Suppression of Growth of *Ralstonia solanacearum*, Tomato Bacterial Wilt Agent, on/in Tomato Seedlings Cultivated in a Suppressive Soil

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Received June 3, 1998; accepted in revised form November 4, 1998

To identify the sites responsible for the suppressiveness of tomato bacterial wilt in a suppressive soil, population dynamics of *Ralstonia solanacearum* in non-rhizosphere soil, roots and stems of tomato plants was compared between a wilt-conducive soil and a suppressive soil both of which were artificially infested with the pathogenic strain SL8. Rhizobacteria were recovered as two fractions; root fraction-1 obtained after four washings in water, which was assumed to correspond to the rhizoplane, and root fraction-2 that was macerated after the washings and was assumed to be the fraction corresponding to the part inside of the roots and the firmly attached cells to the rhizoplane. In the conducive soil, all the tomato seedlings wilted while none wilted in the suppressive soil during the 31-d period of cultivation after transplanting to soils infested with the pathogen at an initial density of 10^3 cfu per g dry soil. The number of pathogens in the conducive soil increased first in root fraction-1, then in root fraction-2 and in stems up to 10^8-10^{10} cfu per g wet plant tissue during the 16-d period of cultivation after transplanting. In contrast, in the suppressive soil, the number of pathogens remained below 10^6 cfu per g tissue both in the two root fractions and in stems for 40 d. Survival rate of the pathogen inoculated into the tomato-free soils was higher in the suppressive soil than in the conducive soil. These results suggest that prior multiplication in root fraction-1 at a density above 10^6 cfu per g root is necessary for the pathogen to multiply in root fraction-2 and stems at a density above 10^6 cfu per g for the occurrence of wilt, and that the primary site for suppressiveness of the disease in tomato plants cultivated in the suppressive soil in this report was located in root fraction-1.

Key Words: **disease suppressive soil, *Ralstonia solanacearum*, rhizoplane, soilborne plant disease, tomato bacterial wilt.**

Ralstonia solanacearum Smith (Yabuuchi et al. 1995) is a soilborne pathogen destroying a variety of plants in the tropical, subtropical, and warm temperate regions of the world. Although a large number of studies have been conducted to control the disease (Hayward 1991; Hartman and Elphinstone 1994) including biological control, little attention has been

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given to the microscale distribution and population dynamics of the pathogen. So far, few studies have been reported on the spatial distribution of the pathogen in plant (Trigalet and Demery 1986; Hara and Ono 1991; Vasse et al. 1995; Nakaho 1997) and in soil (Toyota and Kimura 1996).

It has been known that the incidence of the disease can be reduced in some soils (Ho et al. 1988; Koga et al. 1997) and by several cultural practices such as appropriate cropping systems and soil amendments (Hayward 1991; Hartman and Elphinstone 1994). Information on the dynamics and distribution of the pathogen in soil-plant systems under such disease-suppressive conditions may enable to develop effective methods of control. As for the fungal pathogen, for example, a useful mathematical model was reported which described the behavior of the pathogen in the rhizosphere as well as enabled to interpret the effects of an antagonistic biocontrol agent (Bailey and Gilligan 1997; Gilligan and Bailey 1997).

In an area in Yamaguchi Prefecture, Japan, tomato plants have been cultivated with few outbreaks of bacterial wilt in spite of continuous cropping for more than 10 y. In this report, we compared the population dynamics of *R. solanacearum* in the non-rhizosphere soil, roots and stems of tomato between a wilt-suppressive and a wilt-conducive soil in Yamaguchi to identify the sites for suppression.

MATERIALS AND METHODS

Soils. Samples from two soils, Mutsumi and Yamadai soils, were collected at a depth of 0–20 cm in May, 1996. Mutsumi soil was sampled from a farmer's greenhouse field in Yamaguchi Prefecture. In the sampling field, cow manure had been applied to the soil annually (100 Mg ha^{-1} y^{-1}) and tomato plants had been grown during a period of 8 y, with few outbreaks of bacterial wilt. Yamadai soil was sampled in a ryegrass-grown field in Yamaguchi University. Samples from both soils were sieved (4 mm) and stored at room temperature. Before each experiment, the soil samples were preincubated for 14 d at 30°C under a water potential of −6.2 kPa (pF 1.8). As for the soil samples for the inoculation of *R. solanacearum* strain SL8, a portion of soil water (ca. 50 g water per kg dry soil) was removed by air-drying 3 d before the inoculation in order to adjust the soil water potential at −6.2 kPa after addition of the SL8 inoculum.

Some physicochemical and microbiological characteristics of the soils are shown in Table 1. Bacterial antagonists against *R. solanacearum* indicated in Table 1 were enumerat-

Table 1. Chemical and microbiological properties of soils (before experiment).

Soil	pH (H_2O)	Organic C (%)	Total N (%)	CEC (cmol(+) kg^{-1})	Sand	Silt	Clay
					(%)		
Mutsumi	6.5	4.28	0.42	27.5	48	29	23
Yamadai	4.4	1.62	0.15	13.8	55	25	20

Soil	Bacteria[a] ($\times 10^7$)	Fungi[b] ($\times 10^5$)	Actinomycetes[c] ($\times 10^7$)	Fluorescent pseudomonads[d] ($\times 10^3$)	Antagonistic bacteria[a] ($\times 10^6$)
Mutsumi	5.1 (0.9)[e]	2.1 (0.3)	2.5 (0.4)	220 (11)	3.7
Yamadai	1.5 (0.3)	2.9 (0.2)	0.3 (0.1)	0.4 (0.4)	0.83

[a] On YG agar medium (Soil Microbiological Society of Japan 1992); [b] on Martin's medium (Martin 1950); [c] on 1% starch medium (Takaki et al. 1992); [d] on King's medium B (King et al. 1954); [e] SE in parenthesis.

ed as follows: bacterial colonies grown on YG agar (Soil Microbiological Society of Japan 1992) were transferred to a new YG agar and incubated at 30°C for 10 h. Then, the surface of the YG agar was sprayed on with the suspension of *R. solanacearum* strain SL8 (ca. 10^8 cells mL^{-1}), and the YG agar was kept at 30°C for an additional period of 48 h. The bacterial colonies that formed a clear zone of strain SL8 were counted as antagonists. No mucoidal colonies of *R. solanacearum* were detected from the two soils before the experiments using the selective medium reported by Hara and Ono (1983) (detection limit: ca. 2×10^2 cfu (colony-forming unit) per g dry soil).

Pathogen. *R. solanacearum* strain SL8, isolated from a wilted tomato plants grown in the Mutsumi area in Yamaguchi Prefecture, was used throughout the experiments. The strain was precultured on Hara and Ono's selective medium at 30°C for 2 d and fluidal colonies were suspended in sterile water for use as inoculum. The cell density of the suspension was measured by direct microscopic counting after ethidium bromide staining (Someya 1995).

Severity of bacterial wilt of tomato plants grown in soil. Seeds of wilt-susceptible tomato (*Lycopersicon esculentum* Mill cv. Momotarou) were immersed in sterile water for 1 d, sown in small pots ($2.5\times2.5\times4.5$ cm) (1 seed pot^{-1}) containing preincubated Mutsumi or Yamadai soil, and kept in a growth chamber (30°C, light 12 h/dark 12 h). Ten days after germination, the seedlings together with the soil in the small pot were transplanted to pots 11 cm in diameter (5 seedlings per pot, 8 pots for each soil) containing 300 g (dry wt.) of Yamadai or Mutsumi soil infested with SL8. The infested soils were prepared just before transplanting by spraying a SL8 inoculum suspension (1×10^5 cells mL^{-1}) onto the surface of the preincubated soils and mixing them to reach a final population level of about 10^3 cells per g dry soil. Then the pots containing the seedlings were kept in a growth chamber (30°C, light 12 h/dark 12 h). Soil water potential was maintained at about -6.2 kPa by the addition of sterile water twice a day. The number of wilted plants was recorded during a period of 31 d. Some of the wilted seedlings were tested for the presence of the causal agent for wilting by cutting the stem to identify a white viscous suspension of the pathogen. The experiment was repeated twice with Yamadai soil and three times with Mutsumi soil.

Population dynamics of strain SL8 in soil. The preincubated Mutsumi or Yamadai soil was inoculated with a suspension of strain SL8 (1×10^7 cells mL^{-1}) and mixed well to reach a final population level of about 10^5 cells per g dry soil. Thirty grams (dry wt.) of the inoculated soil sample were packed in 50 mL beakers, incubated at 30°C under a water potential of -6.2 kPa, and the number of *R. solanacearum* was counted periodically using Hara and Ono's selective medium ($n=5$). For each enumeration, three beakers containing the soil sample were analyzed. The detection limit was ca. 2×10^2 cfu per g dry soil in both soils.

Population dynamics of *R. solanacearum* and total number of aerobic bacteria in rhizoplane, roots, and stems. Five plants grown in a pot as described above were combined and soil particles adhering to their roots were removed using sterilized forceps in sterile water. Then, the roots were shaken four times in sterile water (20 times weight to fresh root weight) for 15 min each to obtain the rhizoplane microorganisms. In this report, the combined fraction of the 1st to the 4th shakings was designated as fraction-1. The washed roots were weighed after removal of the excess water on the root surface, macerated using a sterilized mortar and pestle, suspended in 200 mL sterile water, and shaken for 30 min to obtain further microorganisms in/on roots (designated as fraction-2). In a preliminary experiment, we observed that (1) the number of bacteria obtained by shaking decreased

drastically until the 4th shaking, and that (2) the number of bacteria obtained by the 5th shaking was nearly equal to 1% of that by the 1st shaking and to 2–5% of that of the roots macerated after the 4th shaking. The results using tomato plants grown in Mutsumi soil are shown in Fig. 1. A similar trend of change in the bacterial number was observed in Yamadai soil (data not shown). The results suggest that most of the easily extractable rhizobacteria can be obtained until the 4th shaking, in fraction-1. We assumed that fraction-1 corresponded to the rhizoplane. Fraction-2 consisted of hardly extractable rhizobacterial cells, which were assumed to reside inside the roots or to be firmly attached to the surface of the roots. Stems (0–5 cm from the soil surface) of the seedlings were also weighed (fresh wt.), macerated, and shaken in sterile water to obtain the bacterial cells in/on stems. For fractions-1 and -2 and stems, the number of *R. solanacearum* was counted on Hara and Ono's selective medium ($n=5$). The number of total aerobic bacteria was counted in fractions-1 and -2 using YG agar ($n=5$). These bacterial counts were performed at 3, 6, 10, 15, 25, and 40 d after transplanting in the Mutsumi soil and 2, 4, 7, 10, 13, and 16 d in the Yamadai soil using three pots (15 plants) selected randomly at each sampling, and expressed as cfu per g wet roots or stems measured. The detection limit of *R. solanacearum* per g wet plant tissue was about 4×10^3 cfu in fraction-1, and 1×10^3 cfu in fraction-2 and stems. Similar experiments to analyze the bacterial population dynamics were repeated twice.

RESULTS AND DISCUSSION

Severity of bacterial wilt of tomato (Fig. 2)

No symptoms of wilt were observed on tomato plants grown in the infested Mutsumi soil during the 31-d period of cultivation after transplanting. In the Yamadai soil, wilt began to occur 5–7 d after transplanting, and all the plants wilted by 18–31 d. The stems of some of the wilted plants were cut, and a white viscous material was found in all the stems, suggesting the occurrence of infection with *R. solanacearum*. Consequently, we considered

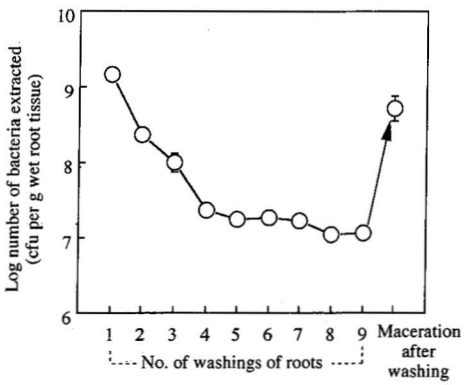

Fig. 1. Number of aerobic bacteria recovered from the roots of tomato plants grown in Mutsumi soil after 9 washings and after maceration of the roots. Aerobic bacteria were counted on YG agar. Standard error ($n=5$) was indicated by a bar or within each symbol.

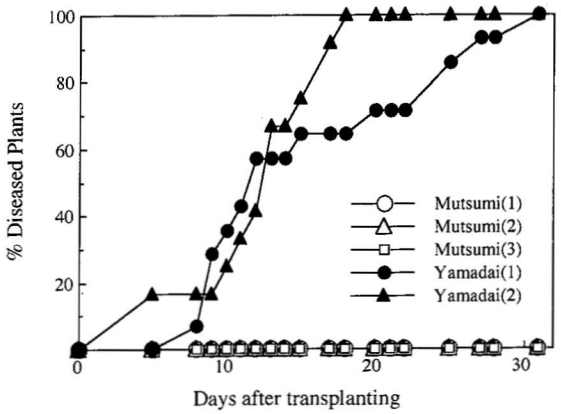

Fig. 2. Severity of bacterial wilt of tomato plants grown in Mutsumi soil and Yamadai soil inoculated with *Ralstonia solanacearum* strain SL8 at an initial density of 10^3 cfu per g dry soil. Number of experiments is indicated in parenthesis. Forty seedlings were used in each experiment.

that the Yamadai soil was a conducive soil and the Mutsumi soil a suppressive soil for tomato bacterial wilt in this report.

Population dynamics of *R. solanacearum* in the non-rhizosphere soil, roots, and stems

Although five plants in a pot were combined when the counts were performed, the number of *R. solanacearum* varied considerably among the pots. Thus, the bacterial numbers obtained from each pot are separately shown in Figs. 3, 4, and 6. In Figs. 3 and 6, a set of data from an identical pot is shown with an identical X-value.

In the tomato plants grown in the Yamadai soil, *R. solanacearum* was detected 4 d after transplanting in fraction-1, then in fraction-2 after 7 d and in stems after 10 d (Fig. 3). The number tended to increase during the 16-d period of cultivation up to 10^8–10^{10} cfu per g wet tissue in the three parts of the plants. In contrast, in the Mutsumi soil, the number remained below 10^6 cfu per g in any of the three parts of the plants throughout the 40-d period of cultivation with one exception (25 d). A similar trend in the population dynamics of the pathogen was observed in another experiment (data not shown).

Relationship between the number of *R. solanacearum* in fractions-1 and -2 and in stems is shown in Fig. 4. In general, the tomato plants with a larger population of the pathogen in stems contained a larger population of the pathogen in root fraction-2, and the plants with a larger population in fraction-2 harbored a larger one in fraction-1. These tendencies appeared to be more evident in the plants with more than 10^5–10^6 cfu of the pathogen per g tissue. Since the pathogen reaches the rhizoplane at first, then the inside part of the roots, and finally the stems, the results suggest that prior multiplication in the rhizoplane at a density above 10^6 cfu per g wet root is necessary for strain SL8 to multiply in root fraction-2 at a density above 10^6 cfu per g and that prior multiplication in fraction-2 is necessary for multiplication in stems at a density above 10^6 cfu per g. Consequently, it was suggested that the suppressiveness of bacterial wilt in the Mutsumi soil was mainly due to the persistence

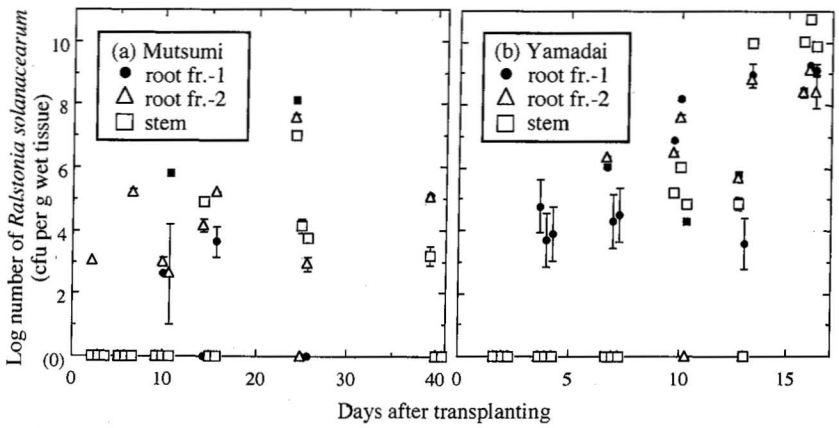

Fig. 3. Population dynamics of *Ralstonia solanacearum* in root fraction-1 (●), in root fraction-2 (△), and in stems (□) in tomato plants grown in Mutsumi soil (a) and Yamadai soil (b). (0) in the Y-axis: under the detection limit. Sampling was performed at 3, 6, 10, 15, 25, and 40 d after transplanting in Mutsumi soil and at 2, 4, 7, 10, 13, and 16 d in Yamadai soil. Three pots each containing five plants were analyzed at each sampling. A set of data from an identical pot corresponds to an identical X-value. Standard errors ($n=5$) are indicated by bars or within each symbol.

of the pathogen in the rhizoplane at a density below 10^6 cfu per g wet root. In addition to the rhizoplane, the persistence of the pathogen at a density below 10^6 cfu per g in fraction-2 and in stems as well as in fraction-1 may indicate the presence of additional suppressiveness in these two fractions.

In the experiments on population dynamics, 6 out of 150 plants in Mutsumi soil (4%) and 141 out of 155 plants in Yamadai soil (91%) wilted during the 40-d period of cultivation.

In tomato-free Yamadai soil, the number of strain SL8 decreased below the detection limit within 21 d of incubation, while in the Mutsumi soil it decreased more slowly to a level of 10^3-10^4 cfu per g soil (Fig. 5). These results suggest that the survival of the pathogen in the non-rhizosphere soil was not directly related to the occurrence of tomato wilt in the two soils. A similar discrepancy was reported previously (Ho et al. 1988; Koga et al. 1997).

Population dynamics of total aerobic bacteria in the root fractions (Fig. 6)

In each soil, the number of total aerobic bacteria did not change appreciably in either

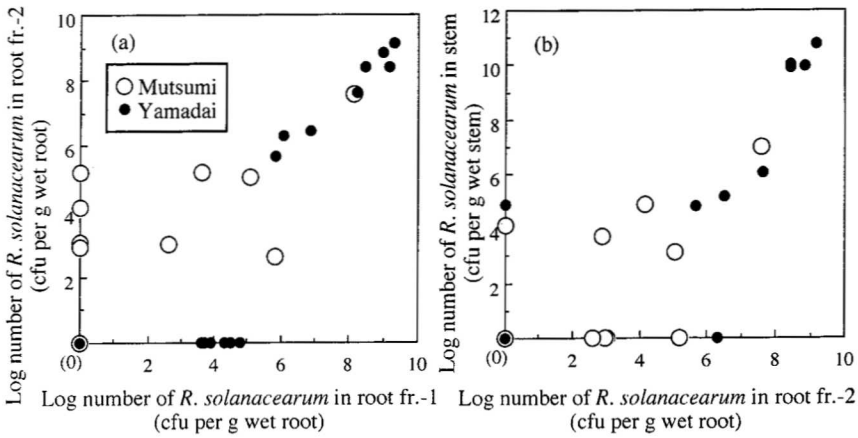

Fig. 4. Relationships between the number of *Ralstonia solanacearum* in root fraction-1 and in root fraction-2 (a) and between the number in root fraction-2 and in stems (b) in tomato plants grown in Mutsumi soil (○) and in Yamadai soil (●). (0): under the detection limit.

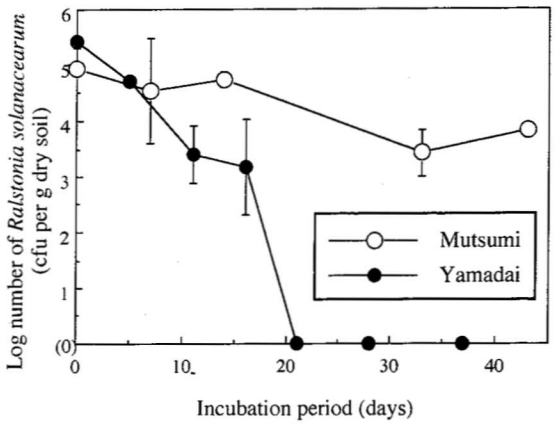

Fig. 5. Survival of *Ralstonia solanacearum* strain SL8 in tomato-free Mutsumi soil (○) and Yamadai soil (●). (0) in the *Y* axis: under the detection limit. Three samples were analyzed at each sampling. Standard errors are indicated by bars or within each symbol.

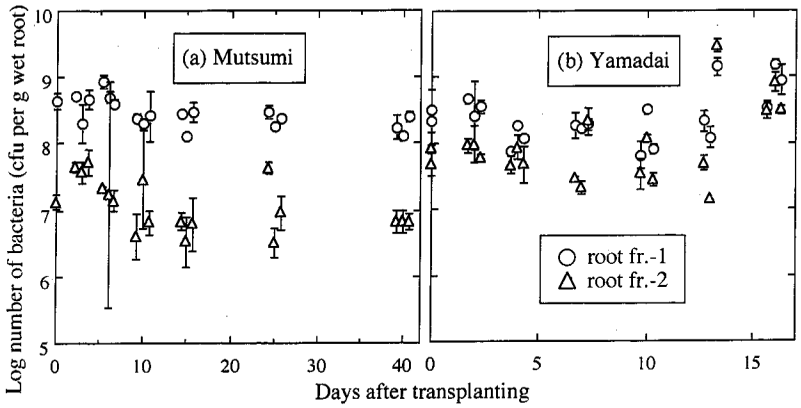

Fig. 6. Population dynamics of total aerobic bacteria in root fraction-1 and in root fraction-2 in tomato plants grown in Mutsumi soil (a) and Yamadai soil (b). Sampling was performed at 3, 6, 10, 15, 25, and 40 d after transplanting in Mutsumi soil and at 2, 4, 7, 10, 13, and 16 d in Yamadai soil. Three pots each containing five plants were analyzed at each sampling. A set of data from an identical pot corresponds to an identical X-value. Standard errors ($n=5$) are indicated by bars or within each symbol.

of the two root fractions during the cultivation period. Mutsumi and Yamadai soils did not differ significantly in the average number of total aerobic bacteria in fraction-1 throughout the cultivation period, while in fraction-2 the average bacterial number was higher in the Yamadai soil than in the Mutsumi soil ($p<0.05$). A similar trend of changes in the bacterial populations was observed in another experiment.

Sites for suppressiveness of tomato bacterial wilt in Mutsumi soil

In this report, we compared the population dynamics of *R. solanacearum* in the non-rhizosphere soil and three parts of the plants between the suppressive (Mutsumi) and conducive (Yamadai) soils. As shown in Figs. 3–5, the primary site for suppressiveness of bacterial wilt in tomato plants grown in the Mutsumi soil was located in root fraction-1, and not in the non-rhizosphere soil. Furthermore, fraction-2 and the stems may contain additional sites for suppression. The detection of such suppressive sites at first and then focusing on those sites may enable to investigate the mechanisms of suppressiveness and to develop effective methods of biocontrol although the sites may be different in each suppressive soil.

As shown in Fig. 1, most of the easily detached rhizobacteria could be recovered in fraction-1. It is reasonable to assume that these bacteria originated from the rhizoplane. However, all the rhizoplane bacteria were not necessarily recovered in fraction-1 because some bacterial strains may remain firmly attached to the root surface. Strain SL8 used in this report forms mucoidal colonies, suggesting the production of exopolysaccharides, by which the cells can become firmly attached. More precise determination of the location of the cells of strain SL8 in fractions-1 and -2 is necessary.

It remains to be determined why the population of the pathogen at a density below 10^6 cfu per g root in fraction-1 in the Mutsumi soil was controlled. It is often considered that root-colonizing microorganisms are responsible for the suppressiveness of plant disease (e.g., Schroth and Hancock 1982; Weller 1988; Furuya et al. 1991; Handelsman and Stabb 1996; Mulya et al. 1996). In this report, no significant differences in the number of total aerobic bacteria in the rhizoplane (root fraction-1) was observed between the two soils. Microbial

community structure in the rhizoplane may affect the suppressiveness in fraction-1. The fact that the population of total aerobic bacteria in the roots or hardly extractable fraction (root fraction-2) was smaller in the Mutsumi soil than in the Yamadai soil (Fig. 6) may also suggest that the microbial community in the Mutsumi rhizoplane inhibited bacterial invasion of roots, or that some endophytic bacteria in the Mutsumi soil may repel strain SL8 through induced resistance.

As shown in Table 1, the two soils used differed in their chemical properties such as total N content and soil pH. These soil properties could affect the nutritional and physiological status of the plants, which may account for the differences in the host resistance. The Yamadai soil appears to be less fertile than the Mutsumi soil. The growth of the roots may be more restricted in the Yamadai soil, i.e., the roots grown in the Yamadai soil may be thinner than those in the Mutsumi soil. Then, the roots grown in the Yamadai soil may have a larger surface area per g root and be attacked more frequently by the pathogen. It is also possible that host resistance was associated with Ca nutrition (Yamazaki and Hoshina 1995; Yamazaki et al. 1998) although the soil Ca content was not determined in this report.

It is interesting to note that the pathogen remained at a density of 10^6 cfu per g root and stem and was restricted to the primary xylem tissues in a resistant cultivar (Nakaho 1997). Similar mechanisms whereby the pathogen remained at a density below 10^6 cfu per g may be involved in the wilt-susceptible cultivar, depending on the soil conditions. Further studies should be carried out to analyze the mechanisms controlling the suppressiveness observed in the Mutsumi soil.

Acknowledgments. We thank Mr. T. Tokunaga, Yamaguchi Agricultural Experiment Station, Mr. T. Fujimoto, Hagi Agricultural Improvement Center, and Mrs. T. Tokunaga, Mutsumi town for their assistance in soil sampling. We also thank Dr. S. Takaki, Fumakilla Ltd. for his helpful discussions. This research was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09760059).

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