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REVIEW
Biological Soil Disinfestation (BSD) of Soilborne Pathogens and Its Possible Mechanisms

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Abstract
Biological soil disinfestation (BSD) is one of the methods for soil disinfestation recently developed and consists of organic amendment, irrigation, and covering the soil surface with plastic film. BSD trials with artificially infested soils effectively killed *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum*. *F. oxysporum* f. sp. *lycopersici* was not detected 9 days after treatment. Application of BSD to *R. solanacearum*-infested soil decreased disease severity 4 weeks after transplantation. Reduction in soil pH and Eh was observed in the BSD-treated soil. HPLC analysis revealed the accumulation of acetic acid and butyric acid in the soil and the disinfestation effect of BSD was almost reproduced by mixing these organic acids with the infested soil. Volatile compound(s) evolved in the BSD-treated soil was also suggested to contribute to disinfestation. PCR-DGGE (PCR-denaturing gradient gel electrophoresis) analysis revealed the increase in the abundance of several bacteria in the BSD-treated soil, and two of them had 100% similarity with potential organic acid producers, *Clostridium* sp. and *Enterobacter* sp.

Discipline: Plant disease
Additional key words: anaerobic bacteria, bacterial wilt, Fusarium wilt, organic acids, soil reduction

Introduction

Methyl bromide had been the most effective chemical fumigant for many plant pests[4,5]. However, it is harmful to human health and destructively damages the ozone layer[4]. Thus it is no longer available for agricultural use other than for some diseases which are hard to control by any other measure. Though alternative chemicals are still available, human health and environmental safety are the largest concerns today, so environmentally safe soil disinfestation measures against soilborne diseases have increasingly been investigated to replace chemical fumigants.

Soil sterilization methods using steam have been practiced in Europe for more than a century[5]. Recently, soil sterilization methods with hot water or steam also have been investigated intensively for practical use in Japan[6,18,19]. Their efficacy against soilborne plant pests is significant and stable but these methods require special apparatus and consume much fuel. Other soil sterilization methods, such as soil solarization and flooding, are also available where favorable conditions are secured[4,5]. They are sometimes not as effective as chemical fumigants and require high temperature and a long incubation period. The efficiency of these methods is improved by an organic amendment, due possibly to soil reduction[11,14,16]. However, little attention has been paid to the condition of soil reduction during the course of technique development.

Shinmura[15,16] developed a method, biological soil disinfestation (BSD) fortified by soil reduction, which required neither high temperature nor a long term incubation and consisted of three steps: (1) introduction of easily decomposable organic materials (e.g. wheat bran, molasses, rice straw, rice bran, etc.) to soil, (2) flooding the soil by irrigation, and (3) covering the soil surface with plastic film to induce reduced soil conditions. Practically, 1 to 2 t of organic material are added to each 1,000 m^2 of soil treated. BSD using wheat bran or molasses proved to be effective against a wide range of soilborne pathogens, such as *F. oxysporum* f. spp. *lycopersici*, *spinaciae* and

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radicis-lycopersici, *F. redolens*, *Phomopsis sclerotioides*, *Pyrenochaeta lycopersici*, *R. solanacearum*, and *Verticillium dahliae*, as well as the nematodes *Meloidogyne incognita* and *Pratylenchus* sp.[15,16,20].

BSD is increasingly used in Japan, but its precise mechanism has not been studied so far. In this paper, using two important soilborne pathogens of tomato as models, i.e. Fusarium wilt pathogen, *F. oxysporum* f. sp. *lycopersici* and bacterial wilt pathogen, *R. solanacearum*, the efficacy of BSD using wheat bran was confirmed, and its possible mechanism was discussed in terms of key microorganisms active in reduced conditions.

Efficacy of BSD

BSD efficacy was determined using artificially infested soil. Soil was infested with *F. oxysporum* f. sp. *lycopersici* CU1 (race 1), the causal agent of Fusarium wilt of tomato. Infested soil (ca. 2.5 kg) in 14 cm-diameter pots was amended with 40 g of wheat bran, irrigated to maximum water holding capacity, and sealed with plastic film. In this system, excess water drained from a hole in the bottom of the pot. The pathogen was not detected 9 days after BSD treatment[8]. When 20 g of wheat bran were amended, significant disinfestation was not induced and the pathogen survived at 1.7×10^4 CFU/g dry soil 14 days after treatment[8]. Survival of the pathogen was not affected in both non-treated soil and wheat bran-amended soil without irrigation; estimated population density was 1.5×10^6 and 1.7×10^6 CFU/g dry soil, respectively. When the soil without wheat bran was irrigated and the pots were sealed, the pathogen decreased to 8.0×10^4 CUF/g dry soil. In the case of *Ralstonia solanacearum* EK1-2 (biovar 4, race 1), the causal agent of bacterial wilt of tomato, when 250 g of infested soil in a glass bottle was treated with BSD using 4 g of wheat bran, population of the pathogen decreased below the detection limit 14 days after treatment[9]. No colony was formed even when a portion of BSD-treated soil was directly spread onto selective media in both cases. Based on these results, BSD is expected to control Fusarium wilt and bacterial wilt of many crops including tomato.

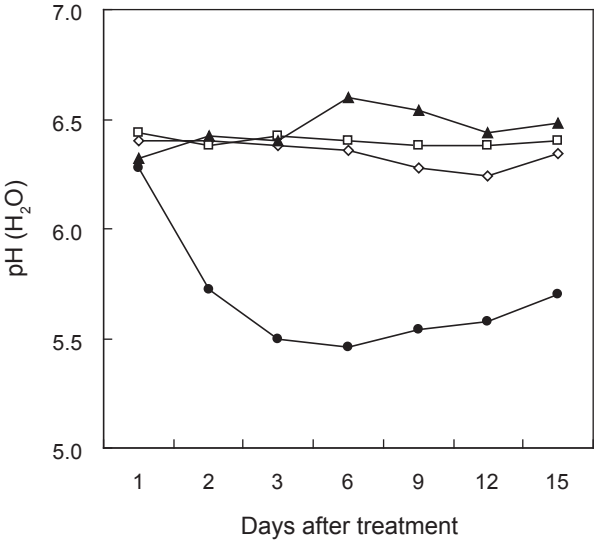

Fig. 1. Effect of BSD on soil pH
Soil samples were collected 1, 2, 3, 6, 9, 12, and 15 days after treatment and soil pH was measured by the conventional method (soil : water = 1 : 2.5; reproduced from Momma et al. (2006) with permission).
—◇—: Control, —□—: Irrigated and covered soil, —▲—: Wheat bran-amended soil, —●—: BSD.

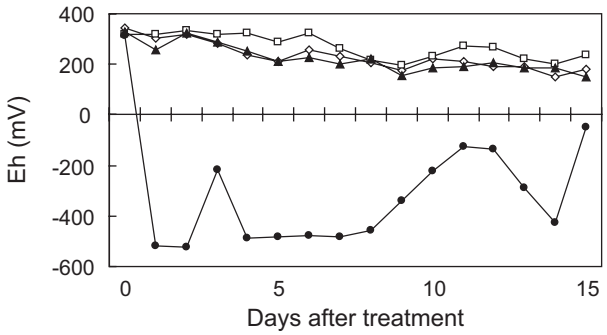

Fig. 2. Effect of BSD on soil Eh
Soil Eh was measured every day for 15 days with an ORP meter; reproduced from Momma et al. (2005) with permission.
—◇—: Control, —□—: Irrigated and covered soil, —▲—: Wheat bran-amended soil, —●—: BSD.

Factors associated with BSD

BSD treatment reduced soil pH and Eh (Figs. 1 and 2). The pH reduction suggested the accumulation of organic acids. Eh reduction implied consumption of oxygen. In addition, a peculiar odor was generated from BSD-treated soil. When wheat bran-amended soil had been heated at 80ºC for 2 h prior to irrigation and sealing, the disinfestation effect of BSD against *F. oxysporum* f. sp. *lycopersici* was not induced[8]. Indigenous microorganisms play an important role in BSD-treated soil.

1. Organic acids

Acetic acid and n-butyric acid were detected in BSD-treated soil by HPLC analysis[9]. Acetic acid accumulated at 2,100 mg/kg dry soil and butyric acid at 1,800 mg/kg dry soil, 15 and 6 days after treatment, respectively. Soil amended with the organic acids could almost reproduce

the pesticidal effect of BSD against the pathogens[9]. When *R. solanacearum*-infested soil was mixed with the mixture of butyric and acetic acid (1,000 mg/kg of dry soil, each), the pathogen decreased below the detection limit 7 days after incubation. In the case of *F. oxysporum* f. sp. *lycopersici*, the survival of the pathogen was estimated to be 0.6% of the non-amended treatment 7 days after incubation in the soil mixed with butyric acid and acetic acid (2,000 mg/kg of dry soil, each). However, the population density of the pathogen was still maintained at 10^3 CFU/g dry soil.

Survival of *F. oxysporum* f. sp. *lycopersici* in acetic acid or butyric acid solution was subsequently determined. Bud cells were completely killed in 0.02% acetic acid or 0.02% butyric acid, but chlamydospores barely survived in 0.14% acetic acid or 0.2% butyric acid solution[9].

These results implied that chlamydospores were tolerant to both acids possibly because of their thick cell wall. However, once chlamydospores germinated, their tolerance should be lowered as were bud cells. In the soil mixed with the organic acids, chlamydospores might remain dormant. The dormancy should have helped the pathogen survive, resulting in incomplete suppression. While in the BSD-treated soil, chlamydospores are expected to germinate in an early, aerobic phase due to stimuli from decomposing wheat bran and then are killed through subsequent organic acid accumulation. Therefore, a germination-lysis mechanism[3] is applicable to BSD-mediated disinfestation. When *F. oxysporum* was incubated under anaerobic conditions, mycelial growth was completely suppressed but mycelia remained viable[8]. These results suggest that the efficacy of BSD is attributed mainly to the accumulation of acetic acid and butyric acid and indirectly to oxygen deficiency.

2. Soil microorganisms

The FDA method[1,13] elucidated the enhancement of microbial activity in BSD-treated soil[8]. Increase in population density of bacteria and actinomyces was also indicated by the dilution plate technique[8]. Thus, the activated bacteria were considered to contribute to the disinfesting effect of BSD.

Six bacteria randomly isolated from BSD-treated soil were inoculated separately into autoclaved soil and the soil was treated with BSD using sterilized wheat bran and distilled water. Three isolates induced significant disinfestation against *F. oxysporum* f. sp. *lycopersici*, one showed moderate disinfestation, and the remaining two had no effect on the pathogen 2 weeks after incubation[10]. The three effective isolates had the same 16S rDNA sequence with *Clostridium* sp. and PCR-DGGE analysis suggested the abundance of this species in BSD-treated soil (Fig. 3). Clostridia are spore-forming, obligate anaerobes that grow under anaerobic conditions, survive under aerobic conditions, and degrade a wide variety of organic compounds to mixtures of acids, alcohols, CO_2 and H_2[7]. Acetate, propionate and butyrate are most commonly produced by Clostridia. For example, *C. butyricum* is generally known as a producer of both acetic acid and butyric acid.

DGGE band patterns in BSD-treated soil drastically differed from those in wheat bran-amended soil[10]. Band sequencing followed by homology search implied the abundance of *Enterobacter* sp., *Klebsiella pneumoniae*, *Acinetobacter* sp., and *Thermodesulfotobacterium commune* in BSD-treated soil. DGGE bands corresponding to *Acinetobacter* sp. and *Enterobacter* sp. could be observed in wheat bran-amended soil, too. In addition to Clostridia, a typical intestinal bacterium *Enterobacter* sp. produces acids from several sugars[2], and the environment of BSD-treated soil seems favorable for the bacterium. Including *Enterobacter* sp. and *Clostridium* sp., numerous bacteria potentially produce acids. Those acid-producers would exist in a wide range of environments and may be able to contribute to the disinfesting activity in the BSD-treated soil. However, contribution of bacteria other than the species regarded as *Clostridium* sp. could not be determined in this study. Isolation of effective bacteria is a future theme for more detailed understanding, and the development of the responsible bacteria in the BSD-treated soil is to be investigated using DGGE and other molecular methods.

3. Volatile compounds

Soil pre-inoculated with the three effective bacteria had an intense, peculiar odor after BSD treatment[10], implying the involvement of volatile compounds in BSD. When mycelial plugs of the fungal pathogen were exposed to volatile compounds evolved from BSD-treated soil, its growth was almost completely suppressed but viability was not affected (Fig. 4). These results indicated that the volatile compounds had fungistatic but not fungicidal activity[10]. It has been well documented that volatile compounds released from cruciferous plant-amended soil had an adverse effect against several soilborne fungal pathogens and that the volatile compounds contained pesticidal isothiocyanate[12,17]. Isothiocyanate is commercially used as an ingredient of chemical fumigants. However, in the case of wheat bran, release of such compounds from wheat bran has not been confirmed so far. Eh and pH declined just after BSD treatment, followed by the generation of the odor. Thus, the generation of the odor seemed to be the result of organic acid production by anaerobic bacteria activated under a reduced soil condition.

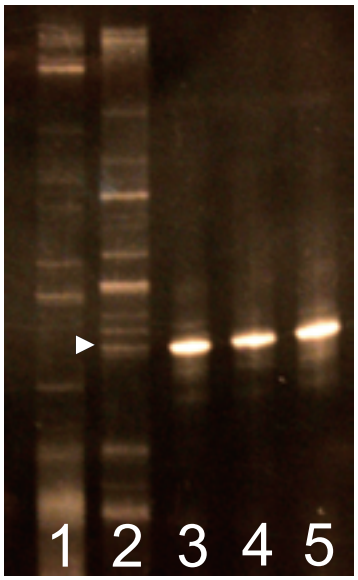

Fig. 3. DGGE bands of bacterial isolates that reproduced a suppressive effect similar to the BSD treatment
Lane 1: wheat bran-amended soil, 2: BSD-treated soil, 3–5: bacterial isolate considered to be *Clostridium* sp. (Reproduced from Momma et al. (2007) with permission). Note that a band considered to be derived from *Clostridium* sp. was present in BSD-treated soil (arrow).

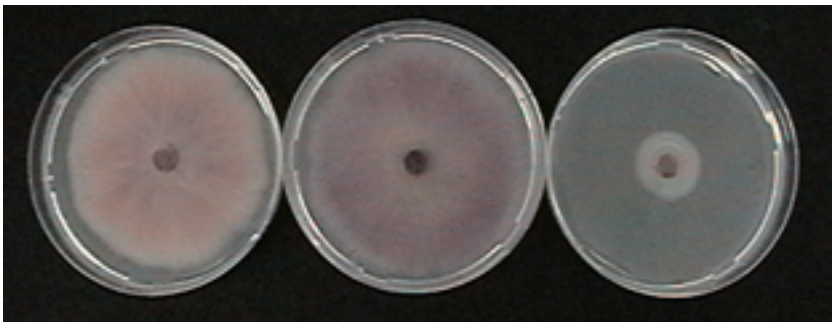

Fig. 4. Effect of volatile compounds from BSD-treated soil
Mycelial plugs of *Fusarium oxysporum* f. sp. *lycopersici* were exposed to the volatile compounds for 10 days. Left to Right: Control, Wheat bran-amended soil and BSD-treated soil.

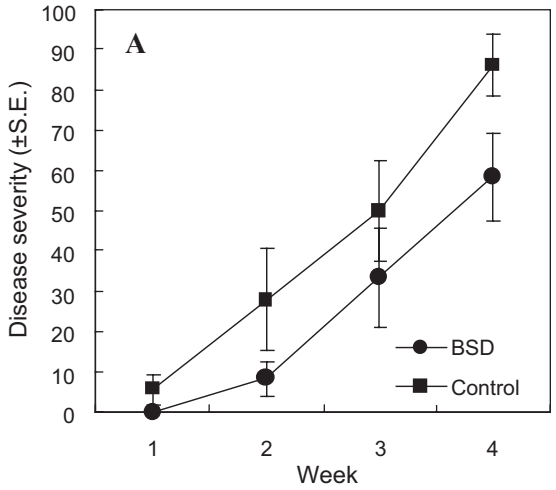

Fig. 5. Effect of BSD on bacterial wilt caused by *Ralstonia solanacearum*
Two-week-old tomato seedlings were transplanted to each plot 8 days after BSD treatment and disease severity was measured every week. Disease severity was determined every week using the following formula; disease severity = Σ (individual disease score) / (3 × total number of plants) × 100. Individual disease scores are as follows: 0 = healthy, 1 = less than 1/3 leaves wilted, 2 = more than 1/3 leaves wilted, 3 = more than 2/3 leaves wilted or plant dead. BSD treatment was done as follows; wheat bran (6.0 kg) was mixed into each artificially infested field plot (3.0 m × 1.0 m), the plots were flooded, covered with plastic film and incubated for two weeks. Tomato plants were also grown in non-treated pathogen-infested plots. A: Disease severity, B: Tomato plants 4 weeks after transplantation.

Identification of active ingredients in BSD volatiles was, however, unsuccessful.

BSD under field condition

In our field experiment, artificially infested soil in a plot (3.0 m × 1.0 m) was treated by BSD. The plot was amended with 6.0 kg of wheat bran, flooded, covered with plastic film and incubated for 2 weeks. The soil was tilled 4 times every 2 days and then, two-week-old tomato seedlings were transplanted to the plot. Disease severity was determined every week using the following formula: disease severity = Σ (individual disease score) / (3 × total number of plants) × 100. Individual disease scores are as follows: 0 = healthy, 1 = less than 1/3 leaves wilted, 2 = more than 1/3 leaves wilted, and 3 = more than 2/3 leaves wilted or plant dead.

BSD treatment delayed the occurrence of bacterial wilt of tomato and decreased disease severity in comparison with the untreated control (Fig. 5). However, the disease control efficacy might not be fully acceptable to growers because resurgence by the surviving pathogen would possibly be a problem.

It is often difficult to achieve sufficient disinfestation deep in the soil. The difficulty in permeation of the BSD effect was partially overcome by using molasses instead of wheat bran because it percolated downward through soil[16]. Even with this improvement, control of soilborne bacteria may still remain difficult. A small number of bacterial cells that survive can multiply explosively and establish quickly under favorable conditions, causing severe disease. To alleviate the risk, BSD should be combined with other disease management strategies such as compost amendment, the use of resistant cultivars, crop rotation, and field sanitation.

In the case of pathogenic *F. oxysporum*, however, this may not be the case, because the pathogen is not as competitive as true saprophytes for organic matter and because it may take longer for population build-up to the threshold for disease occurrence than bacterial pathogens. Nevertheless, there may be a concern about fungal diseases other than Fusarium wilt such as damping-off disease which is enhanced by residual fresh organic matter. This can be overcome by optimizing the amount of organic amendment, taking long enough intervals after BSD treatment until transplantation, and/or choosing an appropriate growth stage of seedlings for transplanting.

Conclusion

The results from Momma et al.[8,9] suggest that BSD is a practical disease management strategy for controlling pathogenic microorganisms. Its efficiency is mainly attributed to the accumulation of acetic acid and butyric acid produced by anaerobic soil bacteria. Involvement of volatile compounds in BSD-mediated disinfestation was also suggested but active ingredients were not clarified. However, BSD should be incorporated into an integrated pest management strategy, because of its efficacy against many plant pests[13,14,18].

Standard methods must be established to apply BSD in different fields. The amount of organic amendment and sufficient water content level are principal determinants of BSD to be optimized. As mentioned above, accumulation of organic acids is the key factor in BSD and is accelerated through the decomposition of organic materials under reduced conditions. A reduced condition is first induced by oxygen consumption by soil microorganisms and then, maintained by plastic film and irrigation water which prevent an oxygen supply from the atmosphere. In addition, free soil water acts as a carrier of active ingredients, such as organic acids. Therefore, disinfestation efficacy may be predicted by determining Eh and/or pH, and these parameters would be useful for developing standard methods adapted for each field. Depending on the scale of a field and the kind of organic amendment, one should choose an appropriate way or tool to exploit and operate BSD in terms of organic material, irrigation and covering of the soil surface. Because molasses can be thinned with water and supplied through irrigation tubes, the use of molasses is promising in that it reduces the grower's labor in applying an organic amendment.

In conclusion, BSD can be used as one of the alternatives to chemical fumigation and should be helpful to promote and maintain environmental safety.

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