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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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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 in 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* dominated in 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*, *Clostridium cylindrosporum*, *Clostridium sufflavum*, and *Clostridium xylanovorans*. 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.

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 crops 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 (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 human health problems (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 to chemical

Abbreviations: BSD, biological soil disinfestation; Exp, experiment; ITC, isothiocyanate; ORP, oxidation–reduction potential; OTU, operational taxonomic unit; PCR-DGGE, polymerase chain reaction-denaturing gradient gel electrophoresis; VFAs, volatile fatty acids.

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fumigation. To implement 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 a 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 designed 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 (Sarwar 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 populations of *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 crops, 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 disease 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. 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 °N, 135.96 °E), 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 2.5×4.0 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. 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 min by data loggers (TR-724, T & D Co. Ltd.). Oxidation–reduction potential (ORP) of soil (at 15 cm depth) was measured at intervals of two or three days at four points of every treatment by electrodes (Ag/AgCl) inserted 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 every week. 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 °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) conducted at the Agricultural Research Center, Yamaguchi, Japan (34.9 °N, 131.3 °E) during June 2010. The soil was gray lowland soil (sandy loam, pH 6.5) and the