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Changes in the Population Density of Bacterial Wilt Pathogen in Soil after Anaerobic Soil Disinfestation with Molasses and Subsequent Tomato Cultivation

Yasuhiro INOUE[1]*, Akira KUMAZAKI[2,3] and Kazuhiro NAKAHO[1]

[1] Institute for Plant Protection, National Agriculture and Food Research Organization, Tsukuba, Japan
[2] Gifu Prefectural Research Institute for Agricultural Technology in Hilly and Mountainous Areas, Nakatsugawa, Japan

Abstract

Anaerobic soil disinfestation (ASD) with water-soluble organic materials such as molasses is used to control bacterial wilt of tomatoes caused by the *Ralstonia solanacearum* complex, but the disease often recurs in post-ASD cultivation. Changes in the soil population density of the pathogen caused by ASD with molasses and subsequent tomato cultivation were investigated in the lower (LL, 30 cm-60 cm) and upper (UL, 0 cm-30 cm) soil layers from three fields with different soil characteristics to understand the causes of the pathogen density suppression and bacterial wilt recurrence. In all fields, the population density decreased in both the LL and UL after ASD. The bacterial density increased in the LL of all fields after post-ASD cultivation, but there was no difference in UL. These results indicate that tomato cultivation can increase the pathogen's survival rate after ASD treatment. Changes in the colonization of the pathogen in soil after ASD were investigated by laboratory tests. The colonization ability of the pathogen improved in both UL and LL soil after ASD but quickly returned to pre-ASD levels with air inflow. This may indicate that aeration can reduce the risk of pathogens surviving in soil after ASD.

Discipline: Agricultural Environment
Additional key words: deep soil layer, soil pathogen population, sustainability of disinfectant effect, water-soluble organic matter

Introduction

Bacterial wilt is one of the most severe diseases affecting tomato cultivation. It is a soilborne disease caused by the pathogenic bacterium *Ralstonia solanacearum* complex, which penetrates deep into the soil and can survive for extended periods (Coutinho 2005, Hayward 1991). The pathogen enters plants via root injuries and natural openings. Once it reaches the primary xylem, it moves to the secondary xylem and migrates to the shoots, where they block the water flow of the plant by propagating in the xylem vessels, thereby causing wilting and eventual death of the plant (Bae et al. 2015, Schell 2000). Large numbers of pathogen cells are released from infected tomato plants into the soil, spreading contamination (Inoue & Nakaho 2018, Inoue et al. 2018). However, the life history of these bacteria remains poorly understood, and the population density required for establishing infection is unclear. Effective disease management requires understanding the ecology of the pathogen in natural environments.

Soil disinfection (or soil disinfestation) is widely used to control bacterial wilt, and several methods are available. In Japan, chemical fumigants, such as chloropicrin, 1,3-dichloropropene, and dazomet, are widely used because they are inexpensive, the treatment method is simple, and the required effect can be obtained even at low temperatures in early spring and late fall. However, these disinfectants only affect the area where the volatilized ingredient penetrates, meaning that the disinfecting effect at a soil depth of approximately 30 cm is high but is lower in the deeper layers (Inoue & Nakaho 2019). Soil solarization is also an inexpensive disinfestation method. However, the killing temperature for the pathogen

*Corresponding author: inoue.yasuhiro845@naro.go.jp
[3] Present address: Sakata Seed Corporation, Yokohama, Japan
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must be 40°C or higher for several days (Date et al. 1993, Kongkiattikajorn & Thepa 2007). Sufficient temperatures cannot be obtained at a depth of 30 cm-50 cm, even during midsummer in warm regions, such as California in the United States and Saga Prefecture in Japan (Pullman et al. 1981, Yamaguchi et al. 1987). Therefore, the effectiveness of this strategy for controlling bacterial wilt is low. Under such circumstances, anaerobic soil disinfestation (ASD), also called biological soil disinfestation, using water-soluble organic substances, has recently attracted attention. ASD reduces the number of bacteria, fungi, and nematodes vulnerable to oxygen loss and organic acid production caused by the degradation of organic matter by soilborne bacteria (Momma 2008, Shennan et al. 2014). This method suppresses the soil population density of the bacterial wilt pathogen at depths of 60 cm (Inoue & Nakaho 2019) because water-soluble organic substances permeate into the deep layers of the soil, where reduction in the soil occurs by the activity of the soilborne bacteria. In Japan, molasses, sugar-containing diatomite, and ethanol are used as water-soluble organics (Inoue & Nakaho 2019, Kawabe et al. 2019, Momma et al. 2013, Otani 2018, Shinmura 2003).

Repetitive soil disinfection is performed in many farm fields. This is thought to be because the disinfecting effects only last for one growing season or because one application cannot completely suppress the onset of the disease. In our study, we observed that ASD with water-soluble organic substances suppressed the occurrence of bacterial wilt, but the incidence increased with repeated cultivations of tomatoes. It is important to determine the duration of the disinfection effects when considering the timing of the next disinfection. In addition, ASD with water-soluble organic substances has been performed in many farm fields. However, the differences in the effects of different soil types in these fields have not been previously investigated. Understanding the differences in effectiveness among different soil types is important when selecting a disinfection method.

The disease onset of bacterial wilt of tomato varies depending on factors such as the weather, the tomato variety, and the amount of available water (Hayward 1991). Therefore, it is difficult to compare the disinfection effects in different regions and terms using only the disease incidence. On the other hand, measuring the population density of the pathogen in the soil is useful for comparing disinfection effects because it directly measures the decrease in the pathogen. In addition, investigating how to change the colonization and population density of the pathogens in the soil after disinfection and post-cultivation may clarify why the disinfection effect does not persist. This study investigated the effects of ASD using molasses and tomato cultivation on the population density of the pathogen in the upper (UL, 0 cm-30 cm) and lower (LL, 30 cm-60 cm) soil layers in three different fields. Our results clarified the effects of suppressing pathogen density using ASD with molasses and how the pathogen density changed with subsequent tomato cultivation.

Materials and methods

1. Surveyed fields

Details of the three surveyed test fields are shown in Table 1. The first field was in Tochigi, Tochigi Prefecture (glass greenhouse, 4,860 m^2), a paddy field conversion site with andosol soil. Tomatoes were planted in the latter half of August and harvested until June of the following year. Until 2012, soil solarization was performed every July by covering water-saturated soil with a polyethylene film (over 0.02 mm thick) and leaving it for four weeks. ASD with molasses was performed three times, in 2013, 2015, and 2017. Soil solarization was performed in 2012, 2014, and 2016, and the field was reset to receive an initial ASD treatment in the following years. Soil samples were collected from six sites in the field.

The second field was in Hamamatsu, Shizuoka Prefecture (vinyl greenhouse, 200 m^2), which was converted from a citrus orchard with red-yellow soil. This field was planted with tomatoes in early September and harvested until early July of the following year. The field was disinfected annually with chloropicrin until 2016. ASD with molasses was performed in July 2017. The soil samples were collected from three sites in the field.

The third field was in Nakatsugawa, Gifu Prefecture (Nakatsugawa field; vinyl greenhouse, 120 m^2). It was a test field in the Nakatsugawa branch of the Gifu Prefectural Research Institute for Agricultural Technology and had brown forest soil. Tomatoes and eggplants were planted in April and harvested annually until November. The field was disinfected with carbamsodium in April 2013. ASD with molasses was administered in April 2014. Soil samples were collected from four sites. Kumazaki (2017) outlined the cultivation tests for this field in 2014.

2. Outline of ASD with molasses processing

ASD with molasses was prepared as follows. A field was plowed and flattened, and irrigation tubes were placed at intervals of 60 cm-80 cm. Then, they were covered with a polyethylene film approximately 0.02 mm thick. A liquid fertilizer injector diluted the molasses 0.4%-0.6% (v/v), and irrigation tubes applied an even

Table 1. Outline of the surveyed fields

Field name	Tochigi			Hamamatsu	Nakatsugawa
Location	Tochigi, Tochigi Prefecture			Hamamatsu, Shizuoka Prefecture	Nakatsugawa, Gifu Prefecture
House type	Glass greenhouse			Vinyl greenhouse	Vinyl greenhouse
Cultivated area	4,860 m^2			200 m^2	120 m^2
Soil type	Andosols			Red-yellow soil	Brown forest soil
Number of plantings	10,500 (until 2014) / 11,200 (after 2015)			212	216
Soil disinfection of the previous year	Solarization	Solarization	Solarization	Chloropicrin	Carbam-sodium[a]
Disease occurrence in pre-ASD[b] cultivation	30% (Green-guard[c])	0.5% (Green-force)	A few (Green-force)	9.4% (Green-guard)	> 3% (Green-guard, B-baria and double grafted of both)
Date of ASD execution	2013.07.19	2015.07.19	2017.07.19	2017.07.26	2014.04.15
Amount of molasses used	4,320 kg (889 g/m^2)	4,320 kg (889 g/m^2)	4,320 kg (889 g/m^2)	192 kg (960 g/m^2)	120 kg (1,000 g/m^2)
Disease occurrence in post-ASD cultivation	None (Green-guard and Green-force)	None (Green-force)	A few (Green-force)	None (B-baria)	None (Green-guard, B-baria and double grafted of both)
Soil collection date before ASD	2013.06.17	2015.06.23	2017.06.01	2017.07.20	2013.12.25
Soil collection date after ASD	2013.08.19	2015.08.27	2017.08.30	2017.08.23	2014.05.12
Soil collection date after post-ASD cultivation	2014.07.02	2016.06.23	2018.06.14	2018.07.26	2014.12.25

[a] Not registered as a disinfectant for bacterial wilt of tomato in Japan
[b] ASD: Anaerobic soil disinfection
[c] Varieties of bacterial wilt-resistant rootstock used in the cultivation

flow throughout the field. Approximately 150-160 L/m^2 of water was used in the Tochigi and Nakatsugawa fields and > 200 L/m^2 in the Hamamatsu field. The film remained in place for more than four weeks after the end of irrigation. In the Tochigi field, the soil blackening was used as the indicator of soil reduction. In the soils of Hamamatsu and Nakatsugawa, the production of ferrous iron was confirmed using the dipyridyl method (Kumada & Asami 1957). In this study, tomato cultivation after ASD with a disease incidence of 0.1% or less was considered successful for disinfestation and was used for analysis. For the Tochigi and Hamamatsu fields, the survey for disease occurrence was conducted by interviewing farmers on soil collection days in June and July in 2014, 2016, and 2018. In the Nakatsugawa field, a survey for disease occurrence was conducted as needed, and cultivation tests were conducted simultaneously. The rootstock varieties used for cultivation and the degree of disease are listed in Table 1.

3. Soil sampling and measurement of soil population densities

Soil was collected using a root auger set (Daiki Rika Kogyo Co., Ltd., Saitama, Japan). An auger, 4 cm in diameter, was inserted to a depth of 30 cm, and the soil was collected as the upper layer (UL, 0 cm-30 cm). Next, a 3 cm auger collected soil at a depth of 60 cm for a lower layer (LL, 30 cm-60 cm) sample. More than 100 g of soil was collected for each sample. The collection site was set within an error of 30 cm before and after ASD and after cultivation, using the pillars of the greenhouse as markers. The collected soil was placed in a plastic bag, as much air as possible was removed, shielded from the light with a cardboard box, and stored at room temperature (approximately 25°C) until analysis because the number of viable *R. solanacearum* cells is reduced at low temperatures (Imazaki & Nakaho 2010). It was confirmed in multiple soils that the detection of *R. solanacearum* does not change for approximately two weeks, even when stored at room temperature; the detection conditions were confirmed effective in previous studies. The analyses were conducted within two weeks of sample collection. The pathogen was detected using the most probable number-polymerase chain reaction (MPN-PCR) method (Inoue & Nakaho 2014) with a quantification range modified to 3-2,400 colony-forming units (cfu)/g. Primers and reaction conditions were described by Inoue et al. (2018).

4. Determining changes in the soil population densities of the bacterial wilt pathogen

Data on the soil population density before and after ASD were taken at 14 points in the Tochigi field, 3 points

in the Hamamatsu field, and 3 points in the Nakatsugawa field, for a total of 20 points. Similarly, data after ASD and post-cultivation were taken at 15 points in the Tochigi field, 3 points in the Hamamatsu field, and 4 points in the Nakatsugawa field, for a total of 22 points. Assuming that the collection sites were the same, the Wilcoxon signed-rank test was performed with the null hypothesis that there was no difference in the detection results and the alternative hypothesis that there was a change. EZR statistical software in the R Commander RT (Kanda 2013) ver. 1.15 software was used for the test, and the value of 0 was set below the detection limit. In addition, the Wilcoxon rank-sum test was performed when there was no correspondence between the soil collection points.

5. Investigation of soil colonization of the bacterial wilt pathogen after ASD

Untreated and ASD-treated soil was inoculated with bacterial wilt pathogen to investigate differences in the pathogen colonization. *Ralstonia pseudosolanacearum* 8107R (a rifampicin-resistant strain derived from MAFF [Ministry of Agriculture, Forestry and Fisheries, Japan] 107632) was suspended in sterile distilled water at an optical density of 0.3 at 600 nm, and the population density of the suspension was measured by plating 10 µl of a 10^5-fold diluted suspension onto YP medium and the average number of colonies formed on three plates was calculated. The soil treated with ASD was prepared in the laboratory using the following method. Andosol collected from a farm field in Tsukuba City, Ibaraki Prefecture was used. The soil was collected separately into upper (0 cm-30 cm, UL) and lower (30 cm-60 cm, LL) layers, and each was sieved through a 4 mm mesh. The soil was filled into 320 mL squeeze bottles, and 0.6% molasses solution was added until the bottles were full. The lid was left slightly loose for the first two days to allow generated gases to escape and then tightened to keep the air out. The bottle was stored at 35°C for at least four weeks. After the treatment, the soil was removed from the bottle, wrapped in gauze, and left overnight in an incubator at 35°C so the water content was almost the same as before ASD. This point was defined as the first day after ASD. The soil was placed in a polyethylene (PE) bag shielded from light using a cardboard box and stored at room temperature. A portion of the soil was placed in a nylon cellulose (NC) bag to remove as much air as possible using a hand pump, and the bag was closed with a sealer. The PE bag allowed air to pass through, whereas the NC bag blocked air. The soils used in the study were soil before ASD, soil one day after ASD, soil placed in PE bags for one, two, three, and four weeks after ASD, and soil placed in NC bags for two or four weeks after ASD. Treated soils, 5 g soil on a dry-weight basis, were placed in a 50 mL tube, inoculated with 50 µL of 10^3-fold diluted bacterial suspension, and stirred. The inoculated soil was placed in the dark at 25°C for one week. The pathogen density in the soil was determined using the MPN-PCR method described above, and 50 µL and 5 µL of the soil extract were spread on YP media containing rifampicin and cycloheximide with two replicates, respectively. When the pathogen density was at least 500 cfu/g, the number of colonies formed on the medium was counted, and the average of the two replicates was calculated, $\times 10^2$ and $\times 10^3$ as the number of the pathogen per g, respectively. Each test was performed in three replications, and the average value was calculated. Results were expressed as the log of the arithmetic mean bacterial population in each independent experiment. The colonization rate was calculated by dividing the number of pathogens detected in the soil by the number of inoculated pathogens.

Results

1. Case study in Tochigi field

Table 2 shows the soil population densities of the pathogen in the Tochigi field. The soil population densities of the pathogen after ASD were measured in July 2013 and decreased in both UL and LL, although they were detected in LL at both sites. Bacterial wilt occurred in approximately 30% of the 10,500 plants in the pre-ASD cultivation, but no disease was recorded post-ASD cultivation. However, the population of pathogenic bacteria increased in the LL at both sites after post-ASD cultivation. ASD was conducted in July 2015, and the pathogen was detected in the LL at all sites and in the UL at four sites before disinfestation but was not detected at any site in either layer after disinfestation. Bacterial wilt occurred in approximately 0.5% of the plants in the pre-ASD cultivation but not in the post-ASD cultivation. The pathogen was detected at only one site in the UL after post-ASD cultivation. ASD was conducted in July 2017, and the pathogen was detected at a density of 3 cfu/g in the LL at one site before disinfestation but not at any site after disinfestation. A few plants exhibited bacterial wilt in pre-ASD cultivation, which was also observed in post-ASD cultivation. At the end of subsequent cultivation, the pathogen was detected in the LL soil at three sites and in the UL soil at one site.

2. Case study in Hamamatsu field

The soil population densities of the pathogens for the Hamamatsu field are shown in Table 3. Before ASD, the pathogen was detected in the LL at two sites but not in the soil after ASD. Bacterial wilt did not occur post-ASD

Table 2. Effects of anaerobic soil disinfestation with molasses against bacterial wilt pathogens in tomato cultivation fields (Tochigi, Tochigi Prefecture)

Point in the field	Depth (cm)	Anaerobic soil disinfestation (ASD)		Post-cultivation
		Before	After	
		cfu/g[a]	cfu/g	cfu/g
ASD in 2013[b]				
West-South	0-30	Nt	< 3	< 3
	30-60	Nt	< 3	< 3
Middle-South	0-30	> 2,400	< 3	< 3
	30-60	> 2,400	3.6	93
East-South	0-30	Nt	< 3	< 3
	30-60	Nt	< 3	< 3
West-North	0-30	Nt	< 3	< 3
	30-60	Nt	9.2	460
Middle-North	0-30	> 2,400	< 3	< 3
	30-60	> 2,400	< 3	< 3
East-North	0-30	Nt	< 3	< 3
	30-60	Nt	< 3	< 3
ASD in 2015[b]				
West-South	0-30	< 3	< 3	< 3
	30-60	43	< 3	< 3
Middle-South	0-30	< 3	< 3	< 3
	30-60	240	< 3	< 3
East-South	0-30	> 2,400	< 3	< 3
	30-60	93	< 3	< 3
West-North	0-30	3.6	< 3	3.6
	30-60	1,100	< 3	< 3
Middle-North	0-30	7.2	< 3	< 3
	30-60	3.6	< 3	< 3
East-North	0-30	7.2	< 3	< 3
	30-60	150	< 3	< 3
ASD in 2017[b]				
West-South	0-30	< 3	< 3	< 3
	30-60	< 3	< 3	9.2
Middle-South	0-30	< 3	< 3	Nt
	30-60	< 3	< 3	Nt
East-South	0-30	< 3	< 3	< 3
	30-60	< 3	< 3	23
West-North	0-30	< 3	< 3	Nt
	30-60	< 3	< 3	Nt
Middle-North	0-30	< 3	< 3	3.6
	30-60	3	< 3	15
East-North	0-30	< 3	< 3	nt
	30-60	< 3	< 3	nt

[a] Estimated population density of the pathogenic bacterium (colony-forming unit/g dry-weight soil) in the soil. < 3: below detection limit; > 2,400: above detection limit in the method of this work; nt: not tested

[b] Anaerobic soil disinfestation with molasses was done in 2013, 2015, and 2017.

Table 3. Effects of anaerobic soil disinfestation with molasses against bacterial wilt pathogens living in tomato cultivation fields (Hamamatsu, Shizuoka Prefecture)

Point in the field	Depth (cm)	Anaerobic soil disinfestation		Post-cultivation
		Before	After	
		cfu/g[a]	cfu/g	cfu/g
Middle-North	0-30	< 3	< 3	< 3
	30-60	93	< 3	< 3
Middle-Middle	0-30	< 3	< 3	< 3
	30-60	< 3	< 3	< 3
East-South	0-30	< 3	< 3	< 3
	30-60	21	< 3	7.2

[a] Estimated population density of the pathogenic bacterium (colony-forming unit/g dry-weight soil) in the soil. < 3: below detection limit

Table 4. Effects of anaerobic soil disinfestation with molasses against bacterial wilt pathogens living in tomato cultivation fields (Nakatsugawa, Gifu Prefecture)

Point in the field	Depth (cm)	Anaerobic soil disinfestation		Post-cultivation
		Before	After	
		cfu/g[a]	cfu/g	cfu/g
West-East	10-30	43	3.6	1,100
	30-60	240	< 3	7.4
West-North	10-30	> 2,400	23	< 3
	30-60	240	23	> 2,400
Middle-Middle	10-30	nt	240	> 2,400
	30-60	nt	< 3	460
Middle-South	10-30	< 3	3	3.6
	30-60	3.6	< 3	< 3

[a] Estimated population density of the pathogenic bacterium (colony-forming unit/g dry-weight soil) in the soil. < 3: below detection limit; > 2,400; above detection limit in the method of this work; nt: not tested

cultivation, although it did occur in 20 of the 212 plants in pre-ASD cultivation. However, the pathogen was detected at only one site in the LL after post-ASD cultivation. The decrease in bacterial density owing to ASD and the increase in bacterial density after cultivation in LL were similar to the results for the andosols in the Tochigi field.

3. Case study in Nakatsugawa field

The soil population densities of the pathogen in the Nakatsugawa field are shown in Table 4. The pathogen was detected in the UL at all times after ASD but in the LL at only one site. No occurrence of bacterial wilt was observed in the post-ASD cultivation, but increased pathogen density was observed in three of four sites in both UL and LL.

4. Assessment of the changes in the soil population densities of the pathogen

Data from 20 sites before and after ASD were used. The soil population density of the pathogen in LL decreased at 14 sites and did not increase at any site. The Wilcoxon signed-rank test determined the significance probability to be $p = 0.00012$, suggesting that the soil population density decreased at a significance level of 1%. Soil population density in the UL decreased at eight sites and increased at one. The Wilcoxon signed-rank test suggested that soil population density decreased at a significance level of 1% ($p = 0.0078$).

Data from 22 sites were used to analyze the changes in soil population density of the pathogen after ASD and post-cultivation. The soil population density of the

pathogen in LL increased at nine sites after post-cultivation. The Wilcoxon signed-rank test suggested that the soil population density of the pathogen had increased at a significance level of 1% (p = 0.0039). In contrast, in the UL, there were increases at five sites and a decrease at one site, and no significant change in soil population density of the pathogen was detected (p = 0.29 by Wilcoxon signed-rank test).

5. Changes in colonization of the bacterial wilt pathogen in soil after ASD

Changes in the colonization and growth of the bacterial wilt pathogen in the soil after ASD were clarified through laboratory tests. Approximately 1/10 to 1/100 of the inoculated pathogen was recovered from the untreated (pre-ASD) soil one week after the inoculation (Fig. 1). The number of pathogens detected in the soil one day after ASD was 10-100 times the number inoculated, suggesting that the pathogen proliferated in the soil. Although there was variation between trials, pathogen colonization in the soil decreased over time and reached the same or lower levels as that pre-ASD about two weeks post-ASD. When the post-ASD soil was stored in NC bags, bacterial colonization and growth were maintained better than when stored in PE bags for four weeks (Table 5). This indicates that the colonization ability of the pathogen improves in the soil after ASD and that the soil quickly returns to its original state with air inflow. In contrast, it has been suggested that the colonization and growth of the pathogen may be promoted in soil without air inflow after ASD.

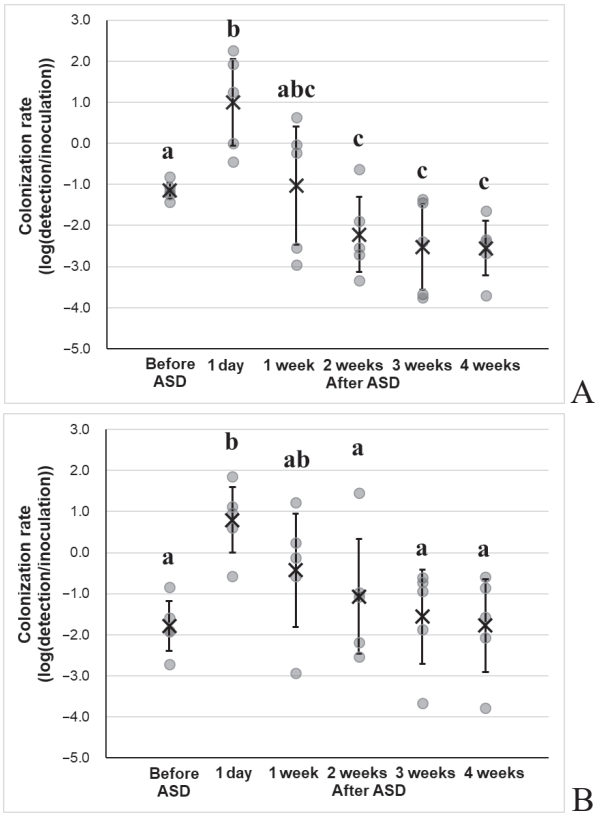

Fig. 1. Scatter plot of colonization rate of *Ralstonia pseudosolanacearum* in soil before and 1 day, 1, 2, 3, and 4 weeks after anaerobic soil disinfestation (ASD)
The number of bacteria detected in the soil was divided by the number of inoculated bacteria, and the logarithm of the result taken. Each data group is shown as a grey circle (n = 5 in each group). Crosses indicate the mean in each group. *Error bars* represent ± standard error of the mean. Means with different letters in the same disease severity index differed significantly (P < 0.05, Tukey-Kramer honestly significant difference test). Panel A: The soil collected from 0 cm-30 cm. Panel B: The soil collected from 30 cm-60 cm.

Table 5. Colonization rate of *Ralstonia pseudosolanacearum* in soil after anaerobic soil disinfestation (ASD) stored in polyethylene (PE) and nylon cellulose (NC) bags

	Trial 1					Trial 2				
		Upper layer soil[a]		Lower layer soil[b]			Upper layer soil		Lower layer soil	
ASD	Inoculated[c]	Detected[d]	log(D/I)[e]	Detected	log(D/I)	Inoculated	Detected	log(D/I)	Detected	log(D/I)
Before	4,000.0	416.7	−0.98	84.1	−1.68	19,700.0	11,383.3	−0.24	150.0	−2.12
After 1 day	5,133.3	30,500.0	0.77	21,333.3	0.62	4,133.3	309,666.7	1.87	111,833.3	1.43
After 2 weeks in a PE bag	5,900.0	21.3	−2.44	35.3	−2.22	16,800.0	3,133.3	−0.73	83.3	−2.30
After 2 weeks in a NC bag		82,333.3	1.14	216.7	−1.44		16,166.7	−0.017	7,166.7	−0.37
After 4 weeks in a PE bag	4,966.7	433.3	−1.06	1.2	−3.62	20,566.7	2,500.0	−0.92	116.7	−2.25
After 4 weeks in a NC bag		62,333.3	1.10	241.0	−1.31		126,833.3	0.79	22,666.7	0.042

[a] The soil collected from 0 cm-30 cm.
[b] The soil collected from 30 cm-60 cm.
[c] The number of bacteria inoculated (cfu/g).
[d] The number of bacteria detected in the soil (cfu/g).
[e] The number of bacteria detected in the soil divided by the number of bacteria inoculated, and then the logarithm of the result.

Discussion

This study showed that ASD with molasses reduced the soil population density of the bacterial wilt pathogen, and the treatment was effective in wide layers—0 cm-60 cm. Inoue and Nakaho (2019) reported the effects of various soil disinfection methods on the soil population density of the bacterial wilt pathogen. Their research suggested that ASD with a water-soluble organic substance was more effective in reducing the soil population density of pathogens at LL than other disinfection methods; however, only a few examples were provided. The ASD with molasses trial in their study was conducted in gray lowland soils. Pathogen control by ASD is affected by many environmental factors, such as soil temperature, soil type, and pathogen type (Bonanomi et al. 2010, Shrestha et al. 2016). This study showed that ASD with molasses reduced the population density of the bacterial wilt pathogen in the soil in three fields with different soil types. In Japan, ASD with sugar-containing diatomite or dried molasses effectively controls wilt pathogens in the lower layers (Kawabe et al. 2019, Maeda et al. 2017, National Agriculture and Food Research Organization 2019, Otani 2018). The tests were conducted on different soil types in various regions. These results indicated that ASD with water-soluble organic substances can decrease the soil population density of the pathogen in many types of farm fields.

Furthermore, this study showed that the soil population of the pathogen increased in LL of ASD-treated soil followed by post-cultivation. In post-ASD cultivations in the Tochigi field, soil solarization was conducted before the next cultivation. During the cultivation after solarization in 2014, bacterial wilt increased. The farmer reported the incidence of bacterial wilt to be about 0.9% by June 2019, following soil solarization in July 2018 (personal communication). As mentioned in the Introduction, the effectiveness of solarization in reducing the population density of the pathogen was high in the UL but low in the LL. The results of field surveys have shown that the presence of the pathogen in LL is related to plant infection (unpublished data). These facts indicate that the pathogens that increased in LL may have affected the occurrence of bacterial wilt in the subsequent cultivation.

It is unclear why bacterial wilt pathogens were not detected after ASD but were detected again after tomato cultivation. Our research group has previously shown that the pathogen migrates from wilted plants to the soil and contaminates the soil (Inoue & Nakaho 2019, Inoue et al. 2018). However, there were no wilted plants around the soil collection sites in this study. Therefore, pathogen detection after cultivation is unlikely due to the migration from infected plants to the soil. In the soil after ASD, where NC bags blocked air inflow, many of the inoculated *R. pseudosolanacearum* survived or grew. However, the number of bacteria decreased rapidly in PE bags, which allowed air inflow. Similar effects were observed in soils from the Tochigi and Hamamatsu fields (Supplementary Table 1). Soils after ASD may be more susceptible to the bacterial wilt pathogen, and the pathogen that remains in the soil may grow. The results of this study suggest that it is important to allow air to pass quickly after ASD treatment.

In ASD, the increased soil temperature due to solar heat increases the disinfestation effect (Blok et al. 2000, Butler et al. 2014). The UL layer was assumed to have a greater soil temperature increase than the lower layer. Therefore, it was predicted that more pathogens would survive in the LL than in the UL. Differences in the number of microorganisms in the soil may also affect the growth of the pathogen (Lee et al. 2017). In any case, to maintain the disinfection effect, it is necessary to thoroughly disinfect the lower layer and promptly return the soil to its pretreatment condition after ASD. Our research group will continue to analyze the dynamics of the bacterial wilt pathogens *R. solanacearum* and *pseudosolanacearum* in soil to improve the available technology for suppressing the occurrence of this disease.

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Supplementary Table 1. Colonization of *Ralstonia pseudosolanacearum* in soil before and 3 days, 3 and 6 weeks after anaerobic soil disinfection

ASD	Hamamatsu[a]		Tochigi	
	UL[b]	LL	UL	LL
Before	< 3[c]	< 3	3	< 3
After 3 days	> 2,400	460	> 2,400	1,100
After 3 weeks	460	43	38	3.6
After 6 weeks	23	< 3	< 3	< 3

[a] soil sampling location

[b] UL: upper layer soil (0 cm-30 cm); LL: lower layer soil (30 cm-60 cm)

[c] Estimated population density of *R. pseudosolanacearum* (colony-forming unit/g dry-weight soil) in the soil. < 3: below detection limit; > 2,400: exceeding detection limit