(X90515)
Verruco
YRB-53
Pirellula staleyi (AJ231183)
YRB-70 (2)
Plancto
Sulfolobus acidocaldarius (D14053)
(B)
0.02
YRBa-90 (2)
YRBa-23 (3)
Bacillus ginsengi (EF371375)
YRBa-51 (6)
Bacillus niacini (AB021194)
Bacillus circulans (AF540984)
YRBa-2 (13)
YRBa-3 (5)
Bacillus pycnus (AB271739)
YRBa-56 (6)
YRBa-48
YRBa-47 (2)
Ureibacillus thermosphaericus (X90640)
YRBa-74
YRBa-63
Paenibacillus ginsengarvi (AB271057)
YRBa-40
YRBa-79 (5)
Bacilli
YRBa-1
C. saccharobutylicum (U16147)
YRBa-46 (4)
YRBa-72 (2)
YRBa-49
YRBa-81
YRBa-50
YRBa-30
YRBa-11
Oxobacter pfennigii (X77838)
YRBa-35 (3)
Clostridia
YRBa-17
Hyphomicrobium sp. M3 (AF098790)
YRBa-87 (2)
Bradyrhizobium japonicum (JN375583)
α -prot
Nitrosospira multiformis (AB070984)
YRBa-69 (3)
Sulfuricella denitrificans (AB506456)
YRBa-16
β -prot
YRBa-33
Cytophaga sp. T2/21.8
(HQ658664)
YRB-32
YRBa-25
Bacter
YRBa-7
Solirubrobacter soli (AB245334)
YRBa-73
Actino
YRBa-55
Symbiobacterium thermophilum
YRBa-18
(AB004913)
Symbio
YRBa-43
YRBa-20
YRBa-52
YRBa-13 (2)
Caldilinea aerophila (AB067647)
YRBa-64 (2)
YRBa-37
Longilinea arvoryzae (AB243673)
YRBa-86
Chlo
Sulfolobus acidocaldarius (D14053)

Fig. 3

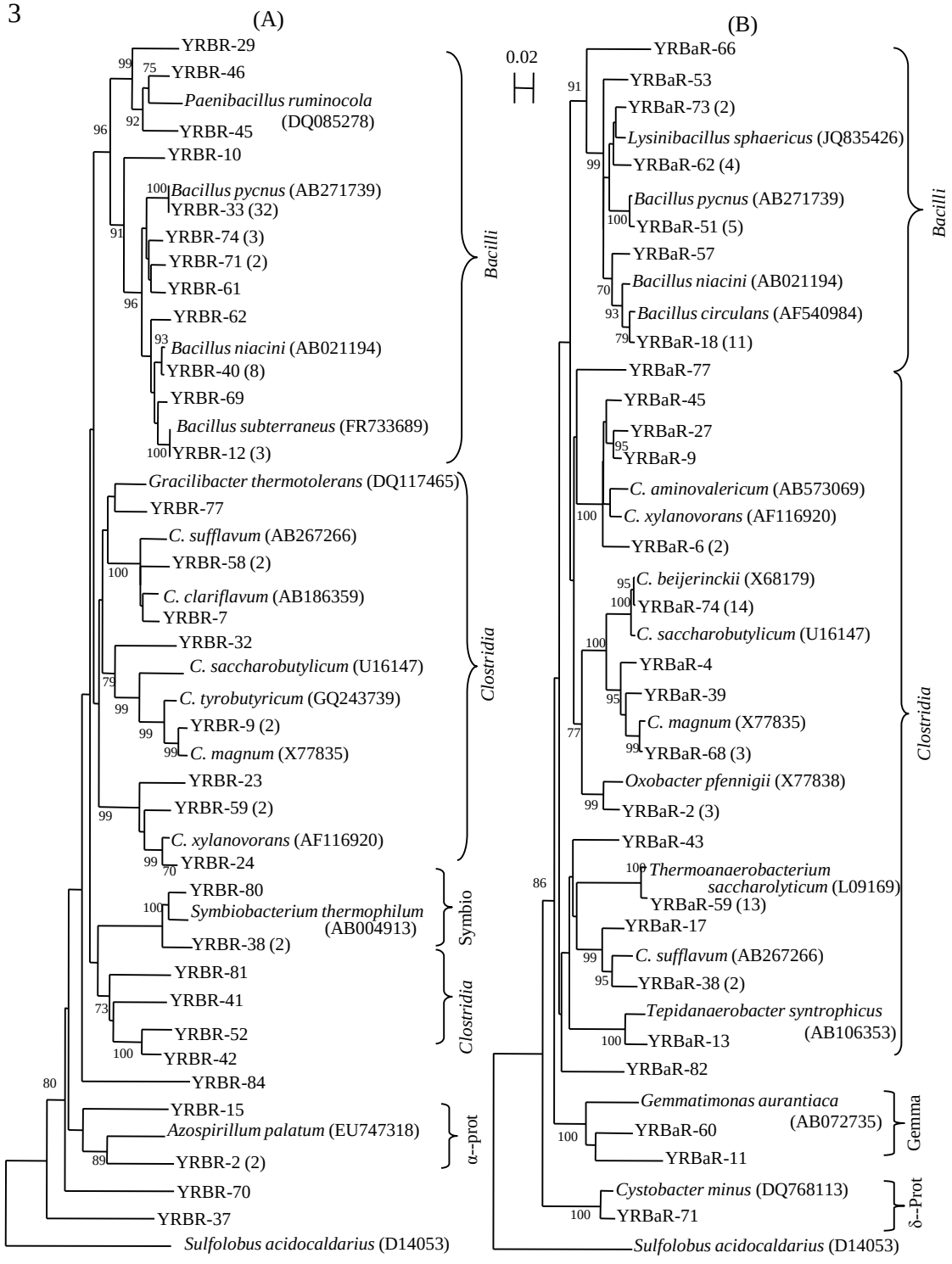
(A)
YRBR-29
YRBR-46
Paenibacillus ruminocola (DQ085278)
YRBR-45
YRBR-10
Bacillus pycnus (AB271739)
YRBR-33 (32)
YRBR-74 (3)
YRBR-71 (2)
YRBR-61
YRBR-62
Bacillus niacini (AB021194)
YRBR-40 (8)
YRBR-69
Bacillus subterraneus (FR733689)
YRBR-12 (3)
Bacilli
Gracilibacter thermotolerans (DQ117465)
YRBR-77
C. sufflavum (AB267266)
YRBR-58 (2)
C. clariflavum (AB186359)
YRBR-7
YRBR-32
C. saccharobutylicum (U16147)
C. tyrobutyricum (GQ243739)
YRBR-9 (2)
C. magnum (X77835)
YRBR-23
YRBR-59 (2)
C. xylanovorans (AF116920)
YRBR-24
Clostridia
YRBR-80
Symbiobacterium thermophilum (AB004913)
YRBR-38 (2)
Symbio
YRBR-81
YRBR-41
YRBR-52
YRBR-42
Clostridia
YRBR-84
YRBR-15
Azospirillum palatum (EU747318)
YRBR-2 (2)
α--prot
YRBR-70
YRBR-37
Sulfolobus acidocaldarius (D14053)
0.02
(B)
YRBaR-66
YRBaR-53
YRBaR-73 (2)
Lysinibacillus sphaericus (JQ835426)
YRBaR-62 (4)
Bacillus pycnus (AB271739)
YRBaR-51 (5)
YRBaR-57
Bacillus niacini (AB021194)
Bacillus circulans (AF540984)
YRBaR-18 (11)
Bacilli
YRBaR-77
YRBaR-45
YRBaR-27
YRBaR-9
C. aminovalericum (AB573069)
C. xylanovorans (AF116920)
YRBaR-6 (2)
C. beijerinckii (X68179)
YRBaR-74 (14)
C. saccharobutylicum (U16147)
YRBaR-4
YRBaR-39
C. magnum (X77835)
YRBaR-68 (3)
Oxobacter pfennigii (X77838)
YRBaR-2 (3)
Clostridia
YRBaR-43
Thermoanaerobacterium saccharolyticum (L09169)
YRBaR-59 (13)
YRBaR-17
C. sufflavum (AB267266)
YRBaR-38 (2)
Tepidanaerobacter syntrophicus (AB106353)
YRBaR-13
YRBaR-82
Gemmatimonas aurantiaca (AB072735)
YRBaR-60
YRBaR-11
Gemma
Cystobacter minus (DQ768113)
YRBaR-71
δ--Prot
Sulfolobus acidocaldarius (D14053)

Fig. 4

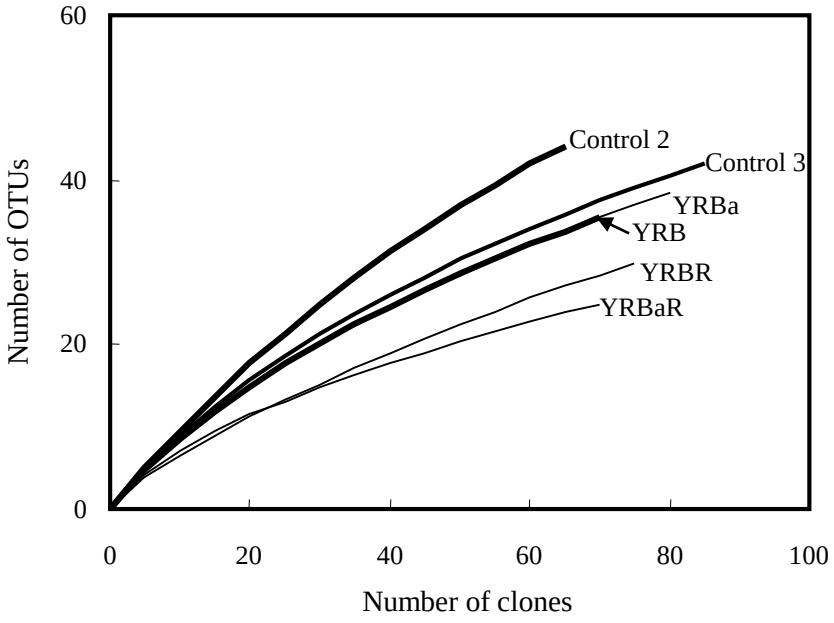
60
40
20
0
Number of OTUs
0
20
40
60
80
100
Number of clones
Control 2
Control 3
YRBa
YRB
YRBR
YRBaR

Table 1. Wilt disease incidence of spinach plants cultivated in pots with differently treated soil

Treatment of pot soil[a]	Disease incidence (%)[b]
Without treatment (Original)	100a
Control (irrigation without biomasss)	100a
Brassica with irigation	31.7 ± 31.8b
Wheat bran with irigation	22.7 ± 6.9b
Radish with irigation	15.4 ± 26.6b

[a] Cells of *Fusarium oxysporum* f. sp. *spinaciae* YSF-1 were inoculated to soil for all treatments. [b]Mean ± SD (n = 4). Means followed by different letters are singnificantly different (P = 0.05) according to Duncan's multiple range test.

Table 2. Composition profiles of phylogenetic groups of bacteria based on 16S rRNA gene sequences for the differently treated soil samples of the model experiment

Phylum or class	Clone library (% of abundance)			
	Control	*Brassica* -treated	Wheat bran-treated	Radish-treated
Alphaproteobacteria	15.9	8.0	10.4	13.9
Betaproteobacteria	6.8	5.7	2.1	1.4
Gammaproteobacteria	2.3	5.7	3.1	2.8
Deltaproteobacteria	4.5	1.1	3.1	-[a]
Acidobacteria	34.1	16.1	5.2	4.2
Verrucomicrobia	-	-	-	-
Bacteroidetes	-	-	2.1	1.4
Planctomycetes	3.4	3.4	3.1	2.8
Firmicutes (*Clostridia*)	-	31.0	32.3	19.4
Firmicutes (*Bacilli*)	-	17.2	16.7	15.3
Gemmatimonadetes	12.5	1.1	4.2	5.6
Actinobacteria	6.8	6.9	9.4	15.3
Chloroflexi	9.1	3.4	4.2	6.9
Others	4.5	-	4.2	11.1

[a]Not detected.

Table 3. Concentrations of VFAs in differently treated soil in the greenhouse

Plot name[a]	Biomass incorporated			VFAs (mmol/l)[b]		
	1	2	3	Acetate	Propionate	Butyrate
Control 2	None	None		-[c]	-	-
YRB	Radish	Mustard		8.7	3.3	3.4
YRBa	Radish	Azamina		9.3	1.7	1.6
Control 3	None	None	None	-	-	-
YRBR	Radish	Mustard	Radish	21.9	3.4	10.6
YRBaR	Radish	Azamina	Radish	20.9	3.2	10.4

[a]Control 2 and 3, irrigated and polythene covered without biomass for each treatment.

[b]Measured after triplicate samples were mixed. [c]Not detected.

Table 4. Effects of different treatments on wilt disease incidence and yields of spinach cultivated in the greenhouse

Plot name[a]	Biomass incorporated			Wilt disease	Yield of spinach
	1	2	3	incidence (%)[b]	(g/m^2)[b]
Control 1	None			7.7 ± 4.9	4150 ± 420b
YR	Radish			-[c]	5070 ± 620a
Control 2	None	None		44.5 ± 30.3a	1062 ± 584d
YRC-B	Radish	None		21.9 ± 15.1b	896 ± 307d
YRB	Radish	Mustard		3.4 ± 3.6c	4086 ± 1329b
YRC-Ba	Radish	None		26.8 ± 11.6b	2462 ± 533c
YRBa	Radish	Azamina		5.7 ± 5.9c	4810 ± 530a
Control 3	None	None	None	58.5 ± 20.5b	1029 ± 763b
YRCC-B	Radish	None	None	79.0 ± 8.8a	489 ± 361bc
YRBR	Radish	Mustard	Radish	6.0 ± 10.7d	4003 ± 635a
YRCC-Ba	Radish	None	None	75.0 ± 22.2a	238 ± 251c
YRBaR	Radish	Azamina	Radish	38.0 ± 18.7c	3512 ± 1209a

[a]Control 1, 2, and 3, irrigated and polythene covered without biomass for each treatment. YRC-B/YRCC-B and YRC-Ba/YRCC-Ba: *Brassica* (Mustard) and *Brassica* (Azamina) plants were cultivated in each field before the second treatment, respectively, but the plants were removed from the field without incorporation into soil. YRB, YRBa, YRBR, and YRBaR were BSD-treated plots with radish and *Brassica* . [b]Mean ± SD (n = 10). Means followed by different letters are singnificantly different (P = 0.05) according to Duncan's multiple range test for each cropping period. [c]Not detected.

Table 5. Composition profiles of phylogenetic groups of bacteria based on 16S rRNA gene sequences for the soil samples differently treated with radish residue in the greenhouse

Phylum or class	Clone library[a] (% of abundance)					
	Control 2[b]	YRB	YRBa	Control 3[b]	YRBR	YRBaR
Alphaproteobacteria	9.1	11.4	3.5	10.0	2.5	-[c]
Betaproteobacteria	9.1	2.9	-	6.7	-	-
Gammaproteobacteria	7.6	-	5.9	-	1.3	-
Deltaproteobacteria	9.1	2.9	-	2.2	2.5	1.3
Acidobacteria	13.6	1.4	2.4	8.9	-	-
Verrucomicrobia	3.0	1.4	-	3.3	-	-
Bacteroidetes	6.1	1.4	2.4	-	-	-
Planctomycetes	4.5	2.9	-	4.4	-	-
Firmicutes (*Clostridia*)	3.0	20.0	25.9	1.1	19.0	62.7
Firmicutes (*Bacilli*)	6.1	44.3	48.2	16.7	69.6	33.3
Firmicutes (*Symbiobacterium*)	-	-	2.4	17.8	3.8	-
Gemmatimonadetes	6.1	5.7	-	7.8	-	2.7
Actinobacteria	12.1	2.9	2.4	6.7	1.3	-
Chloroflexi	9.1	-	7.1	3.3	-	-
Others	1.5	2.9	-	11.1	-	-

[a]For the clone library names, refer to Table 3 and 4. [b]Mowlick et al. (2013c). [c]Not detected.

Table 6. Estimates of bacterial diversity from differently treated soil with radish residue in the greenhouse

Soil sample[a]	Control 2[b]	YRB	YRBa	Control 3[b]	YRBR	YRBaR
No. of total clones	66	72	85	89	79	75
No. of total OTUs	45	36	40	43	31	26
Coverage (%)	57.5	69.4	70.6	73.0	73.4	78.6
Simpson's diversity index	0.98	0.95	0.95	0.96	0.82	0.91
Shannon-Wiener diversity index	5.32	4.71	4.80	5.05	3.70	3.90

[a]For the soil sample names, refer to Table 3 and 4. [b]Mowlick et al. (2013c).