Persistence and Latency of *Clavibacter michiganensis* subsp. *sepedonicus* in Field-Grown Seed Potatoes

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ABSTRACT

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Whole seed tubers were inoculated with different inoculum doses of *Clavibacter michiganensis* subsp. *sepedonicus* and planted in the field. Ring rot symptom development and the persistence of *C. michiganensis* subsp. *sepedonicus*, in the absence of symptoms, was determined for four generations following introduction of inoculum. Seed tubers were not cut during propagation to preclude redistribution of inoculum. Detection of *C. michiganensis* subsp. *sepedonicus* was positively related to inoculum dose ($r = 0.97$) and rapidly declined with increasing generations. Although the frequency of detection was much less following inoculation with $\leq 10^2$ cells versus inoculation with $\geq 10^4$ cells, all inoculum doses established latent infections that were undetected for at least one, and up to three, generations. Latency for three generations, characterized by *C. michiganensis* subsp. *sepedonicus* detection or ring rot symptom development during the fourth generation, was the longest time interval tested. The occurrence of latent *C. michiganensis* subsp. *sepedonicus* infections and the persistence of inadvertent inoculum sources may, at least partially, explain our inability to eradicate ring rot based on visible symptom expression.

Additional keywords: bacterial ring rot

Bacterial ring rot of potato is caused by the gram positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus* (Spieck. & Koth.) Davis et al. Ring rot was first recorded in Germany in 1906 and, since its introduction to North America during the early 1930s, continues to be a major concern of the potato industry (6,10). Infection by *C. michiganensis* subsp. *sepedonicus* can result in catastrophic tuber losses in both the field and storage (10).

The ring rot bacterium infects tubers through wounds, and most *C. michiganensis* subsp. *sepedonicus* spread occurs during seed cutting and handling (10). Planting infected seed for production of next year's crop completes the disease cycle and, thereby, introduces inoculum to the next generation. Because *C. michiganensis* subsp. *sepedonicus* is primarily seedborne and the potential for loss is so great, all certification agencies enforce a zero tolerance for ring rot (7).

Enforcement of the zero tolerance requires reliable assay methods for detection of the pathogen and the development of symptoms. Several factors are known to affect symptom expression, including *C. michiganensis* subsp. *sepedonicus* strain (1), inoculation dose (1,5,16,17), tempera-

ture (9), soil moisture (3), light quality (15), and potato genotype (2,11-13,16,17). In the United States, visual detection of foliar symptoms during field inspections accounts for 72% of seed lot rejections (7). Additional rejections are due to detection of tuber symptoms during harvest or in storage (20%) and during shipping point inspections (8%; 7). A survey of certification agencies revealed that "latency," described as infection by *C. michiganensis* subsp. *sepedonicus* in the absence of symptoms, was the most common explanation offered for failure to reject infected seed lots (7). Latently infected plants and tubers that escape detection, yet carry *C. michiganensis* subsp. *sepedonicus*, serve as inadvertent inoculum sources for the next generation and pose a risk to both the seed grower and seed buyer. Latent infection has been reported to occur in tissue culture plantlets (4) and field-grown plants (5,12,14).

The objective of this study was to determine the persistence of *C. michiganensis* subsp. *sepedonicus* in a population of seed tubers, in the absence of symptoms, for several generations following the introduction of inoculum. The occurrence of latent *C. michiganensis* subsp. *sepedonicus* infections and the persistence of inadvertent inoculum sources may, at least partially, explain our inability to eradicate ring rot based on visible symptom expression.

MATERIALS AND METHODS

C. michiganensis subsp. *sepedonicus* strain SS43 was used in this study. Ring rot development for potato inoculated with

this strain is described in great detail (1,16,17). To avoid selecting for avirulence within the *C. michiganensis* subsp. *sepedonicus* population, inoculum for the study described below was not increased on artificial growth medium. Instead, inoculum applied to seed tubers consisted of cells extracted from symptomatic eggplants.

For each repetition of the experiment, 15 to 20 eggplant seedlings (*Solanum melongena* cv. Black Beauty in the two- to four-leaf stage) were inoculated by injecting stems with a slightly turbid suspension of strain SS43. Injections were accomplished with the aid of a sterile 1-ml tuberculin syringe and a 26G needle. All bacterial suspensions were prepared in 0.05 M phosphate buffer at pH 7.2. The original *C. michiganensis* subsp. *sepedonicus* culture was the source of cells inoculated into eggplant for the first repetition of this experiment (experiment 1) and cell suspensions for subsequent experimental repetitions (experiment 2 and experiment 3) were prepared from symptomatic tubers stored from the previous growing season.

Foliar symptoms developed in eggplants within 10 to 14 days after inoculation. At 15 to 19 days after inoculation, seedlings were uprooted and washed thoroughly in cool tap water, after which roots and leaves were removed and discarded. The stems were cut into 1-cm segments and segments were placed into several liters of sterile, cold (4.5°C) buffer. The buffer and stem segments were held at 4.5°C overnight and slowly stirred to aid extraction of bacterial cells. The turbid suspension was then poured through several layers of cheesecloth to remove segments and large debris, followed by centrifugation at $12,000 \times g$ for 10 min to concentrate *C. michiganensis* subsp. *sepedonicus* cells. Bacterial pellets were resuspended in 250 ml of cold, sterile buffer. Additional debris was removed by vacuum filtration through coarse filter paper.

Serial dilutions (10-fold) of the filtrate were prepared and lightly stained with 1 to 2 drops of 2% crystal violet to increase contrast. Aliquots were placed into a Petroff Hausser counting chamber (Hausser Scientific Partnership, Horsham, PA) and cells were enumerated. Cell pairs, linked by snapping division, were counted as two individual cells. The concentration of *C. michiganensis* subsp. *sepedonicus* cells in the filtrate was calculated and dilutions were prepared to result in preparations of 10^{10} , 10^7 , 10^5 , 10^3 , and 10^2 cells/ml. Serial

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dilutions were spread-plated (0.1 ml) onto nutrient dextrose agar during experiment 3 inoculum preparation. The relationship between viable bacterial counts (CFU) to physical counts was estimated by enumerating the number of colonies visible after 6 days of incubation at 26°C.

Inoculum was applied to Colorado certified seed potatoes (*Solanum tuberosum* L., cvs. Russet Burbank and Centennial Russet). Seed tubers (approximately 180 g) were surface disinfested with 0.05% sodium hypochlorite for 5 min, rinsed with cool tap water, and air dried. Tubers were inoculated by removing a portion of the stolon end with a standard kitchen "melon-baller" (2.8 cm diameter), applying 0.1 ml of inoculum directly into the depression, and immediately returning the tuber portion to its original position. A small piece of sterile wooden toothpick was inserted to hold the tuber portion in place and the entire stolon end (1/4 of the tuber length) was immediately dipped into melted paraffin and, after several seconds, dipped again. The inoculation volume of 0.1 ml was observed to coat the cut surface of the tuber when the excised "melon ball" tuber portion was replaced and slight pressure was applied. The paraffin quickly sealed the excised tuber portion to the seed tuber and prevented inoculum from escaping or evaporating. Buffer-inoculated seed tubers were treated in the same manner. All inoculations were done in the order of most dilute to most concentrated inoculum preparations.

Inoculated seed tubers, designated as "generation 0" (G0), were planted by hand in field plots. Only whole seed tubers were planted and seed was never cut at any time during the study, to preclude the redistribution of inoculum. A randomized complete block experimental design consisting of two cultivars, six inoculum doses, and four replications was used. Individual treatment plots consisted of 10 inoculated tubers planted at 35-cm intervals within the row, followed by 3 non-inoculated seed tubers planted between plots. A red-skinned cultivar was planted between plots to aid in separating plots during data collection and harvest. Row centers were spaced at 1.8 m. All field plots were cultivated by hand and water was applied by furrow-irrigation. Field plots were located near Center, Colorado in the San Luis Valley at 2,300 m above sea level.

Individual G0 plants were inspected at least three times throughout the growing season and the presence or absence of ring rot symptoms within a treatment plot (replication) was recorded. Symptom expression included interveinal chlorosis and necrosis, necrosis of leaf margins, and early-dwarf rosette in cv. Russet Burbank (10). A stem-squeeze and Gram stain for bacterial signs was occasionally performed. At least one inspection was near the end of the growing season and after

plants achieved full maturity. After plant senescence or frost, treatment plots were harvested by hand. At harvest, all progeny (G1) tubers were inspected for any visible external symptoms of ring rot and, if present, were cut and examined for internal vascular symptoms. A total of 20 non-injured and healthy-appearing G1 tubers were randomly selected from each treatment plot and placed into cold storage (7°C) within several days after harvest.

After storage for 7 months, these tubers were randomly sorted into two subsamples of 10 tubers each. One subsample was tested by the ring rot bioassay, performed as described below, and the second subsample was planted back into the field to produce G1 plots during the second growing season. The G1 experimental design and data collection procedure was the same as described above for G0 plots. After G1 plant senescence or frost, treatment plots were harvested as described above and G2 tubers were stored for ring rot bioassay and plant-back for the third growing season. The G2 experimental design and data collection procedure was the same as described above for G0 and G1 plots. The G3 tuber harvest procedure was as described above for prior generations; however, tubers from replications one and two were composited as a sample, as were tubers from replications three and four. Tubers were stored for ring rot bioassay and plant-back during the fourth growing season. For each composite sample, 20 G3 tubers were tested by bioassay and 20 tubers were planted in the field for evaluation. Data for foliar symptom development were collected as described above for prior generations.

The ring rot bioassay was performed each spring on tubers stored from the previous growing season. The bioassay was performed by lightly peeling the stolon end of each tuber and excising a small amount of vascular tissue with the aid of a sterile scalpel (8). Excised tissue from 10 (G1 or G2) or 20 (G3) tubers was combined and macerated with the aid of a sterile mortar and pestle to which several milliliters of sterile buffer was added. The macerate was filtered through cheesecloth and injected into six to eight eggplants in the two- to four-leaf stage. Eggplants were examined for symptom development for up to 40 days after inoculation; a Gram stain of plant sap was performed on a subsample of plants to verify the presence of coryneform cells. The presence of typical ring rot symptoms in tuber vascular tissue during harvest or at the time of bioassay, or symptom development in eggplant, indicated the presence of infectious *C. michiganensis* subsp. *sepedonicus* in tuber vascular tissue.

The entire experiment was repeated three times. The three repetitions (experiments 1, 2, and 3) were initiated in three successive years, respectively. Each

repetition of the experiment required 4 years or growing seasons for completion of the G0 through G3 sequence. Chi-square analysis was performed on the observed number of replicates that were ring-rot positive by symptom expression or the number of replicates positive following detection of *C. michiganensis* subsp. *sepedonicus* by the ring rot bioassay.

RESULTS

Growth from spread-plated inoculum revealed 130 ($\pm$ 39) CFU were detected for each 100 cells physically counted in the cytometer. Colonies were uniform in appearance and no contaminating bacteria were detected. Enumeration by spread-plating is expected to underestimate the number of *C. michiganensis* subsp. *sepedonicus* cells due to the formation of cell pairs and the presence of non-viable cells. Enumeration of cells used to prepare inoculum doses for this experiment agreed closely to the number of viable *C. michiganensis* subsp. *sepedonicus* cells estimated by spread-plating.

The ring rot reaction of plant foliage and tubers for four generations or growing seasons (G0 through G3) following inoculation of G0 tubers for each repetition of the experiment (experiment 1, 2, and 3) are shown (Table 1). All G0 tubers were considered *C. michiganensis* subsp. *sepedonicus*-positive following direct application of inoculum. Detectable ring rot, determined by development of ring rot symptoms in foliage or tubers, or detection of infectious *C. michiganensis* subsp. *sepedonicus* via the eggplant bioassay, resulted for all inoculum doses and symptoms occurred at least once during each generation. No ring rot detection occurred at any time for the buffer-inoculated treatment; these data are not presented in the table.

Data for experiment 1 reveal that all G0 plants failed to develop foliar symptoms (Table 1). However, the ring rot reaction for the next three generations (G1, G2, and G3) reveals that symptoms eventually developed for all inoculum doses except 10^2 cells per G0 tuber. Cv. Russet Burbank G1 tubers had infectious *C. michiganensis* subsp. *sepedonicus* present for treatments that received $\geq 10^4$ cells per G0 tuber. However, foliar ring rot symptoms only developed for the cv. Russet Burbank treatment inoculated with 10^9 cells per G0 tuber. Bioassay and plant-back of cv. Russet Burbank progeny tubers failed to reveal ring rot during the next two generations. Cv. Centennial Russet G1 tubers had infectious *C. michiganensis* subsp. *sepedonicus* present for treatments inoculated with $\geq 10^6$ cells per G0 tuber. However, foliar symptoms were not detected in the field until the following (G2) generation. The symptomatic cv. Centennial Russet G2 treatment initially received 10 cells per G0 tuber and was latently infected during the two preceding generations.

In the second repetition of the experiment (experiment 2), G0 foliar symptoms only developed for cv. Russet Burbank treatments inoculated with $\geq 10^6$ cells, and all cv. Centennial Russet plants were symptomless (Table 1). The tubers of cvs. Russet Burbank G1 and Centennial Russet G1 had detectable levels of infectious *C. michiganensis* subsp. *sepedonicus* present following inoculation with 10^9 cells per G0 tuber. Plant-back of G1 tubers resulted in foliar symptom development for the cv. Russet Burbank treatment inoculated with 10^4 cells per G0 tuber, and all other Russet Burbank treatments appeared healthy. However, tests of G2 tubers revealed that symptomless G1 plants, initially inoculated with 10^9 cells per G0 tuber, produced infected tubers. All other cv. Russet Burbank G2 treatments appeared healthy. During the final generation, infectious *C. michiganensis* subsp. *sepedonicus* was detected in G3 tubers initially inoculated with 10^2 cells per G0 tuber. This particular treatment had no indication of ring rot during the previous three growing seasons. Foliar data for cv. Centennial Russet G1 reveals that symptoms developed for treatments that initially received $\geq 10^6$ cells per tuber and that G2 tubers harvested from these plants also had detectable levels of *C. michiganensis* subsp. *sepedonicus* present. Foliar symptom development occurred for cv. Centennial Russet G2 treatments initially inoculated with 10^9 and 10^2 cells, and all other treatments appeared healthy. No ring rot was detected during the first two growing seasons for the G2 treatment inoculated with 10^2 cells per G0 tuber. Tubers harvested from this treatment also had infectious *C. michiganensis* subsp. *sepedonicus* present.

Experiment 3 data reveal that foliar symptoms developed for plants of cvs. Russet Burbank G0 and Centennial Russet G0 grown from tubers inoculated with $\geq 10^4$ cells (Table 1). Additionally, the cv. Centennial Russet G0 treatment inoculated with 10 cells also developed foliar ring rot symptoms. All other G0 plants appeared healthy. During the next generation, ring rot was detected for cv. Russet Burbank G1 tubers and plants initially inoculated with $\geq 10^2$ cells per G0 tuber. Results were similar for cv. Centennial Russet G1, except that foliar symptoms failed to develop for the 10^2 inoculum dose. The G1 treatment inoculated with 10^2 cells was latently infected during the prior generation. The cv. Russet Burbank G2 generation had detectable ring rot for treatments initially inoculated with 10^9 (tubers), 10^6 (tubers and foliage), and 10^2 (foliage) cells per G0 tuber. There was no indication of ring rot during the following (G3) generation. Ring rot detection for cv. Centennial Russet G2 resulted for initial inoculations with 10^9 (foliage), 10^6 (tubers), and 10^4 (tubers) cells per G0 tuber. Foliar ring rot symptoms were detected for the cv. Centennial

Russet G3 treatment that received 10^6 cells per G0 tuber, even though the prior generation appeared healthy.

Chi-square analysis for the observed number of ring rot-positive replicates revealed that there were no significant cultivar effects or cultivar interactions ($P \leq 0.05$). Experimental repetition was highly significant ($P \leq 0.01$), presumably due to environmental influences on infection and subsequent disease development. The effects of inoculum dose and generation on the frequency of *C. michiganensis* subsp. *sepedonicus* detection were also highly significant ($P \leq 0.01$). Detection was positively related to inoculum dose ($r = 0.97$) and rapidly declined with increasing generation. Data for the percentage of replicates positive for *C. michiganensis* subsp. *sepedonicus*, combined over cultivar and experiment, more clearly show the relationships of inoculum dose and generation to frequency of ring rot detection (Table 2).

However, data summarized in this manner, for the most part, fails to reveal the occurrence of latent infections.

DISCUSSION

Inoculum was introduced into the stolon end of seed tubers in an effort to reproduce the natural infection process, which concentrates inoculum at the stolon end (10). All inoculum doses established latent infections that went undetected for at least one, and up to three, generations. Latency for three generations, characterized by *C. michiganensis* subsp. *sepedonicus* detection or ring rot symptom development during the fourth generation, was the longest time interval tested.

The eggplant bioassay is a sensitive test for detection of infectious *C. michiganensis* subsp. *sepedonicus* and only living cells are detected by this method (8). Dose-response curves revealed that as few as 4.2 bacterial cells are needed to produce a ring

Table 1. Bacterial ring rot symptom development and/or infectious *C. michiganensis* subsp. *sepedonicus* detection following inoculation of G0 seed tubers

Experiment ^b	Dose ^c	Generation and ring rot reaction ^a							
		G0		G1		G2		G3	
		T ^d	F	T	F	T	F	T	F
Exp. 1, RB	9	4	0	2	2	0	0	0	0
	6	4	0	2	0	0	0	0	0
	4	4	0	1	0	0	0	0	0
	2	4	0	0	0	0	0	0	0
	1	4	0	0	0	0	0	0	0
Exp. 1, CR	9	4	0	2	0	0	0	0	0
	6	4	0	2	0	0	0	0	0
	4	4	0	0	0	0	0	0	0
	2	4	0	0	0	0	0	0	0
	1	4	0	0	0	0	1	0	0
Exp. 2, RB	9	4	3	2	0	1	0	0	0
	6	4	1	0	0	0	0	0	0
	4	4	0	0	1	0	0	0	0
	2	4	0	0	0	0	0	1	0
	1	4	0	0	0	0	0	0	0
Exp. 2, CR	9	4	0	4	1	1	1	0	0
	6	4	0	0	1	2	0	0	0
	4	4	0	0	0	0	0	0	0
	2	4	0	0	0	0	1	1	0
	1	4	0	0	0	0	0	0	0
Exp. 3, RB	9	4	4	4	4	1	0	0	0
	6	4	4	4	1	1	1	0	0
	4	4	2	3	2	0	0	0	0
	2	4	0	1	1	0	1	0	0
	1	4	0	0	0	0	0	0	0
Exp. 3, CR	9	4	4	4	2	0	1	0	0
	6	4	4	4	3	1	0	0	1
	4	4	4	2	1	1	0	0	0
	2	4	0	1	0	0	0	0	0
	1	4	1	0	0	0	0	0	0

^a Generation and ring rot reaction for tubers (T) and foliage (F). Generation 0 (G0) tubers were inoculated, planted, and produced G0 plants. Progeny (G1) tubers were harvested and then planted during the following growing season. The harvest and planting sequence was repeated to produce the succeeding generations, as indicated. All buffer-inoculated controls remained healthy. Columns indicate the number of replications exhibiting at least one tuber or one plant with ring rot symptoms, detectable levels of *C. michiganensis* subsp. *sepedonicus* by the eggplant bioassay, or both. A total of 10 tubers or 10 plants were evaluated for each of four replications (maximum value = 4). For G3 data, 20 tubers and 20 plants were rated for each of two replications (maximum value = 2).

^b Experimental repetition and cultivar. Three repetitions of the experiment were conducted (Exp. 1, Exp. 2, and Exp. 3) with cultivars RB = Russet Burbank and CR = Centennial Russet.

^c Inoculum dose = cells/tuber ($\log_{10}$).

^d All G0 tubers are indicated as *C. michiganensis* subsp. *sepedonicus*-positive, following inoculation of stem-end vascular tissue.

Table 2. Combined data for bacterial ring rot symptom development and infectious *C. michiganensis* subsp. *sepedonicus* detection following inoculation of G0 seed tubers

Dose ^b	Generation and ring rot reaction for tubers (T) and foliage (F) ^a							
	G0		G1		G2		G3	
	T ^c	F	T	F	T	F	T	F
9	100	46	75	38	13	8	0	0
6	100	38	50	21	17	4	0	8
4	100	24	25	17	4	0	0	0
2	100	0	8	4	0	8	16	0
1	100	4	0	0	0	4	0	0
Buffer only	0	0	0	0	0	0	0	0

^a Generation 0 (G0) tubers were inoculated and planted to produce G0 plants. Progeny (G1) tubers were harvested, assayed, and planted. The harvest and planting sequence was repeated to produce the succeeding (G2 through G3) generations, as indicated. Cultivar effects and cultivar interactions were not significant ($P = 0.05$). Columns indicate the percentage of replications exhibiting at least one tuber or one plant with ring rot symptoms, detectable levels of *C. michiganensis* subsp. *sepedonicus* by the eggplant bioassay, or both. For G0 through G2 data, 10 tubers and 10 plants were evaluated for each replication; each data point represents 24 replications. For G3 data, 20 tubers and 20 plants were evaluated for each replication; each data point represents 12 replications.

^b Inoculum dose = cells/tuber ($\log_{10}$).

^c All G0 tubers are indicated as *C. michiganensis* subsp. *sepedonicus*-positive, following inoculation of stem-end vascular tissue.

rot reaction in 50% of the inoculated eggplants (8). In the study reported here, bioassay of vascular tissue taken from asymptomatic tubers revealed the presence of infectious *C. michiganensis* subsp. *sepedonicus*. Tuber tests generally revealed *C. michiganensis* subsp. *sepedonicus* more frequently than did the development of foliar symptoms.

Nelson (14) demonstrated that both ring rot symptom development and latent infection were positively correlated with inoculum dose. His research revealed that, when seed tubers were cut into seed pieces and inoculum was redistributed at the start of each growing season, infectious *C. michiganensis* subsp. *sepedonicus* persisted in a population of plants for three generations. Symptom expression increased with generation, presumably because the bacterium was annually redistributed among seed tubers during seed cutting. Also, a portion of the plant population was latently infected during each generation (14). In contrast, only whole tubers were propagated for the study reported here. The sole source of infectious *C. michiganensis* subsp. *sepedonicus* was from parent plants because there was no annual redistribution of inoculum during seed cutting and no reintroduction of inoculum to seed tubers after the G0 inoculations. Buffer-inoculated controls remained healthy throughout the course of the study. As stated above,

the incidence of ring rot decreased with increasing generation. Self-elimination of ring rot may be possible if whole seed is planted and cutting is avoided.

These data demonstrate that increased effort is needed to attempt eradication of ring rot. For example, improved assays must be developed that permit reliable and consistent detection of *C. michiganensis* subsp. *sepedonicus* and seed certification programs will need to develop regulations that permit rejection of infected propagation material in the absence of symptoms. Research is also needed to determine *C. michiganensis* subsp. *sepedonicus* population dynamics in tissue culture plantlets and minitubers, as well as the factors that affect bacterial replication and symptom expression. Ultimately, this information could be used to improve sampling and assay methods for early *C. michiganensis* subsp. *sepedonicus* detection and to reduce the potential for inadvertent dissemination of infected propagation material.

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