Determination of Population Densities of *Corynebacterium sepedonicum* in Potato Stems During the Growing Season

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ABSTRACT

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Estimates of population densities of *Corynebacterium sepedonicum* in potato stems grown from inoculated seed tubers were made during the growing season with a quantitative immunofluorescence procedure. Population densities, calculated as immunofluorescing units per gram fresh weight (IFU/g), reached a maximum of 10^8 to 10^{10} in the cultivars Red Pontiac, Russet Burbank, and Desiree grown in growth rooms and in field plots. The population density increased most rapidly in Red Pontiac during the growing season, and this cultivar also expressed the highest incidence of bacterial ring rot symptoms. Desiree generally did not develop symptoms and had the lowest bacterial population densities in the stems. Russet Burbank expressed moderate symptoms and developed variable population densities. Within 80 days after planting, *C. sepedonicum* could be detected in symptomless stems of all three cultivars by immunofluorescence with a cell wall-specific monoclonal antibody and by enzyme-linked immunosorbent assay with an extracellular polysaccharide-specific monoclonal antibody. The ELISA values were positively correlated (correlation coefficient = 0.85) with estimates of population densities.

Corynebacterium sepedonicum (Spieck. & Kotth.) Skapt. & Burkh. (syn. *Clavibacter michiganense* subsp. *sepedonicum* (Spieck. & Kotth.) Davis et al) causes the bacterial ring rot disease of potato. The bacterium invades the vascular tissue of stems and tubers and can cause severe wilting of the foliage and decay of the tubers. The bacterium, however, can also be present in potato stems and tubers without manifestation of macroscopic symptoms (8). Such symptomless, or latent, infections are of particular concern because the only control for the disease is avoidance of the pathogen (9). Because the etiologic agent is chiefly borne by tubers used for planting, avoidance is achieved by planting tubers that do not exhibit ring rot symptoms and that were harvested from ostensibly healthy plants.

Visual inspection to determine the presence of ring rot infections is limited to detection of symptoms and cannot be used to determine whether asymptomatic infections are present. Visual inspection for symptoms also is frequently inadequate because symptoms are generally not expressed until late in the growing season when natural senescence (23) and diseases such as Verticillium wilt and early blight are present. In addition, expression of bacterial ring rot symptoms varies with environmental factors, inoculum concentration, and cultivar differences (16). Differences in the tendency of cultivars to express symptoms is especially troublesome for field inspectors. Although some cultivars consistently express symptoms when infected with *C. sepedonicum*, other cultivars such as the resistant cultivars, Teton and Merrimack, never or very seldom express symptoms (2,21). High population densities of *C. sepedonicum*, however, have been reported to be present in symptomless potato stems and tubers, and these infections could serve to spread the disease agent to other seed lots (8).

Various procedures, such as the examination of tubers under ultraviolet light (14) and Gram staining of tuber and stem extracts (15), have been implemented from time to time in an attempt to determine whether a crop of symptomless potatoes is free of the ring rot pathogen. More recently, serological and DNA-probe procedures have been considered (18,22,24,26). The immunofluorescence procedure has received the greatest amount of attention since it is one of the most sensitive for detecting a specific bacterium. The specificity of this procedure, which had been limited by nonspecificity of antisera reactions (4,17), has been considerably enhanced by the availability of monoclonal antibodies specific for *C. sepedonicum* (11). Recently we have

also developed a procedure for quantifying bacterial cell populations with the immunofluorescence technique (7). This technology provides a means whereby population densities of *C. sepedonicum* can be monitored in plants even when symptoms are not expressed. Characterization of population density dynamics of *C. sepedonicum* in potato plants during the growing season will assist in determining whether the pathogen can be detected in the field before harvest and in the absence of plant symptoms.

The objective of this study was to monitor the development of populations of *C. sepedonicum* in potato plants quantitatively during the growing season, and, in particular, to explore whether the bacteria can be detected in a potato crop by serological procedures during the growing season when disease symptoms are absent. Studies were carried out over 2 yr in field and growth room experiments with potato plants grown from artificially inoculated seed pieces.

MATERIALS AND METHODS

Bacterial culture. Strain R8 of *C. sepedonicum*, isolated in 1984 from an infected potato tuber (cv. Russet Burbank) grown in Alberta, was used throughout this study. The culture was maintained at −80 C and grown on yeast extract, glucose, mineral salts (YGM) medium (6). Inoculum, when required, was prepared by washing 5–7-day-old bacterial cells from the medium with one-quarter strength Ringer's solution and adjusting the bacterial concentration turbidimetrically. Aliquots of decimal dilutions were spread on YGM medium to obtain counts of colony-forming units (cfu). The suspension was used as the stock from which dilutions were made for inoculation.

Inoculation of tuber seed pieces. Tuber seed pieces, each containing one eye, were cut with a melon-baller for the 1987 growth room experiment. These were inoculated by inserting a plastic pipet tip containing 20 μl of inoculum at 1×10^8 cfu/ml into the base of the eye to a depth of about 5 mm. Tuber seed pieces, cut in the same way as in 1987, were inoculated by vacuum infiltration for the 1988 growth room experiment. Seed pieces were placed in a vacuum desiccator and immersed in a bacterial suspension containing 1×10^6 cfu/ml of one-quarter strength Ringer's solution. A vacuum of 96–100 kPa was created in the desiccator with a faucet aspirator for 15 min and then released to force the inoculum into the cut tubers. After inoculation the tubers were air dried and then planted within 1 day.

For the field experiments, tubers were cut in half longitudinally and inoculated by vacuum infiltration. Tubers for the 1987 and 1988 experiments were inoculated with 1×10^8 and 1×10^6 cfu/ml, respectively. Control seed pieces were treated with Ringer's solution alone. Inoculated and control tuber seed pieces were held at room temperature for a week before planting.

Growth room experiments. The cultivar Russet Burbank was used in the 1987 growth room experiment. Ten inoculated seed pieces were planted in soil in 20-cm round plastic pots, and plants were grown with a 14-hr photoperiod with illumination provided by incandescent bulbs and fluorescent tubes to 127 μE m^{-2} sec^{-1} at 30 cm above the bench. Day temperature was 26 C; night temperature was 18 C.

The cultivars Russet Burbank, Red Pontiac, and Desiree were used in the 1988 growth room experiment. Twenty seed pieces of each cultivar were inoculated at the one inoculum level. Growth conditions were the same as in the 1987 experiment, except that the photoperiod was extended to 16 hr. Plants were arranged randomly on the growth room bench.

Field experiments. The cultivar Russet Burbank was used in the field experiment conducted in 1987. Four rows of 15 inoculated and four rows of 15 uninoculated seed pieces were planted. Seed pieces were spaced 30 cm apart within a row, and rows were 2 m apart. The cultivars Russet Burbank, Red Pontiac, and Desiree were used in the 1988 field experiment. Rows of 30 inoculated seed pieces of each cultivar were planted in a randomized complete block arrangement with four replications. Two rows of 30 uninoculated seed pieces of each cultivar served as controls and were separated from the inoculated plants by two guard rows to prevent cross-contamination. Seed pieces were again spaced 30 cm within rows, but rows were 1 m apart.

Sampling. All the plants in the growth room experiments were sampled when the experiment was terminated 80 days after planting. The field experiments were sampled at approximately 2-wk intervals throughout the growing season. On each sampling date during the growing season four stems were collected from each row of the field plot experiments. Samples were initially cut as 15–20-cm sections of stem, which included the portion of stem at soil level. Subsequently, a 2-cm section of stem at soil level was cut aseptically from the initial sample, weighed, and placed in a small plastic bag. One or two milliliters of distilled water was added to the bag, and the stem tissue triturated by tapping the bag with a hammer.

Quantitative immunofluorescence. Tissue homogenates were diluted with distilled water in a decimal series, and 20 μl of four dilutions were applied to each of two individual wells of multi-well microscope slides. The preparations were air dried at 50 C and fixed by immersion in acetone for 10 min. They were stained by the indirect immunofluorescence procedure with monoclonal antibody 9A1 as the primary antibody (11). The preparations were scanned with a fluorescence microscope, and the number of fluorescing bacterial cells counted at the most appropriate dilution. The automated microscope system as described previously was used to make the counts, except when the number of fluorescing cells was <1 per microscope field, in which case manual counts were made (7). Automatic counts were made in two scan areas of each of the duplicate preparations. Each scan area consisted of 81 microscope fields. When manual counts were required 30–81 microscope fields were counted in each scan area. The number of fluorescing cells present in a sample was calculated as the number of immunofluorescing units (IFU)/g fresh weight of stem tissue.

Enzyme-linked immunosorbent assay. Samples from the 1988 field experiment were also tested by ELISA with monoclonal antibody 1H3. The procedure was the same as used previously (10) except that 50 μl of tissue homogenate was mixed with 50 μl of sample buffer in the ELISA-plate wells rather than preparing the samples directly in the buffer. In this way the same samples prepared for immunofluorescence could be used for the ELISA test. ELISA results were quantified as the mean of the absorbance (405 nm) of two duplicate tests.

Statistical analyses. Analysis of variance was carried out on the immunofluorescence and ELISA 1988 field plot data. For analyzing the proportion of serological tests results that were positive, an arc sine, square root transformation was done before analysis.

RESULTS

In the 1987 growth room experiment, some fluorescing cells were found in all the stems that were sampled (Table 1). The population of bacterial cells detected, however, varied widely from a few cells per microscope field (< 10^5 IFU/g) to many cells

TABLE 1. Population densities of *Corynebacterium sepedonicum*, in potato stems grown in a growth room, estimated as immunofluorescence units/g (IFU/g) fresh weight

Cultivar	Percent plants infected[a]	Log_{10} IFU/g in infected plants	
		Mean	Range
1987			
Russet Burbank	100	8.13	4.28–10.90
1988			
Red Pontiac	100	9.09	7.46–9.70
Russet Burbank	83	8.47	4.81–9.08
Desiree	58	7.60	4.43–8.38

[a] Plants were considered infected if *C. sepedonicum* was detected. Ring rot symptoms were absent except for in some Red Pontiac stems.

at 1/1,000 dilution of the preparation (>10^{10} IFU/g). In the 1988 growth room experiment the inoculum was applied by vacuum infiltration rather than by injection as in 1987, and the inoculum concentration was 100-fold lower. Nevertheless, most of the Russet Burbank stems contained fluorescing bacteria, and they were present at levels similar to those found in the 1987 experiment (Table 1). All the Red Pontiac stems contained fluorescing bacteria, and their mean populations tended to be higher by at least an order of magnitude than those in Russet Burbank. In contrast, bacteria were detected in just over one half of the Desiree stems, and the populations were generally lower by an order of magnitude than in Russet Burbank. Bacterial ring rot symptoms only developed in some Red Pontiac plants; all others remained symptomless.

Fluorescing bacteria were detected in all of the stems at the first sampling date, 21 days after planting, of the 1987 field plot experiment, but the number of cells detected was few. The population density of bacteria in the stems increased at each sampling date until day 91, after which the population stabilized (Fig. 1). Bacterial ring rot symptoms also were first observed on the 91-day sampling date and became more pronounced as the season progressed. The variability in bacterial population among stems was greatest during the first part of the growing season when populations were generally below 10^8 IFU/g.

The inoculum concentration used for the 1988 field experiment was 100-fold less than that used for the 1987 experiment. The number of Russet Burbank stems with bacteria remained low, as did the population density (< 10^5 IFU/g) during the first 70 days of the growing season. It then increased sharply, although the population density remained lower in many of the stems than was detected during 1987 (Fig. 2B). Only 4% of the Russet Burbank plants expressed bacterial ring rot symptoms (Table 2). Red Pontiac, on the other hand, expressed more severe symptoms, and 95% of the plants had typical ring rot symptoms by the 67th day after planting. The high bacterial population density detected by immunofluorescence in Red Pontiac anticipated the high level of symptom expression (Fig. 2A). Overall, stems of cultivar Desiree had lower bacterial populations than Red Pontiac and Russet Burbank, although the population in one stem was as high as 10^{10} IFU/g (Fig 2C). At each sampling date at least some of the Desiree stems did not contain sufficient bacterial cells to be detected by immunofluorescence. During the entire growing season only one plant of Desiree expressed symptoms that resembled the bacterial ring rot disease (Table 2).

The differences in population densities, averaged over all the sampling dates, were statistically significant ($P = 0.00004$) among all three cultivars. The proportion of stems with immunofluorescing cells (Table 2) was not significantly different between Russet Burbank and Desiree, but the number of stems with bacteria were significantly lower than those of Red Pontiac at the 5% level of confidence.

The ELISA test results, combined from all the 1988 field data, were positively correlated with the bacterial populations estimated by the immunofluorescence procedure. The correlation was best described by $\log_{10}$ IFU/g versus the $\log_{10}$ transformation of the ELISA values (Fig. 3). ELISA values of ≥0.1 were obtained for 75% of the samples that had ≥10^5 IFU/g, and 89% of the samples in which no cells were found by immunofluorescence had ELISA

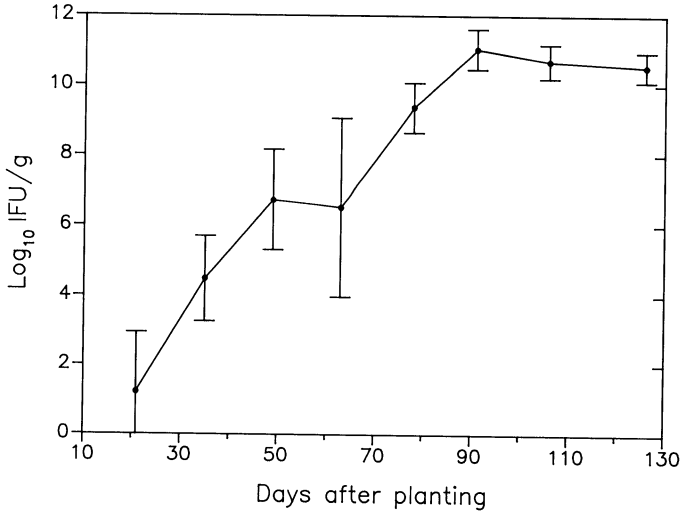

Fig. 1. Population density of *Corynebacterium sepedonicum* in Russet Burbank stems during the 1987 growing season. Each data point represents the mean population of 16 stems. Vertical bars represent the standard deviations.

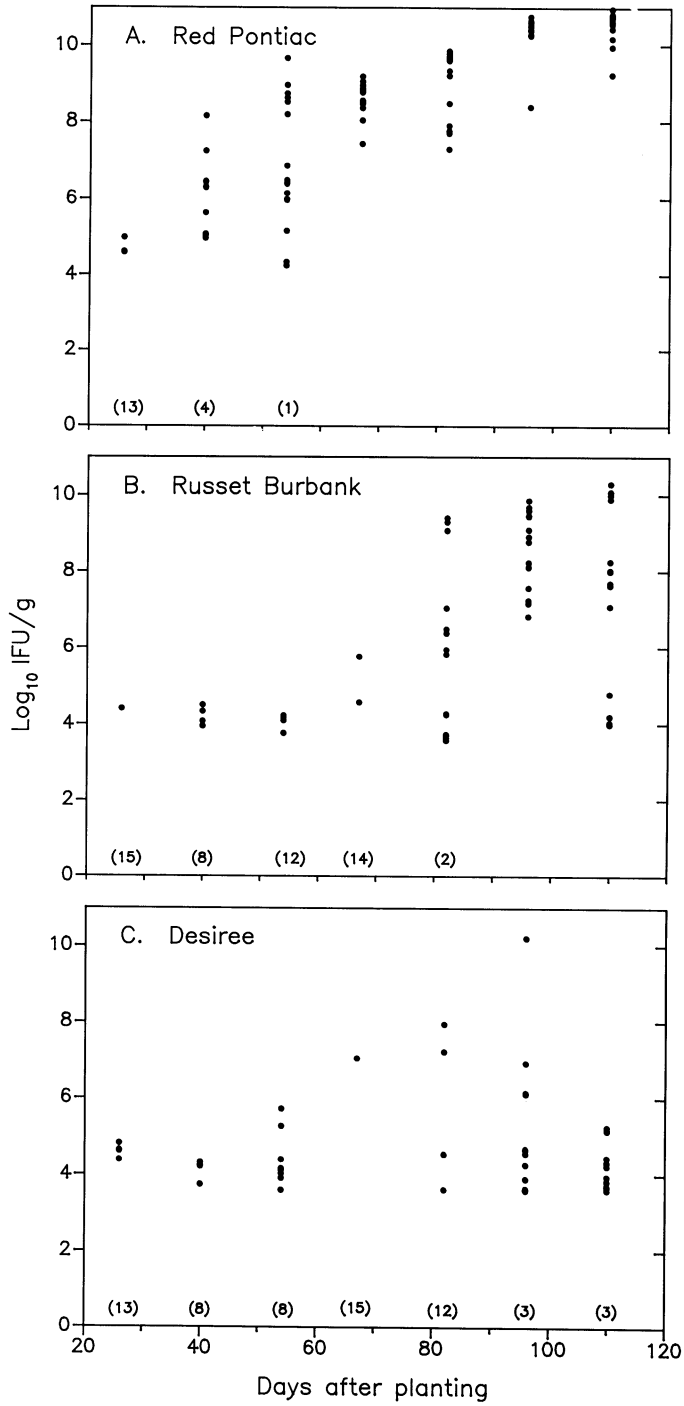

Fig. 2. Population density of *Corynebacterium sepedonicum* in potato stems of **A,** Red Pontiac; **B,** Russet Burbank; and **C,** Desiree during the 1988 growing season. Sixteen stems were tested on each sampling date except on the 40-day sampling date when 12 stems were tested. Each data point represents the population density of an individual stem. The numbers in parentheses above the *X*-axis indicate the number of stems (if any) in which no fluorescing bacteria were detected.

values of <0.1. The mean ELISA values at each sampling date of the 1988 field experiment are given for each cultivar in Table 3. The ELISA values for Red Pontiac, averaged over all the sampling dates, were significantly higher than those for Russet Burbank and Desiree ($P = 0.00048$).

None of the control plants grown from uninoculated tubers developed symptoms. Although stems from the uninoculated plants had consistently low ELISA values, mean ELISA values for stems from uninoculated plants at two sampling dates for both Russet Burbank and Desiree were >0.1. In each of these cases this was due to a single high reading for one sample that was not reproduced in the duplicate sample. Fluorescing cells were not detected by immunofluorescence in stems from uninoculated plants except for in nine stems (data not shown). Five of these had one or two cells per 324 microscope fields scanned; the remaining four stems had 6–47 cells per 324 microscope fields.

TABLE 2. Percentage of plants in which bacterial ring rot was detected by symptom expression, immunofluorescence test, and enzyme-linked immunosorbent assay (ELISA) during the 1988 growing season

	Days after planting						
Cultivar and test	26	40	54	67	82	96	110
Red Pontiac							
Visual symptoms[a]	0	0	0	95	98	100	ND[b]
Immunofluorescence[c]	19	50	94	100	100	100	100
ELISA[d]	0	25	56	100	100	100	100
Russet Burbank							
Visual symptoms	0	0	0	0	0	4	ND
Immunofluorescence	6	25	25	13	88	100	100
ELISA	0	0	0	25	44	94	81
Desiree							
Visual symptoms	0	0	0	0	0	1	ND
Immunofluorescence	19	25	50	6	25	81	81
ELISA	0	0	13	63	25	25	25

[a]Based on all 30 plants in each of four replications.
[b]ND = no data available since senescence prevented accurate determination of symptoms.
[c]Based on test of 16 stems, i.e., four stems from each of four blocks; stems were considered positive if any fluorescing bacteria were found.
[d]Based on test of 16 plants, i.e., four stems in each of four blocks; stems were considered positive if mean absorbance of two determinations was >0.1.

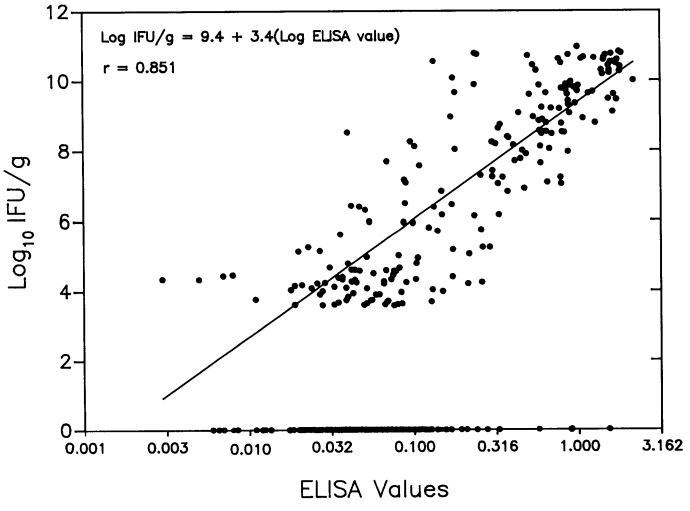

Fig. 3. Relationship between estimates of population densities of *Corynebacterium sepedonicum* by immunofluorescence and ELISA tests of potato stems. Data from three cultivars tested on seven sampling dates are included. Data on stems in which no cells were found by immunofluorescence (i.e. IFU/g = 0) were excluded from the regression calculations.

The presence of *C. sepedonicum* was detected in potato stems by immunofluorescence with greater frequency than by ELISA (Table 2). Both serological procedures, however, detected *C. sepedonicum* in potato plants that did not exhibit foliar ring rot symptoms.

DISCUSSION

The entire cell population of *C. sepedonicum*, including viable and nonviable cells, was quantified by immunofluorescence. Although the inability to distinguish viable from nonviable cells is a disadvantage for some research objectives, it was not of concern in this study because the objective was to detect the presence of the bacterium and the relative increases in total cell number during the growing season. With immunofluorescence, about 1×10^4 IFU/g fresh weight of tissue could be detected. Population density estimates at the lower level of detection, however, were considered inaccurate because they were based on very few cells, that is, <1 cell per microscope field. Obstruction of cells by plant tissue debris contributed to the difficulty in making accurate counts in the undiluted and 1/10 dilutions of the samples. When bacterial populations were $>1 \times 10^5$ the number of fluorescing cells could be counted at higher sample dilutions where tissue debris was not a problem.

The sensitivity threshold of our immunofluorescence procedure was very similar to that reported for other bacteria-plant systems. The sensitivity threshold for detection of *Corynebacterium michiganense* in composite tomato seed preparations was estimated to be 7×10^3 (25). Similarly, *Erwinia chrysanthemi* and *Pseudomonas caryophylli* could be detected at 8.7×10^4 and 3.3×10^4 cells per carnation stem sample, respectively (19). Sensitivity of the ELISA procedure for detecting pathogenic bacteria in plant tissue has generally been of a similar order of magnitude to that of immunofluorescence. For example, sensitivity of ELISA was 1×10^4 cells per milliliter for detecting the ratoon stunt bacterium in sugarcane (20) and for detecting *Pseudomonas phaseolicola* in bean (3).

The monoclonal antibody that was used for immunofluorescence in this study to quantify population densities reacted with a cell wall antigen (11), whereas the monoclonal antibody used for the ELISA test reacted with an extracellular polysaccharide antigen of *C. sepedonicum* (10). Hence, ELISA sensitivity cannot be expressed in terms of bacterial numbers.

TABLE 3. Mean[a] absorbance readings (405 nm) for enzyme-linked immunosorbent assays of potato stems from inoculated and uninoculated plants during the growing season

	Days after planting						
Cultivar and treatment	26	40	54	67	82	96	110
Red Pontiac, inoculated							
Mean	0.05	0.13	0.14	0.65	0.75	1.41	0.96
Standard deviation	0.02	0.12	0.11	0.15	0.25	0.40	0.55
Red Pontiac, uninoculated							
Mean	0.04	0.04	0.02	0.07	0.08	0.05	0.06
Standard deviation	0.01	0.01	0.01	0.02	0.01	0.03	0.03
Russet Burbank, inoculated							
Mean	0.05	0.05	0.02	0.09	0.23	0.82	0.42
Standard deviation	0.01	0.02	0.01	0.05	0.27	0.59	0.51
Russet Burbank, uninoculated							
Mean	0.05	0.04	0.02	0.06	0.15	0.05	0.12
Standard deviation	0.01	0.00	0.01	0.01	0.17	0.02	0.08
Desiree, inoculated							
Mean	0.05	0.05	0.04	0.18	0.18	0.20	0.09
Standard deviation	0.01	0.01	0.04	0.18	0.25	0.37	0.09
Desiree, uninoculated							
Mean	0.05	0.05	0.02	0.09	0.17	0.23	0.05
Standard deviation	0.01	0.01	0.01	0.03	0.28	0.49	0.05

[a]Mean is based on two determinations per stem. Sixteen plants were tested for each cultivar of the inoculated plants at each sampling date (i.e., four plants from each of four blocks), and eight plants were tested for each cultivar of the uninoculated plants at each sampling date (i.e., four plants from each of two blocks).

Nevertheless, there was a significant correlation between the immunofluorescence and ELISA test results, as would be expected. Inconsistencies between the immunofluorescence and ELISA results were most frequently of the type in which high ELISA values were obtained, while only a few or no cells were observed in immunofluorescence. Experimental error including cross contamination of samples account for some of the discrepancies, particularly those in which the duplicate ELISA values were dissimilar. Discrepancies between the two methods may also have occurred, however, if the bacterial metabolites diffused or were transported beyond the tissue in which bacteria were present. It is conceivable that only extracellular polysaccharide moved from cells of *C. sepedonicum* in the seed piece or basal stem segments into the sampled portion of some of the stems. Such a scenario has been described by De Kam (12) for *Erwinia salicis* antigen that was transported through the xylem of willow (*Salix* spp.) shoots into the leaves where it could be detected by ELISA.

The maximum population density of *C. sepedonicum* achieved in the potato stems ranged from 10^8 to 10^{11} IFU/g among the experiments and cultivars in this study. The mean bacterial populations in Russet Burbank stems reached 10^8 IFU/g in both growth room experiments (Table 1) and in the 1988 field plot experiment (Fig. 2B). The 100-fold higher bacterial population detected in the 1987 field experiment may have been due, in part, to the higher level of inoculum used and to differences in growing conditions (Fig. 1). Nevertheless, some Russet Burbank stems from the growth room experiments and 1988 field plot also had populations as high as those in 1987, but they were fewer in number, resulting in an overall lower mean value. The bacterial populations in Red Pontiac and Desiree in the growth room experiment were in the same range as those in the field experiment. The reproducibility of these results confirms that high population densities may be achieved in symptomless plants under different growing conditions.

The potato cultivars that were used in this study were locally grown cultivars that had shown differences in degree of symptom expression in previous field experiments (unpublished data). Red Pontiac consistently exhibited typical bacterial ring rot symptoms when grown from inoculated seed pieces, Russet Burbank showed moderate symptoms in only a few stems at the same inoculum level, and Desiree only very occasionally expressed field symptoms. In this study, the frequency and severity of symptom expression of all three cultivars were consistent with those previously observed (Table 2). The tendency of the cultivars to express symptoms was reflected by the mean population densities and ELISA values attained. The cultivar, Red Pontiac, which expressed the highest incidence of symptoms also had the highest bacterial population density and the highest ELISA values and, vice versa, the cultivar, Desiree, expressed the lowest incidence of symptoms and had the lowest population density and ELISA values. The cultivar Desiree also had the greatest proportion of stems that were negative in the serological tests in both the growth room (Table 1) and field plot experiments (Fig. 2). Perhaps tolerance to bacterial ring rot not only involves development of a lower population density of bacteria in the plant but also a lower level of stem infection from the seed tuber. These results are strikingly similar to those observed with the taxonomically related bacterium *Clavibacter xyli* subsp. *xyli* by Davis et al (5) in sugarcane. They also found that population densities of the pathogen were lower in resistant than in susceptible host cultivars and that stalks of resistant cultivars often did not contain detectable levels of bacteria when vegetatively propagated from infected plant material. The lower bacterial densities in resistant cultivars were related to the number of vascular bundles that were invaded by the bacterium (13). Although the organization of the dicotyledonous potato vascular tissue is considerably different than that in the monocotyledonous sugarcane, it would be of interest to determine if the number of xylem vessels that were invaded by the bacterium also differed among susceptible and tolerant or resistant potato cultivars.

Our results provide evidence that *C. sepedonicum* can be detected in potato stems during the growing season in symptomless plants. Although the rate at which the bacterial populations developed in the plant varied among the cultivars, readily detectable population densities occurred in all of them at least by 80 days after planting (Fig. 2, Table 3). In most instances bacteria were detected in some of the stems by both immunofluorescence and ELISA before this time. It may be possible, therefore, to field test for bacterial ring rot in much the same way as field tests are being conducted for potato virus diseases (27). It still needs to be established how such field testing will compare to testing of composite seed tuber samples as currently practiced in some regions (1).

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