Validating Immunoassay Test Performance in the Detection of *Corynebacterium sepedonicum* During the Growing Season

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This work supported in part by USDA grant 58-32U4-8-048.
We gratefully acknowledge the technical assistance of P. Nolte, C. Breyer, J. Salas, A. Harris, and N. Fylling.
Published with the approval of the director of the North Dakota Agricultural Experiment Station as Journal article 1874.
Accepted for publication 31 December 1990 (submitted for electronic processing).

ABSTRACT

Gudmestad, N. C., Baer, D., and Kurowski, C. J. 1991. Validating immunoassay test performance in the detection of *Corynebacterium sepedonicum* during the growing season. Phytopathology 81:475-480.

The growth and serological detection of *Corynebacterium sepedonicum* in potato stems was studied in the growth chamber and in the field. Populations of *C. sepedonicum* were similar in two susceptible cultivars, Norchip and Russet Burbank, which differ in seasonal maturity. The maximum population of the ring rot bacterium in these cultivars was between 10^8 and 10^9 cfu/g fresh weight, depending on the growing season. Populations of *C. sepedonicum* in stems of field-grown plants of Belrus developed more slowly and were generally lower when compared to the two susceptible cultivars. Development of bacterial ring rot symptoms in Norchip and Russet Burbank was associated with bacterial populations $\geq 10^7$ cfu/g fresh weight. Indirect fluorescent antibody staining (IFAS) and enzyme-linked immunosorbent assay (ELISA) were studied for their effectiveness in detecting populations of *C. sepedonicum* during the growing season. Specificity and efficiency of both serological tests were high, >94% and >90%, respectively, in both years of the study. IFAS and ELISA were capable of detecting 10^6 cfu/g fresh weight with a detection sensitivity of approximately 80%. Based on the development of populations of *C. sepedonicum* in planta and the overall efficiency of IFAS and ELISA, these serological tests could be used to detect symptomless ring rot infections during the growing season.

Additional keywords: *Clavibacter michiganense* subsp. *sepedonicum*, *Solanum tuberosum*.

Control of bacterial ring rot of potato, caused by *Corynebacterium sepedonicum* (syn. *Clavibacter michiganense* subsp. *sepedonicum*), has been centered around the use of disease-free, certified seed potatoes. A zero tolerance for ring rot exists in all certified seed potato production areas of North America (14); no level of the disease is tolerable and a certified field or seed lot is rejected if any plants or plant parts infected with ring rot are found during the inspection process.

A field inspection, performed by trained field inspectors, is conducted during the latter part of the growing season primarily to visually detect bacterial ring rot symptoms (14). Because a number of plant pathogens can cause wilting in potato, a plant visually suspected of being infected with *C. sepedonicum* is usually confirmed in the laboratory, frequently by a serological technique (15). Unfortunately, the difficulty of this inspection process is increased by the presence of confounding factors, such as plant senescence, Verticillium wilt, Fusarium wilt, and other diseases that destroy plant foliage.

A primary factor contributing to the persistence of ring rot in the potato industry is that this disease can exist as symptomless (latent) infections (11); the bacterium can be present in potato plants and tubers but no visible disease symptoms are expressed. A number of variables can influence whether or not a ring rot infection will be asymptomatic. Inoculum dosage (1,11), potato cultivar (1,8), and environmental conditions (9) can affect the development of ring rot disease symptoms. Additionally, the frequency of ring rot disease expression in a field may be so low that its visual detection during field inspections is extremely difficult (2), if not improbable.

The objective of this study was to determine the populations of recoverable *C. sepedonicum* in stems of potato cultivars that differ in their seasonal maturity and ability to express bacterial ring rot symptoms. In addition, we wanted to determine the efficiency of serological detection of these bacterial populations.

MATERIALS AND METHODS

Bacterial cultures. Two strains of *C. sepedonicum* were used in the course of these studies. Strain Cs 5-4, which is resistant to 100 ppm streptomycin (courtesy of G. A. Nelson, Agriculture Canada, Lethbridge, Alberta), was used in growth-chamber studies. Strain NDCs 50/100, which is resistant to 50 ppm rifampin and 100 ppm streptomycin, was used in all field experiments. The double antibiotic-resistant strain of *C. sepedonicum* was a spontaneous mutant obtained by using standard gradient plate techniques (16). This strain was selected because of its high virulence characteristics compared to the parental strain, NDCS AS-1, and for stability of the antibiotic resistance.

Cultures of each strain were stored at −80 C and streaked onto nutrient broth-yeast extract medium (NBY) (13) amended with the appropriate antibiotics when needed. Inoculum was prepared by placing two loopfuls of bacteria from solid NBY-medium plates into flasks that contained NBY broth amended with antibiotics. Cultures of bacteria were grown at room temperature (23 ± 2 C) on an orbital shaker for 48 hr. Bacteria were harvested by centrifugation (7,000 × *g* for 15 min), and the bacterial pellet was resuspended in quarter-strength NBY broth. Populations were determined spectrophotometrically based on a previously established standard curve at 640 nm.

Plant inoculations. Stem cuttings of *Solanum tuberosum* L. 'Norchip' and 'Belrus,' used in growth-chamber studies, were obtained by removing the primary growing point of each cultivar to stimulate axillary bud development. When axillary stems were 5–8 cm long, they were removed and rooted in moist Sunshine Mix (Fisions Blend #1, Western Corp. Vancouver, B.C., Canada). After 21–28 days, rooted stem cuttings were removed and excess potting mixture was washed off under running tap water. Plants of each cultivar were inoculated by placing the roots in a suspension of 10^6 cfu/ml of *C. sepedonicum* for 1 hr. After inoculation, plants were individually transferred to a 10-cm-diameter clay pot containing Sunshine Mix and were placed in the growth chamber. Uninoculated plants of each cultivar served as controls.

Potato cultivars Norchip, Russet Burbank, and Belrus were used in field experiments in 1988 and 1989. Single-eye seed pieces (approximately 25–30 g) were inoculated by vacuum infiltration with 10^4 or 10^9 cfu/ml of *C. sepedonicum*. Seed pieces of each cultivar, vacuum-infiltrated with quarter-strength NBY, served as controls.

Growth-chamber experiments. Potato plants were grown under a 10-hr photoperiod with a total light energy of 73 mW/cm^2/hr to encourage ring rot symptom development (12). Day temperature was 23 C and night temperature was 18 C. A minimum of 70 inoculated and 10 uninoculated plants of each cultivar were grown. Treatments were arranged in a completely random design. Twelve inoculated and two uninoculated plants were destructively sampled at weekly intervals for population determinations beginning 21 days after inoculation. The presence or absence of disease symptoms characteristic of bacterial ring rot were noted at each sampling date. The experiment was repeated twice.

Field experiments. Treatments (cultivar × inoculum dosage) were arranged in a randomized complete block design with three replications. A treatment in each replication consisted of a single row containing 30 seed pieces of a cultivar-inoculum dosage combination. Rows were 1 m apart and seed pieces were planted 0.66 m apart. Plants of each treatment were sampled at biweekly intervals beginning 49 days after planting in 1988 and 47 days after planting in 1989. Five plants/treatment/replication were randomly sampled at each sampling date. Potato plants were removed from the soil and a section with 5–7 cm of stem above and below the soil surface was retained for further testing. Development of bacterial ring rot symptoms was monitored on a weekly basis beginning at midseason.

Population determinations. All samples were stored in an ultrafreezer at −80 C until processed. Stem samples were washed in tap water, surface-disinfested in a 0.5% solution of NaOCl for 1 min, and rinsed three times with sterile 0.02 M phosphate-buffered saline (PBS), pH 7.2. A 0.5-g section of stem from near the soil line was aseptically removed and placed in a small plastic bag containing 1.0 ml of PBS. The stem section was crushed by tapping with a rubber mallet. Populations of *C. sepedonicum* were determined by using standard dilution-plating techniques. Aliquots of each 10-fold serial dilution were placed on three replicate plates containing NBY medium amended with the appropriate antibiotics (50 ppm rifampin, 100 ppm streptomycin).

Serological testing. Indirect fluorescent antibody staining (IFAS) (6) and enzyme-linked immunosorbent assay (ELISA) (7) were done with only slight modifications. IFAS was done on plant samples collected in all growth-chamber and field experiments. In all cases, the sample obtained from stem sections used for population determinations also was used in IFAS testing. Four dilutions, undiluted to 10^{-3} for each sample, were examined by IFAS. ELISA was done only on plant samples collected from field experiments conducted in 1988 and 1989. ELISA was done on a 0.5-g stem segment immediately above the segment used for population determinations and IFAS testing.

IFAS was done with monoclonal antibody 9A1, supplied by S. H. De Boer, Agriculture Canada. Crude ascitic fluid containing this antibody was suspended in 50% NH_4SO_4 (1:1). A plant sample tested with IFAS was considered positive if an average of ≥1 fluorescing cells was observed per microscope field. A minimum of 10 randomly selected microscope fields were examined per dilution/sample.

ELISA was done with De Boer's monoclonal antibody 1H3 (7) as either a coat (trap) or sandwich antibody. This monoclonal antibody was treated as described above before its use in serological tests. Two polyclonal antibody sources were tested in reciprocal steps (coat or sandwich) with 1H3 in the indirect double-antibody sandwich ELISA. A rabbit polyclonal anti-*C. sepedonicum* antibody was used in 1988. Blood serum from rabbits was treated with saturated NH_4SO_4, and the resulting pellet was resuspended in PBS with 0.2% sodium azide as preservative. In 1989, a chicken polyclonal antibody (courtesy of S. A. Slack, Cornell University) was used after laboratory tests demonstrated that it had a higher specificity for the target bacterium. The chicken polyclonal antibody had been purified by passage through a DEAE column. A sample from an inoculated plant was considered positive if the absorbance value (A_{405nm}) was greater than two standard deviation units above the mean absorbance value obtained for samples from uninoculated plants of a specific potato cultivar. Mean absorbance values were the result of two duplicate tests.

Specificity, sensitivity, efficiency, and predictive values for negative and positive results were calculated for all serological tests done in these studies (20). A serological test was considered a true positive (TP) when culturing yielded recoverable populations of *C. sepedonicum*. Conversely, a true negative (TN) was obtained when all tests were negative. False positives (FP) resulted when a serological test yielded a positive result but the culturing of stem samples yielded no ring rot bacteria. False negatives (FN) were the result of a negative serological test when culturing yielded populations of *C. sepedonicum*.

Statistical analyses. Analysis of variance and regression analysis were performed by using the Statistical Analysis System (SAS Institute, Inc., Raleigh, NC). A significance value of $P = 0.05$ was used in all statistical tests.

RESULTS

Stem colonization of root-inoculated potato stem cuttings with ring rot bacteria was very rapid under growth-chamber conditions. Populations of *C. sepedonicum* were ≥10^7 cfu/g fresh weight, 21 days after inoculation in both potato cultivars. The maximum population of ring rot bacteria recovered in either cultivar was 10^9 cfu/g fresh weight. Levels of ring rot bacteria were significantly different in Belrus and Norchip only on day 35 in the first growth-chamber study (Fig. 1A). In the second growth-chamber experiment (Fig. 1B), the relationship between populations of ring rot bacteria in the two cultivars was nearly opposite that found in the first experiment. All inoculated plants sampled from both cultivars were positive when tested with IFAS, regardless of the presence or absence of ring rot disease symptoms.

Over the course of the field studies, populations of *C. sepedonicum* in the three cultivars ranged from 0 to 10^9 cfu/g fresh weight (Fig. 2). Maximum populations were reached at 105 and 117 days after planting in 1988 and 1989, respectively.

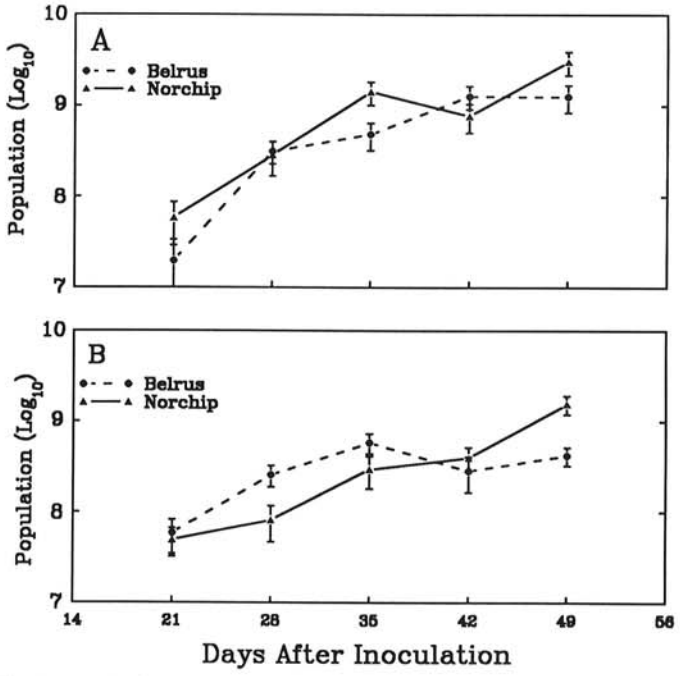

Fig. 1. Population densities of *Corynebacterium sepedonicum* in stems of Norchip and Belrus grown in a growth chamber. **A,** First experiment and **B,** second experiment. Each data point represents the mean population of 12 stems. Vertical bars represent the standard error of the mean.

Symptoms of bacterial ring rot became evident in Norchip and Russet Burbank at 91 and 89 days after planting in 1988 and 1989, respectively. Approximately 15–25% of the lower plant foliage on Russet Burbank plants displayed disease symptoms of wilting and interveinal chlorosis compared to 20–35% of the foliage in Norchip. Symptom development (at approximately 90 days after planting) corresponded to bacterial populations in potato stems of 10^7–10^8 cfu/g of stem tissue (Fig. 2). Typical symptoms of bacterial ring rot, other than stunting, were not evident in Belrus at any time during these field studies. Populations in Belrus varied from 10^2 to 10^9 cfu/g of stem tissue at this time interval, depending on the year of the study. The overall development of *C. sepedonicum* in seed-piece inoculated plants grown in the field tended to be lower than that of root-inoculated plants grown in the growth chamber.

Development of populations of *C. sepedonicum* in Norchip and Russet Burbank was very similar regardless of the original inoculum concentration in both years of the field study (data not shown). The bacterial populations recovered from these two cultivars were not statistically different at any sampling date in either year of the study. In contrast, populations of *C. sepedonicum* in Belrus developed more slowly and were significantly lower ($P = 0.05$) than populations recovered from plants of Norchip and Russet Burbank in 1988 (Fig. 2A). In 1989, there was no cultivar or inoculum effect on stem populations of *C. sepedonicum* (Fig. 2B).

IFAS was very effective in detecting populations of *C. sepedonicum* in stems of all cultivars. Specificity ($TN/[TN + FP] \times 100$) was 98% in both years of the field study. Populations as low as 10 cfu/g fresh weight were detected with this technique but sensitivity was low (<40%) (Fig. 3A). An acceptable level of sensitivity ($TP/[TP + FN] \times 100$) of >80% was not obtained unless populations were $>10^6$ cfu/g (Fig. 3A).

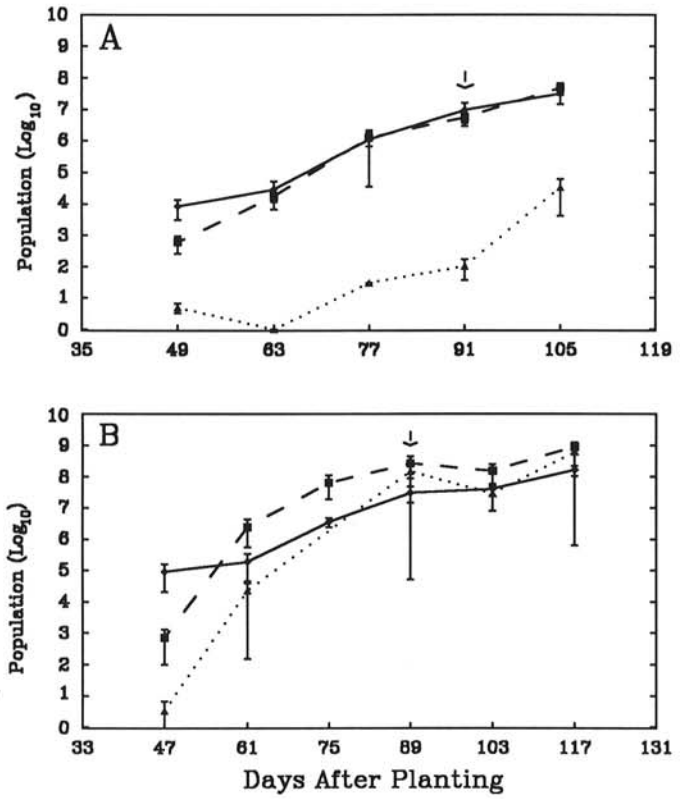

Fig. 2. Populations of *Corynebacterium sepedonicum* in potato stems of Norchip (■), Russet Burbank (♦) and Belrus (▲) grown in the field in **A,** 1988 and **B,** 1989. Each data point represents the mean population in a minimum of 15 stems. Plants inoculated with 10^4 or 10^9 cfu/ml were pooled to develop these growth curves. Vertical bars represent the standard error of the mean. Arrows indicate the initial visual detection of bacterial ring rot symptoms.

Efficiency ($TP + TN/[TP + TN + FP + FN] \times 100$) was >90% regardless of the number of bacteria present in the stem. Predictive values for negative results ($TN/[TN + FN] \times 100$) were lower (88 and 39%) than predictive values for positive results ($TP/[TP + FP] \times 100$) (92 and 98%) in both years.

Mean optical densities (OD_{405nm}) obtained in ELISA tests for the three cultivars with populations of *C. sepedonicum* are illustrated in Figure 4. ELISA test results were more variable in 1988 than in 1989. In 1988, optical densities were much higher when 1H3 monoclonal antibody was used as the coat antibody in ELISA tests. The lack of specificity obtained with the rabbit

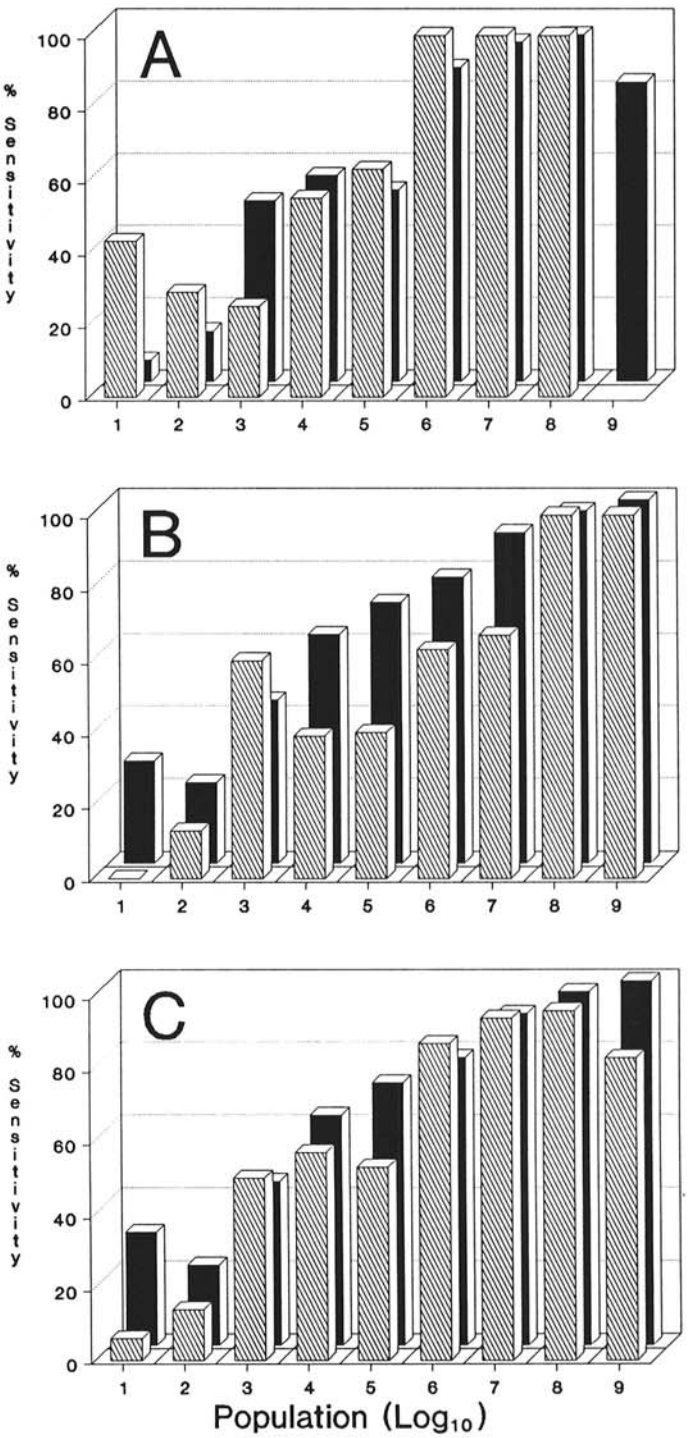

Fig. 3. A, Sensitivity of indirect fluorescent antibody staining (IFAS) in 1988 (striped bars) and 1989 (solid bars). **B,** Enzyme-linked immunosorbent assay (ELISA) in 1988 (striped bars) and 1989 (solid bars). **C,** Comparison of sensitivity of IFAS (striped bars) and ELISA (solid bars) in 1989.

anti-*C. sepedonicum* polyclonal antibody caused us to evaluate other polyclonal antibodies for subsequent studies (Fig. 4A). Chicken polyclonal antibody obtained from S. A. Slack gave superior results and was used in the 1989 field experiment. Chicken polyclonal antibody was used in reciprocal steps (i.e., as either a coat or a sandwich antibody in indirect double-sandwich antibody ELISA) (Fig. 4B). Absorbance values for ELISA tests were generally higher when the chicken polyclonal was used as the coat antibody and the mouse monoclonal was used as the sandwich antibody.

ELISA test results, combined for all cultivars and sampling dates in 1989, were positively correlated with the population of ring rot bacteria recovered from the stems by dilution plating (Fig. 5). Correlations were significant regardless of whether or not the coat antibody used in the ELISA procedure was the chicken polyclonal ($r = 0.676$, $P = 0.05$) or the mouse monoclonal antibody ($r = 0.733$, $P = 0.05$).

Test performance data were nearly as high for ELISA as those obtained for IFAS. Sensitivity of ELISA for detecting *C. sepedonicum* at any population level was generally higher in 1989 than 1988 (Fig. 3B). Specificity and overall efficiency of the ELISA procedure in 1989 with the chicken coat/mouse sandwich antibody combination were improved to 95 and 80%, respectively. Efficiency of ELISA in detecting populations of *C. sepedonicum* $>10^3$ cfu/g was 94%. The predictive value for positive results was also 94% in 1989.

Sensitivity of ELISA and IFAS was similar in 1989 (Fig. 3C). ELISA tests had acceptable levels of sensitivity when populations of *C. sepedonicum* were $\geq 10^6$ cfu/g fresh weight. ELISA tended to be more sensitive than IFAS at lower bacterial populations ($<10^6$ cfu/g) (Fig. 3C).

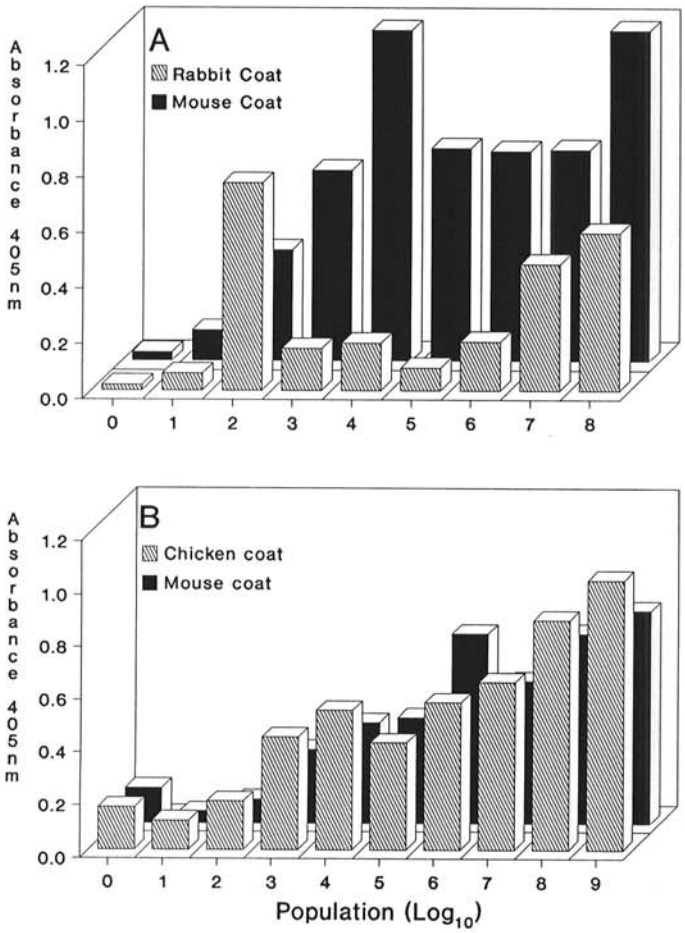

Fig. 4. Mean absorbance values (A_{405nm}) for enzyme-linked immunosorbent assays conducted on inoculated and uninoculated stems of three potato cultivars with different anti-*Corynebacterium sepedonicum* antibody combinations in **A**, 1988 and **B**, 1989.

DISCUSSION

Use of antibiotic-resistant strains of *C. sepedonicum* facilitated our ability to determine low populations of the bacterium during the early stages of the infection process. This bacterium, as well as other taxonomically related bacteria, are notoriously slow-growing in culture (19) and are frequently overgrown when their populations are low relative to the population of other microflora. Hence, *C. sepedonicum* population determinations by dilution-plating techniques have been hindered. Results of the growth-chamber studies demonstrated that the recovery of ring rot bacteria, with little competition from other endophytic microorganisms, would make field studies feasible. However, we selected a spontaneous mutant strain of *C. sepedonicum* that had better virulence characteristics than Cs 5-4 and higher antibiotic resistance to further reduce competition from other microorganisms. Strain NDCs 50/100 proved to be highly virulent and was recovered occasionally from stem samples at levels as low as 1 cfu/g fresh weight.

The potato cultivar Belrus was included in these studies because of its reported inability to express bacterial ring rot symptoms in the field (8). We have found that this cultivar, while not expressing typical wilt symptoms as a result of a *C. sepedonicum* infection, does display considerable stunting and marginal leaf necrosis at the end of the growing season (N. C. Gudmestad, *unpublished data*). Although we were interested in determining if the ring rot reaction of Belrus was one of resistance or tolerance, our experiments were not specifically designed for this purpose. Therefore, whether the reaction of Belrus to bacterial ring rot is one of resistance or tolerance is unresolved by the experiments reported here. It is clear from our data, however, that high populations of *C. sepedonicum* are capable of developing in this cultivar. Because ring rot is difficult to visually detect in plants of Belrus grown in the field, this cultivar is capable of playing an important role in the epidemiology of ring rot on a seed potato farm where it is grown.

The maximum population of *C. sepedonicum* recovered from potato stems in growth-chamber and field experiments by dilution plating was 10^8–10^9 cfu/g fresh weight. This population is 10- to 100-fold lower than populations detected by De Boer and McCann (4) with immunofluorescence. While cultivar differences in the two studies cannot be excluded to explain this apparent discrepancy, it is more likely that the immunofluorescence procedure used by these researchers detected both viable and nonviable bacterial cells. Only viable cells of *C. sepedonicum* would be capable of growing in vitro, which was the basis of population estimates in the studies reported here. Additionally, these researchers (4) reported the population of *C. sepedonicum*

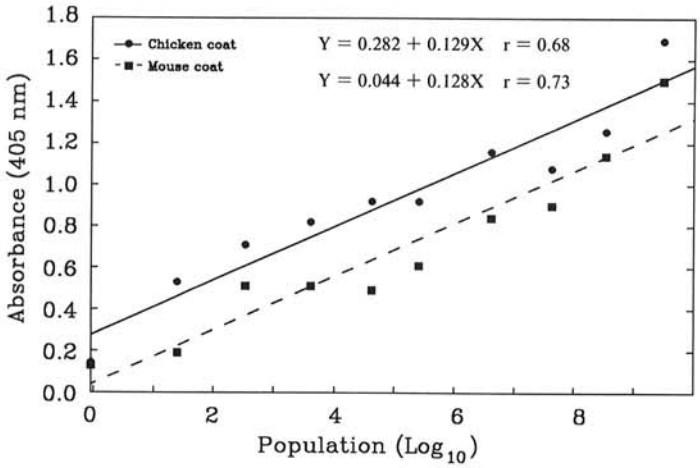

Fig. 5. Relationship between mean absorbance values (A_{405nm}) for enzyme-linked immunosorbent assays with two antibody sources and populations of *Corynebacterium sepedonicum* in inoculated stems of three potato cultivars grown in the field in 1989.

as immunofluorescing units (IFU)/g detected by an automated microscope system (3). In our studies, we frequently observed nonspecific fluorescent staining of cellular debris and other microflora. This phenomenon also may partially explain the higher population densities reported in other studies.

The sensitivity of a pathogen-detection procedure, in clinical terms, describes how well a particular test will recognize an infected individual in a population (20). The overall sensitivity of IFAS tests was comparable in the two years of the study, whereas the sensitivity of ELISA was considerably higher in 1989 than in 1988 (Fig. 3B). Surprisingly, ELISA was generally more sensitive than IFAS in detecting ring rot bacteria in potato stems (Fig. 3C). Populations as low as 10 cfu/g fresh weight could be detected; however, the sensitivity of IFAS and ELISA at this population was extremely low (≤31%). Sensitivity of IFAS and ELISA was not at an acceptable level, >80%, until populations of ring rot bacteria were $\geq 10^6$ cfu/g fresh weight.

Sensitivity of IFAS for *C. sepedonicum* detection has been previously reported (4,15). De Boer and McCann (4) reported that approximately 10^4 IFU/g fresh weight could be detected by using an IFAS procedure and an automated microscope system (3). While it is difficult to ascertain how they arrived at this number, we were able to detect this level of *C. sepedonicum* with a sensitivity of about 57% with IFAS and a manual system. Slack et al (15) reported IFAS as capable of detecting 10^2 cfu/ml. However, this sensitivity estimate was generated with bacteria from pure culture and the detection efficiency at this population level was not determined. We feel that the tedious nature of manually examining large numbers of IFAS slides with a microscope may introduce unacceptable levels of human error, thereby lowering detection limits.

Test performance values for IFAS assays also may have been higher had we accepted a lower threshold number of fluorescing cells to determine a positively infected plant. We chose to designate a test plant as positively infected when an average of ≥1 bacterial cells fluoresced per microscope field. While a lower threshold number would have raised the level of sensitivity, this would have occurred at the expense of specificity levels, which describe how well a test recognizes uninfected individuals (20). When dealing with a disease with a zero tolerance such as bacterial ring rot, it is extremely important to limit the amount of false positives. The erroneous detection of ring rot on a seed potato farm could be financially catastrophic to the grower.

A high correlation between ELISA test results and the population of ring rot bacteria recovered from potato stems was somewhat surprising (Fig. 5). The monoclonal antibody used in the ELISA procedure is one that reacts with the extracellular polysaccharide (EPS) antigen of *C. sepedonicum* (7). As a result, De Boer and McCann (4) have pointed out that the sensitivity of an ELISA procedure with this monoclonal antibody cannot be expressed in terms of bacterial numbers, presumably because EPS produced by the bacterium is not proportional to the population. Nevertheless, based on data reported here, it appears that 10^5 cfu/g fresh weight of *C. sepedonicum* can be detected with a sensitivity of 72% with this procedure (Fig 4C). A relationship between bacterial numbers and EPS production has been established for *Clavibacter michiganense* subsp. *insidiosum* (17), which is taxonomically related to the ring rot bacterium.

The serological procedures used in these studies were sensitive enough to detect populations of *C. sepedonicum* in the absence of symptom expression. Populations of ring rot bacteria $<10^6$ cfu/g fresh weight could be detected with a relative sensitivity of 50–75%. This means that a testing program conducted during the growing season could be initiated as early as 60 days after planting for susceptible cultivars such as Norchip (Fig. 2). Symptom development in Russet Burbank and Norchip was associated with populations of $\geq 10^7$ cfu/g, at approximately 90 days after planting. Although the development of species-specific hybridization probes for the detection of *C. sepedonicum* is eminent (10,18), no information exists as to their sensitivity for detecting bacterial populations in planta. Until this kind of information is available, serological assays will be the primary tools used to detect ring rot bacteria in diagnostic and latent infection assays.

Results reported here and elsewhere (4,5) demonstrate the feasibility of using a serological procedure to detect symptomless infections of bacterial ring rot during the growing season. An immediate application would be in the detection of *C. sepedonicum* in certified potato seed lots that did not receive a ring rot field inspection because of a mitigating factor, such as an early field frost. Additionally, a number of states and Canadian provinces do field testing for potato viruses (generally Potato Virus X [PVX]) that are latent in the plant (14). ELISA is generally used to detect PVX in these tests and would, therefore, be relatively easy for certification agencies to use in another pathogen testing program. While the IFAS procedure has been reported as being more sensitive than ELISA in detecting symptomless populations of ring rot bacteria (4), results reported here indicate that ELISA can be as sensitive as IFAS. However, we found IFAS to be more tedious and much slower in terms of the amount of time required to process large numbers of samples. For these reasons, ELISA may be a procedure that can be more readily adapted for field testing to detect symptomless ring rot infections during the growing season. A number of production areas currently use a postharvest test for this purpose. However, a field test conducted during the growing season may have the advantage in that the samples to be tested are less bulky and populations in the test samples would be higher and, therefore, more efficiently detected.

LITERATURE CITED

1. Bishop, A. L., and Slack, S. A. 1987. Effect of cultivar, inoculum dose, and strain of *Clavibacter michiganense* subsp. *sepedonicum* on symptom development in potatoes. Phytopathology 77:1085-1089.
2. Clayton, M. K., and Slack, S. A. 1988. Sample size determination in zero tolerance circumstances and the implications of stepwise sampling: Bacterial ring rot as a special case. Am. Potato J. 65:711-723.
3. De Boer, S. H., and Hall, J. W. 1988. An automated microscope system for estimating the population of *Corynebacterium sepedonicum* cells labelled with monoclonal antibodies in immunofluorescence. Can. J. Plant Pathol. 10:215-220.
4. De Boer, S. H., and McCann, M. 1989. Determination of population densities of *Corynebacterium sepedonicum* in potato stems during the growing season. Phytopathology 79:946-951.
5. De Boer, S. H., and McNaughton, M. E. 1986. Evaluation of immunofluorescence with monoclonal antibodies for detecting latent bacterial ring rot infections. Am. Potato J. 63:533-543.
6. De Boer, S. H., and Wieczorek, A. 1984. Production of monoclonal antibodies to *Corynebacterium sepedonicum*. Phytopathology 74:1431-1434.
7. De Boer, S. H., Wieczorek, A., and Kummer, A. 1988. An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. Plant Dis. 72:874-878.
8. Manzer, F. E. 1981. Reaction of the potato variety Belrus to bacterial ring rot infections. Maine Agric. Exp. Stn. Misc. Rep. No. 256. 2 pp.
9. Manzer, F. E., Gudmestad, N. C., and Nelson, G. A. 1987. Factors affecting infection, disease development and symptom expression of bacterial ring rot. Am. Potato J. 64:671-675.
10. Mogen, B. D., Oleson, A. E., and Gudmestad, N. C. 1987. Dot-blot detection of the ring rot pathogen: Evaluation of cloned *Corynebacterium sepedonicum* DNA sequences as potential hybridization probes. (Abstr.) Am. Potato J. 64:701.
11. Nelson, G. A. 1982. *Corynebacterium sepedonicum* in potato: Effect of inoculum concentration on ring rot symptoms and latent infection. Can. J. Plant Pathol. 4:129-133.
12. Nelson, G. A., and Kozub, G. C. 1983. Effect of total light energy on symptoms and growth of ring rot-infected Red Pontiac potato plants. Am. Potato J. 60:461-468.
13. Schaad, N. W. 1988. Identification schemes of common genera. Pages 1-15 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. N. W. Schaad, ed. The American Phytopathological Society, St. Paul, MN. 164 pp.
14. Shepard, J. F., and Claflin, L. E. 1975. Critical analyses of the principles of seed potato certification. Annu. Rev. Phytopathol. 13:271-293.
15. Slack, S. A., Kelman, A., and Perry, J. B. 1979. Comparison of three serodiagnostic assays for detection of *Corynebacterium sepedonicum*. Phytopathology 69:186-189.

16. Szybalski, W., and Brison, V. 1952. Genetic studies on microbial cross resistance to toxic agents. J. Bacteriol. 64:489-499.
17. Van Alfen, N. K., McMillan, B. D., and Wang, Y. 1987. Properties of the extracellular polysaccharides of *Clavibacter michiganense* subsp. *insidiosum* that may affect pathogenesis. Phytopathology 77:501-505.
18. Verreault, H., Lafond, M., Asselin, A., Banville, G., and Bellemare, G. 1988. Characterization of two DNA clones specific for identification of *Corynebacterium sepedonicum*. Can. J. Microbiol. 34:993-997.
19. Vidaver, A. K. 1982. The plant pathogenic corynebacteria. Annu. Rev. Microbiol. 36:495-517.
20. Zweig, M. H., and Robertson, E. A. 1987. Clinical validation of immunoassays: A well-designed approach to a clinical study. Pages 97-127 in: Immunoassay: A Practical Guide. D. W. Chan, ed. Academic Press, New York. 167 pp.