J Gen Plant Pathol (2006) 72:247–252
DOI 10.1007/s10327-006-0274-z

FUNGAL DISEASES

Short communication

**Noriaki Momma · Kazuhiro Yamamoto · Peter Simandi
Masahiro Shishido**

Role of organic acids in the mechanisms of biological soil disinfestation (BSD)

Received: May 18, 2005 / Accepted: December 9, 2005

Abstract Biological soil disinfestation (BSD), or reductive soil disinfestation, achieved by amendment with organic materials such as wheat bran followed by flooding and covering the soil surface, has been used to control some soilborne diseases including Fusarium wilt and bacterial wilt of tomato. During a BSD treatment, accumulation of acetic acid and/or butyric acid was detected with high-performance liquid chromatography. Survival of *Fusarium oxysporum* f. sp. *lycopersici* or *Ralstonia solanacearum* was suppressed by these organic acids. Amendment of these organic acids into soil suppressed the survival of *R. solanacearum* at lower concentrations than the maximum detected in BSD treatment, indicating that production of these organic acids is one of the mechanisms of control. However, *F. oxysporum* f. sp. *lycopersici* in soil survived with the maximum concentrations of these organic acids achieved by BSD; thus, involvement of factors other than organic acids may be involved.

Key words *Fusarium oxysporum* f. sp. *lycopersici* · *Ralstonia solanacearum* · Biological soil disinfestation (BSD) · Acetic acid · Butyric acid · Tomato

To control soilborne diseases, soil fumigation with methyl bromide has been one of the most effective measures. However, its use is highly restricted because of its destructive impact on the stratospheric ozone layer (Subbarao 2002). Although other chemical disinfestants for soil treatment such as chloropicrin, 1,3-dichloropropene, and methyl isothiocyanate, are still available, soil disinfestation using these chemicals has become widely recognized as measures incompatible with sustainable agriculture. Therefore, it is imperative to find alternatives to chemical fumigation for disinfesting soils.

A number of soilborne pathogens can be suppressed under anaerobic conditions (Cook and Baker 1983; Blok et al. 2000). For example, populations of soilborne pathogens in rice paddies can decrease during the flooding period, and this suppression is often enhanced by the incorporation of readily decomposable organic matter such as rice straws (Shinmura 2004). Similarly, although usually sporadic and locally confined, soilborne diseases have also been suppressed in crop fields under anaerobic conditions after the incorporation of large amounts of organic matter and/or intensive rainfalls (Cook and Baker 1983).

Applying the concept of enriching organic materials such as fresh broccoli or grass under anaerobic conditions in the soil, Blok et al. (2000) proposed a new technique of biological soil disinfestation (BSD), or reductive soil disinfestation, for the control of soilborne pathogens including *Fusarium oxysporum* f. sp. *asparagi*, *Rhizoctonia solani*, and *Verticillium dahliae*. Shinmura et al. (1999) also developed a soil disinfestation method that combined soil enrichment with organic materials to achieve reduced conditions and soil solarization for controlling root rot of Welsh onion caused by *Fusarium redolens*. This method has commonly been used in Japan against a wide range of soilborne diseases and involves three steps: (1) amending soil with wheat bran, (2) flooding the soil by irrigation, and (3) covering the soil surface with a plastic film to induce reducing soil conditions. Although Shinmura's method uses a wheat bran amendment rather than broccoli or grass, it also requires anaerobic conditions for its effectiveness. Therefore, Shinmura's soil disinfestation may be considered a modification of the BSD technique.

Momma et al. (2005) demonstrated that chlamydospores of *F. oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato, were effectively killed only when

N. Momma · M. Shishido (✉)
Faculty of Horticulture, Chiba University, 648 Matsudo, Chiba 271-8510, Japan
Tel. +81-47-308-8824; Fax +81-47-308-8824
e-mail: shishido@faculty.chiba-u.jp

K. Yamamoto
Technology Development Group, Keiyo Gas Corporation, Ichikawa, Japan

P. Simandi
Faculty of Agriculture, Water and Environmental Management, Tessedik Samuel College, Szarvas, Hungary

the soil conditions were changed as follows: (1) a wheat bran amendment to activate soil microorganisms, with subsequent oxygen depletion, and (2) the soil surface was covered with a plastic film to establish anaerobic conditions by preventing reoxygenation from the soil surface. In addition, it is interesting to note that successful BSD is always correlated with a decrease in soil pH and emission of an unpleasant odor (Momma et al. 2005). Kubo et al. (2005) also implicated organic acids such as acetic acid, as well as antagonistic activity of soil microorganisms in the mechanisms of BSD.

Fusarium oxysporum f. sp. *lycopersici* and *Ralstonia solanacearum* are casual agents of Fusarium wilt and bacterial wilt of tomato, respectively, and cause extensive economic losses worldwide. Because BSD is effective against both of these diseases (Momma et al. 2005), we investigated the production of organic acids during the course of BSD and evaluated the effect of organic acids on the survival of these pathogens.

Preparation of inocula. *Fusarium oxysporum* f. sp. *lycopersici* CU1 (race 1) was grown on potato dextrose broth for 7 days at 25°C with shaking. The fungal culture was passed through cheesecloth and centrifuged at 3000 × *g* for 5 min. The bud cells in the resulting pellet were resuspended in sterile distilled water (bud-cell suspension). For chlamydospores of *F. oxysporum* f. sp. *lycopersici*, a homogenized mycelial suspension of a 7-day-old, static culture in potato sucrose broth (PSB) was added to sterilized soil-water extract (1:1, w/w) and incubated at 25°C. The soil-water extract was prepared by adding 100 g of field soil (andosols; 58.0% sand, 35.2% silt, 6.8% clay) collected from the experimental farm of Chiba University to 100 ml of tap water, autoclaving for 20 min, and filtering the supernatant through a 0.44-μm filter. After a 3-week incubation, only chlamydospores were observed under a microscope. *Ralstonia solanacearum* EK1–2 (biovar 4, race 1) was grown in potato semisynthetic broth (Wakimoto et al. 1993) for 1 day at 28°C. The bacterial culture was centrifuged at 10000 × *g* for 10 min, and the resulting pellet was resuspended in sterile distilled water.

Survival of *R. solanacearum* in soil after BSD treatment. A cell suspension of *R. solanacearum* (5×10^8 cells/ml) was thoroughly mixed into the field soil at a rate of 1% (v/v) to infest the soil. To enumerate the initial viable population, a series of tenfold dilutions of the infested soil prepared with 0.1% water agar was spread onto petri plates with Hara and Ono's selective medium (Wakimoto et al. 1993) and incubated for 3–4 days at 28°C before counting bacterial colonies. The initial cell density was estimated at 2×10^5 CFU/g dry soil.

The infested soil (250 g dry soil) was placed into a glass bottle (600 ml) with a screw cap, amended with 2.5 g of wheat bran, irrigated to field capacity, and the bottle cap was tightly sealed with Parafilm (PM-996, Wako, Osaka) (BSD treatment). Field capacity was defined as the amount of water held in soil after excess water drained away (Veihmeyer and Hendrickson 1949) and was previously determined as soil water content after filtration using a funnel with filter paper for 12 h. In addition, nonamended soils with and without irrigation were prepared as control treatments. The gravimetric water content of the nonirrigated soil was approximately 0.5. After a 2-week incubation at 28°C in the dark, the population size of surviving bacteria was determined by a standard dilution method (Wollum 1982). This experiment was conducted twice.

In the first experiment, survival of *R. solanacearum* was significantly suppressed in the BSD treatment, i.e., no colony formed even in the nondiluted soil sample (Table 1). In contrast, in the control (untreated) and the irrigation (without wheat bran amendment), the viable population of *R. solanacearum* increased approximately ten times over the initial cell density. Similarly, the viability of this pathogen was significantly reduced by BSD in the second experiment. Previously, we reported that survival of chlamydospores of *F. oxysporum* f. sp. *lycopersici* was significantly restricted by BSD (Momma et al. 2005). The present work suggests that the applicability of BSD may be extended to the control of soilborne bacterial pathogens.

Organic acids produced in BSD treatment and their effects on soil pH. A plastic pot (Wagner pot, 1/5000 a) was filled with field soil (ca. 9 kg dry soil) after sieving (<2 mm). Four combinations of wheat bran amendment and irrigation treatments were used: wheat bran amendment with irrigation followed by covering the soil surface (BSD), wheat bran amendment without irrigation or covering (wheat

Table 1. Survival of *Ralstonia solanacearum* in wheat bran-amended and irrigated soil

Plot code	Treatment		log CFU[c] (/g dry soil)	
	Wheat bran[a]	Irrigation[b]	Experiment I[d]	Experiment II[e]
Control	No	No	6.2	NT
Irrigation	No	Yes	6.3	3.6 (0.02)
BSD	Yes	Yes	ND	ND

BSD, biological soil disinfestation; CFU, colony-forming unit; NT, not tested; ND, not detected
[a] Wheat bran was amended to field soil at a concentration of approximately 1.6% (w/w)
[b] Water was added to field capacity, and the test bottle was sealed with plastic film
[c] Viable cell number of *R. solanacearum* was measured after 2-week incubation by a standard dilution method on Hara and Ono's medium. The initial population in the infested soil was 2×10^5 CFU/g dry soil
[d] Two replications
[e] Three replications. Value in parentheses is standard error of the mean

bran), no wheat bran amendment with irrigation and covering (irrigation), and no wheat bran amendment without irrigation or covering (control). Wheat bran was incorporated into the soil at 40 g per pot (equivalent to 1 ton per 5 acres of field). For irrigation treatments, tap water was added to field capacity. The pots in the BSD and irrigation treatments were covered with transparent plastic film (PVC film, 0.05 mm thick, Tatsuno, Tokyo) and sealed with vinyl cords. The pots were arranged with a completely randomized design with three replicates and kept for 15 days on a bench in a greenhouse where temperature ranged from 18° to 44°C.

Soil samples were collected from all pots at 1, 2, 3, 6, 9, 12, and 15 days after treatment, and equal amounts of subsamples from three pots were pooled for each treatment. Soil pH was measured by the conventional method (soil:water = 1:2.5) using a pH meter (M-8, Horiba, Kyoto). Accumulated organic acids in the soil was measured as described by Lawongsa et al. (1987) using a high-performance liquid chromatograph (HPLC; LC-10AS, Shimadzu, Kyoto) equipped with an ultraviolet (UV) detecter (210 nm; SPD-10AV, Shimadzu) and connected to an integrator (Chromatopack, C-R6A, Shimadzu). The column (8 mm i.d. × 30 cm; ULTRON PS-80H, Shinwa, Kyoto) was operated at 60°C. The liquid carrier consisted of distilled water, adjusted to pH 2.1 with perchloric acid. The flow rate was 1 ml/min. Injection volume was 20 µl via an automatic injection loop. In this system, acetic, butyric, citric, lactic, formic, propionic, and valeric acids were detectable.

In both irrigated (BSD) and nonirrigated (wheat bran) treatments, acetic acid was detected when wheat bran was amended (Table 2). Although BSD treatment caused the accumulation of butyric acid in addition to acetic acid, the wheat bran treatment produced only acetic acid until 2 days after the start of the treatment. In addition, the concentration of acetic acid was lower than the BSD treatment in which the concentration kept increasing until the end of the incubation period. The concentration of butyric acid peaked at 6 days after the start of the BSD treatment and gradually decreased to 83% of the maximum concentration after 15 days (Table 2). In the HPLC analyses, of the seven organic acids tested, only acetic and butyric acids were detected in BSD treatment, and no substantial peak other than these two organic acids appeared in the HPLC charts (data not shown). In the irrigation treatment, none of these organic acids was detected.

The BSD treatment decreased soil pH to 5.5 and stayed at this level for 15 days after the start of the treatment (Fig. 1A). To evaluate the effect of organic acid accumulation on soil pH, we added acetic and/or butyric acid to the soil in a plastic vial (50 ml) at concentrations equivalent to that detected by HPLC after the BSD treatment (200–2000 mg/kg dry soil). When acetic acid or butyric acid was amended with 2000 mg/kg dry soil, soil pH lowered to 5.7 and 5.5, respectively (Fig. 1B). When both acids were added together, soil pH decreased to as low as 5.2 with 1900 mg acetic acid and 1700 mg butyric acid per kilogram of dry soil (Fig. 1C). However, this pH seemed to be the lowest pH obtainable with this combination of organic acids, because higher concentrations of these acids did not provide any additional decrease in soil pH.

Acetic and butyric acids were the two organic acids detected in the soil treated by BSD. The decrease in soil pH was correlated with accumulation of these acids (r^2 = 0.96 and 0.98, respectively). Although a small amount of acetic acid was detected early in the wheat bran treatment, the acid disappeared quickly, probably by volatilization. Amendments with these organic acids at amounts equivalent to those detected for the BSD resulted in pH values lower than the levels obtained during the course of the BSD. This is not surprising because biological processes of BSD are more complicated than a simple production of organic acids, and probably involve a variety of metabolic processes that negatively affect the decrease in pH. In addition, it is well recognized that soil organic materials have a buffering effect because of their high ion exchange capacity (Bohn et al. 1985). Therefore, acute soil acidification might have been prevented by the amendment with wheat bran.

Table 2. Accumulation of acetic acid and butyric acid in soil over time after amendment with wheat bran followed by irrigation and covering with film

Plot code	Treatment		Day						
	Wheat bran[a]	Irrigation and cover[b]	1	2	3	6	9	12	15
Acetic acid[c]									
Control	No	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Irrigation	No	Yes	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wheat bran	Yes	No	16.0	14.8	0.0	0.0	0.0	0.0	0.0
BSD	Yes	Yes	206.4	741.7	1142.7	1744.0	1901.2	1884.6	2134.0
Butyric acid[c]									
Control	No	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Irrigation	No	Yes	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wheat bran	Yes	No	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BSD	Yes	Yes	101.9	830.7	1203.1	1843.2	1703.7	1777.3	1514.9

[a] Wheat bran (40 g) was amended per pot (1/5000 a)
[b] Water was added to field capacity, and pots were covered with plastic film. Each treatment had three replications
[c] Measured in units of milligrams per kilogram of dry soil

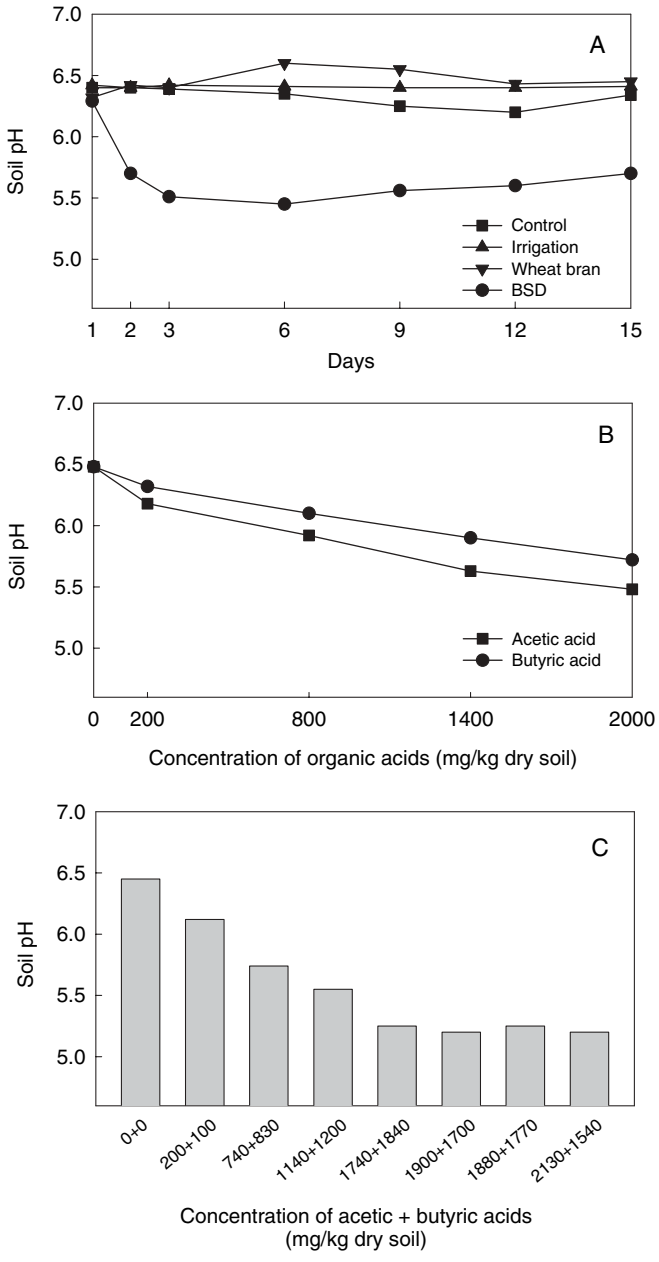

Fig. 1A–C. Change in soil pH during biological soil disinfestation (BSD) treatment and effect of organic acid amendment on soil pH (soil:water, 1:2.5). **A** Soil pH was traced during the course of the treatments. *Wheat bran*, wheat bran was added to field soil at ratio of approximately 1.6% (w/w); *Irrigation*, water was added to field capacity and the test bottle was sealed with plastic film. **B** Acetic acid or butyric acid was amended to soil at 200–2000 mg/kg soil. **C** A solution of acetic acid and butyric acid was added to the soil at the same rate as detected by the HPLC analysis of soils after BSD

Effect of organic acids on survival of pathogens. Survival of the pathogen propagules in organic acid solutions and in soils amended with organic acids was examined. One milliliter of a spore (3×10^6 spores/ml) or chlamydospore (3×10^6 chlamydospores/ml) suspension of *F. oxysporum* f. sp. *lycopersici* or a cell suspension (3×10^8 cells/ml) of *R. solanacearum* was mixed with 29 ml of acetic acid or butyric acid solutions of various concentrations, ranging from 200 to 2000 mg/l. The mixture in 50-ml plastic vials was incubated for 24–48 h at 25°C and 28°C for *F. oxysporum* f. sp. *lycopersici* and *R. solanacearum*, respectively. Viable populations of the propagules after the incubation were counted using the standard dilution method.

Bud cells (spores) of *F. oxysporum* f. sp. *lycopersici* were not viable in either acetic acid or butyric acid solution at 800 mg/l after 24 h of incubation. The fungal chlamydospores, on the other hand, were viable in acetic acid solutions of 800 mg/l and 1400 mg/l after 24 h of incubation but not after 48 h. They also survived in butyric acid solution at 2000 mg/l after a 48-h incubation, although fewer survived as the concentrations of the organic acids increased (Table 3). The population of *R. solanacearum* significantly decreased in 200 mg/l acetic acid or butyric acid solutions after a 24-h incubation and was totally diminished after 48 h (Table 3).

To examine survival of these pathogens in soils amended with these organic acids, autoclaved field soil (30 g dry soil) was infested with 4 ml of the chlamydospore suspension of *F. oxysporum* f. sp. *lycopersici* or cell suspension of *R. solanacearum*. Organic acid (acetic acid, butyric acid, or a mixture of these acids) was added to the infested soil at 200–2000 mg/kg dry soil, and the soil was watered to field capacity. Three replicates of the soil–acid mixtures were transferred to plastic bags (25 × 28 cm) and incubated at 25°C and 28°C for *F. oxysporum* f. sp. *lycopersici* and *R. solanacearum*, respectively. After 2 and 7 days of incubation, viable population sizes of the pathogens were determined by the standard dilution method.

Ralstonia solanacearum did not survive after 7 days of incubation when the concentrations of acetic acid and butyric acid were higher than 2000 and 1400 mg/kg dry soil, respectively (Table 4). When these organic acids (1000 mg/kg dry soil) were added together, *R. solanacearum* was not viable after 7 days. When the concentration of each organic acid was 2000 mg/kg dry soil, survival of *R. solanacearum* was completely suppressed within 2 days (Table 4). In contrast to the relatively high sensitivity of *R. solanacearum* to these organic acids, most of *F. oxysporum* f. sp. *lycopersici* propagules survived well when acetic and/or butyric acids were amended separately at 2000 mg/kg dry soil, even though viable propagules were reduced to approximately 1% of the initial density by the mixture of these acids (Table 4). Okazaki and Nose (1986) demonstrated that acetic acid and *n*-butyric acid accumulated in glucose-amended, flooded soil and that these acids played an important role in killing the chlamydospores of *F. oxysporum* f. sp. *raphani*, the radish yellows fungus. In their experiment, LC_{50} values of acetic acid and *n*-butyric acid were 1506 and 1522 mg/kg dry soil, respectively. In our study, chlamydospores of *F. oxysporum* f. sp. *lycopersici* were almost all killed in the 1400 mg/l acetic acid and the 2000 mg/l butyric acid solutions. However, when these organic acids were added to soil at 2000 mg/kg, near-maximum concentrations were detected in the soil after BSD by HPLC analysis, and their suppressive effects on these bacterial and fungal pathogens were reduced in comparison with the pure organic acid solutions. This implies that for practical application of BSD

Table 3. Survival of *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum* in acetic and butyric acid solutions for 24 or 48 h

Acid solution (mg/l)	*F. oxysporum* f. sp. *lycopersici*								*R. solanacearum*				pH	
	Bud cells (spores)				Chlamydospores								Acetic acid	Butyric acid
	Acetic acid		Butyric acid		Acetic acid		Butyric acid		Acetic acid		Butyric acid			
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h		
0	3.4 (0.01)	2.5 (0.01)	3.4 (0.01)	2.5 (0.01)	5.0 (0.10)	4.8 (0.01)	5.0 (0.10)	4.8 (0.01)	6.1 (0.29)	7.0 (0.12)	6.1 (0.29)	7.0 (0.12)	7.2	7.2
200	2.4 (0.01)	2.3 (0.01)	3.0 (0.03)	2.8 (0.22)	–	–	–	–	2.3 (0.06)	ND	2.0 (0.04)	ND	3.4	3.5
800	ND	ND	ND	ND	2.0 (0.27)	ND	3.7 (0.06)	3.2 (0.03)	ND	ND	ND	ND	3.1	3.2
1400	–	–	–	–	1.0 (0)	ND	2.9 (0.01)	1.2 (0.10)	–	–	–	–	3.0	3.1
2000	–	–	–	–	ND	ND	1.7 (0.05)	1.0 (0.39)	–	–	–	–	2.9	2.9

Survival of the pathogens in the organic acid solutions was determined after 24 and 48 h of incubation. Data given as log CFU/ml (±SE). Each treatment used three replications. ND, not detected

Table 4. Effect of acetic acid and butyric acid amendment on the survival of *Fusarium oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum*

Incubation (days)	Number of survived pathogen propagules										
	Nonamended control	Acetic acid (mg/l)				Butyric acid (mg/l)				Acetic acid + butyric acid	
		200	800	1400	2000	200	800	1400	2000	1000 + 1000	2000 + 2000
R. solanacearum											
2	7.0 (0.13)	6.6 (0.08)	5.7 (0.08)	2.4 (0.16)	4.0 (0.08)	5.7 (0.17)	5.3 (0.06)	2.3 (0.10)	3.9 (0.14)	4.9 (0.31)	ND
7	4.8 (0.14)	5.8 (0.17)	4.4 (0.05)	2.5 (0.14)	ND	4.9 (0.17)	3.4 (0.05)	ND	ND	ND	ND
F. oxysporum. f. sp. *lycopersici*											
2	5.7 (0.06)	6.0 (0.03)	6.0 (0.05)	6.0 (0.02)	5.8 (0.01)	5.8 (0.02)	5.9 (0.03)	5.9 (0.03)	5.6 (0.01)	5.7 (0.02)	5.9 (0.01)
7	5.6 (0.04)	5.9 (0.04)	5.9 (0.02)	5.9 (0.02)	5.7 (0.02)	5.9 (0.05)	5.9 (0.00)	6.0 (0.03)	6.0 (0.02)	5.9 (0.01)	3.4 (0.06)

Acetic acid and/or butyric acid was amended into the soil at 200–2000 mg/kg dry soil. Data given as log CFU/g dry soil (±SE). ND, not detected

to control soilborne pathogens, we need to consider the complexity of the soil environment, which may counteract the suppressive effect of the organic acids. For example, plant residues in soil may protect pathogen propagules from direct contact with the organic acids. Nevertheless, their efficacy may be reduced under field conditions; the results obtained in this study strongly suggest that both acetic acid and butyric acid are important factors contributing to suppression of these soilborne pathogens by BSD.

In organic acid solutions, survival of these pathogens was suppressed even at low concentrations. Similarly, in the soil amended with these organic acids, the survival of *R. solanacearum* was completely suppressed at concentrations lower than the maximum detected after BSD. This is not a surprise considering the antibiotic effect of acetic acid on some bacteria, including *Salmonella typhimurium*, *Escherichia coli* O157, and *Listeria monocytogenes* (Delaquis et al. 1999). However, *F. oxysporum* f. sp. *lycopersici* survived even at the maximum concentrations of these organic acids, suggesting that factors other than organic acids may be involved in suppression of *F. oxysporum* f. sp. *lycopersici*. For example, volatile antifungal compounds were released when cruciferous plants were incorporated into soil, thereby leading to disease suppression (Gamliel and Stapleton 1993). In our previous study, denaturing gradient gel electrophoresis analysis suggested that some soil bacteria specifically proliferating in the BSD treatment might play an important role in the suppression of *F. oxysporum* f. sp. *lycopersici* (Momma et al. 2005). Because many microorganisms produce antibiotics of various types (Handelsman and Stabb 1996; Raaijmakers and Weller 1998), we should also determine if BSD promotes production of antifungal compounds that are effective against *F. oxysporum* f. sp. *lycopersici*.

Acknowledgments This study was supported in part by Grants-in-Aid for Scientific Research (14560037) from the Japan Society for the Promotion of Science. We thank Bio-Trace Service of Keiyo Gas Co., Chiba, for assistance in the analyses of organic acids. We also thank Drs. Y. Amemiya, K. Inubushi, T. Usami, and T. Takeuchi for their valuable suggestions on the experiments.

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