Serological Detection of Nonmucoid Strains of *Clavibacter michiganensis* subsp. *sepedonicus* in Potato

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ABSTRACT

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Serological detection of several strains of *Clavibacter michiganensis* subsp. *sepedonicus* was studied in vitro and in two potato cultivars grown in the field in 1989 and 1990. Colony morphology of these strains was categorized as mucoid, intermediate, or nonmucoid. Enzyme-linked immunosorbent assay (ELISA) detected mucoid and intermediate strains, but not nonmucoid strains, grown in culture. ELISA performed on infected potato stem samples using six combinations of anti-*C. m. sepedonicus* antibodies did not detect the three nonmucoid strains tested but did detect the other strains. Lack of sensitivity of ELISA to detect nonmucoid strains was not a function of bacterial population but may have resulted from lack of diffusible antigen recognizable by available antibody sources. In contrast, all strains were detected in infected potato stems using indirect fluorescent antibody staining.

Additional keywords: bacterial ring rot, *Corynebacterium michiganensis* subsp. *sepedonicum*, exopolysaccharide, glycocalyx, serology, *Solanum tuberosum*.

Bacterial ring rot of potato is caused by *Clavibacter michiganensis* subsp. *sepedonicus* (syn. *Corynebacterium sepedonicum, C. m. sepedonicum*). Management of this disease is based on the use of disease-free seed. Production of pathogen-free seed is accomplished via meristem tip culture accompanied by rigorous laboratory testing. Seed stocks are maintained at relatively low disease levels by seed certification programs.

Diagnosis of bacterial ring rot in certified seed fields is done by the visual observation of symptoms in the field during routine inspections. Development of ring rot symptoms in the field is influenced by inoculum dose, potato cultivar, environmental conditions, and the strain of the bacterium causing the disease (2,3,16).

Plants with visible symptoms of bacterial ring rot are confirmed in the laboratory (18,20). Confirmation is by observation of gram-positive, small, irregular, coccobacilli by Gram stain; isolation of slow-growing, mucoid, and irregular colonies in culture; symptom expression in inoculated test plants; and serological procedures (9). Serological procedures used within the last 15 yr include immunodiffusion, latex agglutination, indirect fluorescent antibody staining (IFAS), and enzyme-linked immunosorbent assay (ELISA)(7,10,13,20). With the advent and use of monoclonal antibodies, the reliability of IFAS and ELISA has been increased. ELISA is as sensitive and as specific as IFAS and more rapid than IFAS when large numbers of specimens need processing (16). However, problems in diagnosing ring rot due to antigenic variation among pathogenic strains of *C. m. sepedonicus* have been reported recently (1,7).

Variant strains of *C. michiganensis* have been identified from natural field sources (1,21). These strains vary in colony morphology and growth rate on commonly used culture media. The purpose of this study was to investigate the specificity and sensitivity of serological techniques for the detection of mucoid, intermediate, and nonmucoid strains of *C. m. sepedonicus* under in vitro and in planta conditions.

MATERIALS AND METHODS

Bacterial strains. *C. m. sepedonicus* strains used in this study are listed in Table 1. Mucoid strain CsOFF (OFF) was isolated from *C. m. sepedonicus* infected potato plants from Minnesota. OFF was used to represent a typical mucoid strain of the pathogen

by culture morphology. Strains CsND009 (ND9) and CsWi002 (Wi2) were isolated from infected potato plants from North Dakota and Wisconsin, respectively. Colonies of strains ND9 and Wi2, which were termed "intermediate" in their morphology, initially appeared nonmucoid but eventually expressed both mucoid and nonmucoid forms after an extended culture period (>14 days). *C. m. sepedonicus* strains CsAK014 (AK14), CsIdNM-1 (INM-1), and CsSDHanke (SD1) were chosen for their slow growth rate and nonmucoid, round colony morphology on nutrient broth/yeast extract (NBY) agar (18). Strain AK14 and SD1 were isolated from infected tubers from Alaska and South Dakota, respectively. A culture of INM-1 was provided courtesy of S. A. Slack (1). Gram stain, IFAS, and eggplant pathogenicity (3) were typical of *C. m. sepedonicus* for all six strains (8).

In vitro experiment. The six *C. m. sepedonicus* strains OFF, Wi2, ND9, INM-1, AK14, and SD1 were grown in NBY broth to late log phase (3–4 days) except for Wi2 (6–7 days). Turbidity of each culture was measured at 540 nm (peak absorbance for NBY) on a UV-visible scanning spectrophotometer (UV-1201, Shimadzu Scientific Instruments, Inc., Columbia, MD). Turbidity of each culture was adjusted to approximate absorbances (A_{540nm}) of 0.3, 0.2, 0.1, 0.05, and 0.025. Plate counts by the spread plate method were performed on each strain at each turbidity (22). Absorbance readings were matched with the plate counts of the same culture to correlate bacterial population estimates with turbidity. ELISA was performed on the same sample using the polyclonal chicken anti-*C. m. sepedonicus* as the coating antibody, and mouse monoclonal 1H3 anti-*C. m. sepedonicus* as the sandwich antibody. The experiment was performed twice.

Preparation of inoculum. Bacteria were cultured at room temperature (~ 23 C) in NBY broth on an orbital shaker for 48–72 h. Bacteria were harvested by centrifugation at 7,000 *g* for 15 min. The pellet was suspended in 0.05 M phosphate buffer (pH 7.2) plus 0.85% saline (PBS). Suspensions were adjusted with PBS to turbidity of $A_{600nm} = 0.1$ (~ 10^8 colony forming units per ml (cfu/ml) estimated by dilution plating) and diluted by two 10-fold serial dilutions to achieve 10^6 cfu/ml (3).

Field experiments. Potato cultivars Norchip and Russet Burbank were used in a field experiment in 1989 and 1990. Vacuum infiltration of seed pieces was performed as previously described (12,16). Inoculum contained 10^6 cfu/ml for each bacterial strain based on previously established standard growth curves (A_{600nm}).

Strains of *C. m. sepedonicus* in 1989 were mucoid OFF, intermediate ND9, and nonmucoid AK14 and INM-1. Strains in 1990 were mucoid OFF, intermediate Wi2, and nonmucoid AK14, INM-1, and SD1. Seed pieces vacuum infiltrated with PBS alone were used as a healthy control.

The experiment was a factorial set of treatments (cultivar × inoculum strain) arranged in a completely random design. Treatments were replicated three times. Seed pieces were spaced 0.66 m apart in rows on 1-m centers. Each replicate treatment was planted with 50 seed pieces.

Plants were sampled biweekly from 75–116 days after planting in 1989. In 1990, plants were sampled biweekly from 56–98 days after planting. Five stem samples of each treatment replication were collected at each sampling date. Stem sections, 5–7 cm above and below the soil line were placed in plastic bags and transported on ice to the laboratory. Stems were refrigerated if processing occurred within 7 days, or frozen at −80 C if processing was delayed.

TABLE 1. Origin and properties of *Clavibacter michiganensis* subsp. *sepedonicus* strains

Strain[w]	State[x]	Year isolated	Cultivar[y]	Colony morphology[z]
AK14	Alaska	1988	Green Mountain	N
INM-1	Idaho	1982	Russet Burbank	N
ND9	North Dakota	1988	Atlantic	I
SD1	South Dakota	1988	Atlantic	N
Wi2	Wisconsin	1988	Atlantic	I
OFF	Minnesota	1984	Russet Burbank	M

[w] CsAk014 = AK14; CsIdNM-1 = INM-1; CsND009 = ND9; CsSDHanke = SD1; CsWi002 = Wi2; and CsOFF = OFF.
[x] Area from which strain was originally isolated.
[y] Potato cultivar from which strain was originally isolated.
[z] Colony morphology: N = nonmucoid, M = mucoid, I = intermediate.

Serological testing. ELISA and IFAS were performed as previously described (9,16). Two 1-g sections of stem tissue ground in 2 ml of PBS or modified Agdia ELISA sample buffer were used in IFAS and ELISA, respectively.

IFAS was performed using monoclonal 9A1 mouse anti-*C. m. sepedonicus* (13)(commercially available, Agdia, Inc., Elkhart, IN) and goat anti-mouse (IgG and IgM) affinity purified, polyclonal, fluorescein labeled antibody (Boehringer Mannheim Corp., Indianapolis, IN), following the package insert instructions. Three 10-fold serial dilutions of each sample were made for IFAS testing following maceration. Ten microliters of the sample or its dilution were placed on a well (5 mm) of a slide (Medical Packaging, Panorama City, CA). A plant sample was considered positive with IFAS if more than one bacterial cell fluoresced per oil immersion high power field (IFU/hpf) (total magnification ×1,250). Ten random microscope fields were examined per well, and the number of fluorescing bacterial cells in 10 fields was averaged. A positive control slide was included, consisting of three 10-fold serial dilutions of ~ 10^8 cfu/ml suspension of a *C. m. sepedonicus* strain. A negative control slide consisted of *Erwinia carotovora* prepared in the same manner.

Anti-*C. m. sepedonicus* sera, used for ELISA testing, were obtained from several sources. AS52 rabbit polyclonal anti-*C. m. sepedonicus* (S. H. De Boer, Agriculture Canada, British Columbia) and chicken polyclonal anti-*C. m. sepedonicus* (S. A. Slack, Cornell University) were used as coating (capture) or sandwich antibodies in ELISA. 1H3 mouse monoclonal anti-*C. sepedonicus* (14) was used as the sandwich antibody in combination with one of the polyclonal antibodies used for coating. Commercially available conjugated antibodies were used at their recommended working dilution. Goat anti-mouse IgG and IgM antibody-alkaline phosphatase (calf intestine) and goat anti-rabbit IgG, antibody-alkaline phosphatase (calf intestine) conjugates were purchased from Boehringer Mannheim Corp. Rabbit anti-chicken IgG (whole molecule) alkaline phosphatase conjugate A-9171 was purchased from Sigma (St. Louis, MO).

The anti-*C. m. sepedonicus* antibodies used in indirect double antibody ELISA were applied in reciprocal steps as a coat or sandwich antibody in all possible combinations in 1989. Each antibody was titred to endpoint, and the optimum working dilution was determined using the appropriate commercially available anti-IgG alkaline phosphatase conjugated antibody. Optimum working dilution was 1:1,000 for the rabbit and chicken polyclonal antibodies and 1:2,000 for the monoclonal antibody.

Mean absorbances were calculated from replicate wells, each performed in duplicate. A plant sample was considered positive by ELISA if the absorbance value at 405 nm was greater than two standard deviations above the mean of the uninoculated plant samples of each variety.

In 1990, ELISA was performed using the chicken polyclonal as the coat and the mouse monoclonal antibody as the sandwich based on the performance of this antibody combination the previous year.

Data collection and analysis. A direct microscopic count method was used to determine bacterial population numbers (17,22). IFAS was used to determine the population size of each *C. m. sepedonicus* strain present in each stem. Populations were expressed as immunofluorescing units per gram fresh weight (IFU/g) of stem tissue.

Specificity of ELISA was calculated using the formula:

$$\text{Specificity} = TN \div (FP + TN) \times 100 \quad (1)$$

where TN = true negatives and FP = false positives. True negatives represent uninoculated plants with negative serological (IFAS and ELISA) test results. False positives represent uninoculated plants with positive serological test results.

Sensitivity was calculated using the formula:

$$\text{Sensitivity} = \text{TP} \div (\text{TP} + \text{FN}) \times 100 \quad (2)$$

TP (sum of true positives) represents stem samples with IFAS results ≥1 IFU/hpf and ELISA absorbance values greater than two standard deviations above the mean of the negative control (uninoculated) samples. False negatives (FN) represent stems with ≥1 IFU/hpf by IFAS and ELISA absorbance values less than two standard deviations above the mean of the negative controls. Sensitivity and specificity were used to determine the best antibody combination in 1989.

Efficiency of the test system also was evaluated. Efficiency was calculated in the following formula:

$$\text{Efficiency} = (\text{TN} + \text{TP}) \div \text{total population} \times 100. \quad (3)$$

Analysis of variance, Duncan's multiple range tests, and regression analysis were performed using Statistical Analysis System procedures (Raleigh, NC). When appropriate, the $\log_{10}$ of the arithmetic number of bacterial cells was used for data presentation.

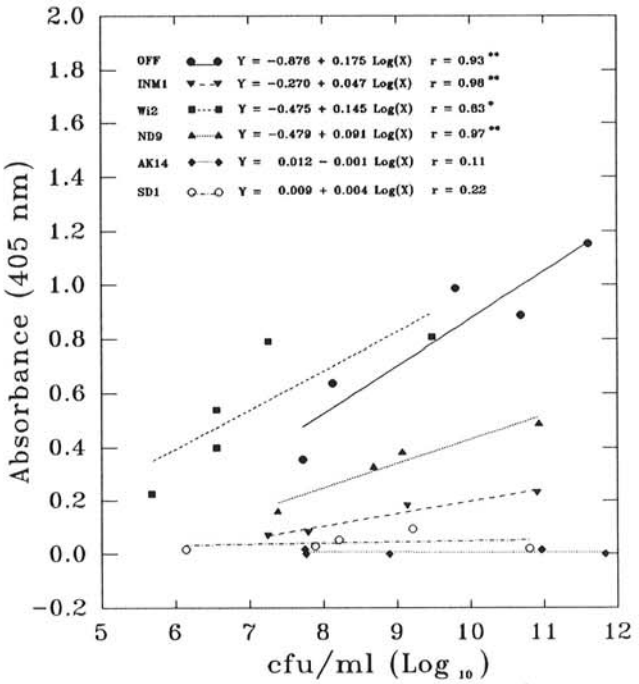

Fig. 1. Relationship of ELISA absorbance values to cfu/ml of *Clavibacter michiganensis* subsp. *sepedonicus* grown to specific turbidities in NBY broth culture. Each data point is the mean of four samples. * and ** represents significance at $P = 0.05$ and 0.01, respectively.

RESULTS

In vitro experiment. An in vitro experiment was performed on the six strains of *C. m. sepedonicus* to determine ELISA absorbance levels for increasing populations of each strain. High absorbance values by ELISA corresponded to high population counts of mucoid strain OFF and intermediate strain Wi2 (Fig. 1). Nonmucoid strains AK14 and SD1 had negative ELISA values with high population numbers. Nonmucoid strain INM-1 at populations above 10^9 cfu/ml had borderline positive ELISA absorbance values. Strain ND9 had ELISA absorbance values ranging from 0.2 to 0.5 when the population count ranged from 10^7 to 10^{11} cfu/ml. Although these values indicate a positive ELISA result, the absorbance value for 10^{11} cfu/ml of ND9 is roughly equivalent to the absorbance resulting from populations of 10^6 to 10^7 cfu/ml of strains OFF or Wi2.

A highly significant ($P = 0.01$) correlation existed between the cfu/ml of *C. m. sepedonicus* and ELISA absorbance values for strains OFF, INM-1, Wi2, and ND9 (Fig. 1). As the bacterial population increased, ELISA absorbance values increased. This relationship was not significant for nonmucoid strains AK14 and SD1. Furthermore, slopes (b_o) for nonmucoid strains AK14 and SD1 were significantly different ($P = 0.05$) from slopes for the other strains.

Field experiment. Immunofluorescent units of *C. m. sepedonicus* per gram of stem tissue were analyzed to determine if there were differences between cultivars. Cultivar × strain interactions were not significant in either year of the study at any sampling date. Because no significant differences in bacterial populations were detected between Russet Burbank and Norchip, data for the two cultivars were combined in each year for further analysis.

C. m. sepedonicus strains differed in their ability to colonize potato stems. Significant differences among strains in IFU/g were evident at 75 and 116 days after planting (DAP) in 1989 (Table 2). Nonmucoid strain AK14 had lower population sizes than the other strains in the first and last sampling date, and this difference was sometimes significant ($P = 0.05$). Stem populations of OFF and Wi2 were significantly higher than all other strains at 70 and 98 DAP in 1990 (Table 3). Populations of intermediate strain Wi2 were significantly higher than all other strains at 56 and 84 days after planting. Populations of mucoid, intermediate, and nonmucoid strains in stems were not significantly different in 1989 and 1990 when comparing similar sampling dates (i.e., 70 and 75, 84 and 89, 98 and 103 DAP) (Tables 2 and 3).

The cultivar × strain interaction was nonsignificant for ELISA absorbance values at any sampling date in 1989, but in 1990 there was a significant cultivar × strain interaction for 56 and 70 DAP. Stems infected with mucoid strain OFF had significantly higher mean ELISA absorbance values than the nonmucoid strains INM-1 and AK14 at all sampling dates in 1989 and 1990 (Tables 2 and 3). Intermediate ND9 had ELISA values similar to the nonmucoid strains at 75 DAP in 1989 but absorbance values similar to the mucoid OFF at 89 and 103 DAP. At 116

TABLE 2. Mean populations and ELISA absorbance values of strains of *Clavibacter michiganensis* subsp. *sepedonicus* strains in 1989 potato stems

	Days after planting							
	75		89		103		116	
Strain[w]	IFU/g[x]	A_{405nm}[y]	IFU/g	A_{405nm}	IFU/g	A_{405nm}	IFU/g	A_{405nm}
OFF	2.0×10^8 a	0.971 a	2.6×10^8 a	0.852 a	5.2×10^8 a	1.390 a	8.2×10^8 a	0.551 b
ND9	1.8×10^7 bc	0.448 b	4.5×10^8 a	0.899 a	2.5×10^8 a	1.444 a	2.2×10^8 b	0.902 a
INM-1	1.4×10^8 ab	0.127 b	4.4×10^8 a	0.132 b	6.4×10^8 a	0.316 b	1.1×10^9 a	0.166 c
AK14	1.2×10^5 c	0.009 b	8.1×10^7 a	0.032 b	1.7×10^8 a	0.092 b	4.0×10^6 b	0.137 c
Control[z]	...	0.048 b	...	0.029 b	...	0.064 b	...	0.106 c

[w] CsOFF = OFF; CsND009 = ND9; CsIdNM-1 = INM-1; and CsAk014 = AK14.

[x] Populations determined as IFU/g fresh weight of stem tissue. Population means within columns followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

[y] ELISA absorbance values (405 nm) means followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

[z] Uninoculated plants were used as control. ELISA absorbance values for uninoculated plants were included in statistical analysis.

DAP, absorbance values for this strain were significantly higher than the other strains (Table 2). ELISA absorbance values for stems infected with intermediate strain Wi2 were significantly higher than the mucoid strain OFF and all nonmucoid strains at all sampling dates in 1990 (Table 3).

ELISA absorbance values for stems infected with mucoid strain OFF generally increased as bacterial populations increased, although this relationship was not always significant (Figs. 2A and 3A). A similar relationship existed for intermediate strain ND9 in Russet Burbank in 1989 (Fig. 2B) and nonmucoid strain INM-1 in Norchip in 1990 (Fig. 3C). However, ELISA absorbance values for nonmucoid strain AK14 did not increase as populations increased in infected potato stems in either year (Figs. 2D and 3D). The same lack of a relationship was seen with nonmucoid INM-1 in 1989 (Fig. 2C), nonmucoid SD1 (Fig. 3E), and intermediate Wi2 (Fig. 3B) in 1990. Slopes (b_o) and intercepts (a) of regression lines for infected stems of each cultivar were significantly different among strains of *C. m. sepedonicus* in 1989 and 1990 ($P = 0.001$).

Sensitivity, specificity, and efficiency were calculated for ELISA for mucoid and nonmucoid strains (Table 4). Populations of 10^5–10^9 IFU/g of each strain, as determined by IFAS, were used to compare ELISA test performance. In 1989, one monoclonal and five polyclonal antibodies were used in all possible combina-

TABLE 3. Mean populations and ELISA absorbance values of *Clavibacter michiganensis* subsp. *sepedonicus* strains in 1990 in potato stems

	Days after planting							
	56		70		84		98	
Strain[w]	IFU/g[x]	A_{405nm}[y]	IFU/g	A_{405nm}	IFU/g	A_{405nm}	IFU/g	A_{405nm}
OFF	2.7×10^6 b	0.246 b	8.9×10^6 a	1.050 b	1.3×10^7 b	0.651 b	2.9×10^8 a	0.500 b
Wi2	1.9×10^7 a	0.540 a	1.2×10^7 a	1.320 a	6.5×10^7 a	1.100 a	4.3×10^8 a	1.140 a
INM-1	3.4×10^6 b	0.015 c	8.0×10^6 a	0.081 c	2.5×10^7 b	0.068 c	5.4×10^7 b	0.069 c
AK14	4.7×10^6 b	0.020 c	2.6×10^5 b	0.028 d	5.7×10^5 b	0.062 c	5.8×10^6 b	0.058 c
SD1	3.6×10^6 b	0.013 c	8.3×10^3 b	0.040 d	1.3×10^6 b	0.048 c	3.0×10^5 b	0.058 c
Control[z]	...	0.012 c	...	0.029 d	...	0.039 c	...	0.084 c

[w] CsOFF = OFF; CsWi002 = Wi2; CsIdNM-1 = INM-1; CsAk014 = AK14; and CsSDHanke = SD1.

[x] Populations determined by IFU/gm fresh weight of stem tissue. Population means within columns followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

[y] ELISA absorbance values (405 nm) means followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

[z] Uninoculated plants were used as control. ELISA absorbance values for uninoculated plants were included in statistical analysis.

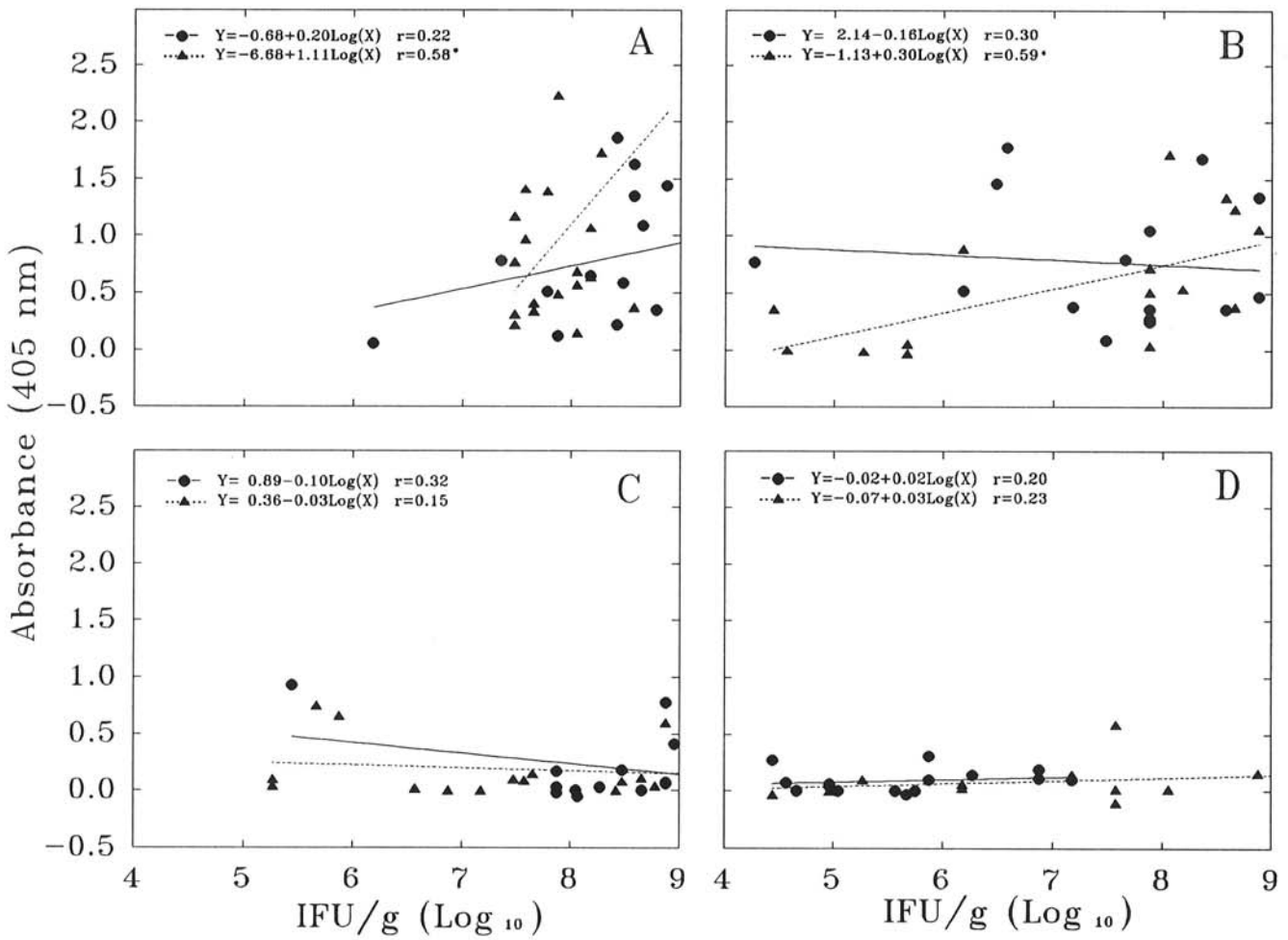

Fig. 2. Relationship of ELISA absorbance values to IFU/g of *Clavibacter michiganensis* subsp. *sepedonicus* in infected stem samples of Norchip (-●-) and Russet Burbank (-▲-) potato plants in 1989. Each data point is the mean of four ELISA samples. **A,** Strain OFF; **B,** strain ND9; **C,** strain INM-1; and **D,** strain AK14. * represents significance at $P = 0.05$.

tions for testing ELISA as a system. The best antibody combination was chosen according to the sensitivity-specificity pair with the optimum specificity (>93%). An ELISA system consisting of chicken polyclonal anti-*C. m. sepedonicus* coated (capture) antibody and mouse 1H3 monoclonal anti-*C. m. sepedonicus* antibody as the sandwich performed with the highest degree of sensitivity and specificity. Specificity calculated for the other antibody combinations was less than 90%. In 1989, ELISA exhibited a sensitivity of 95% in detecting mucoid strain OFF infected stems. The ELISA test exhibited an acceptable level of 89% against the intermediate strain ND9. Sensitivity for nonmucoid strains INM-1 and AK14 (36% and 24%, respectively) was not sufficiently high for this serological detection system.

In 1990, the ELISA was performed in the same manner using the chicken polyclonal/mouse monoclonal antibodies. Acceptable levels of sensitivity (81% and 89%) and specificity (97%) were achieved in detecting mucoid OFF and intermediate Wi2 strains of *C. m. sepedonicus*, respectively, in infected stems. In comparison, the nonmucoid strains were detected with much less sensitivity. Calculations revealed 0–3% sensitivity with 97% specificity in detecting the nonmucoid strains INM-1, AK14, and SD1. The test system was more efficient in both 1989 and 1990 in detecting the mucoid and intermediate strains than the nonmucoid strains of *C. m. sepedonicus* in stem tissue.

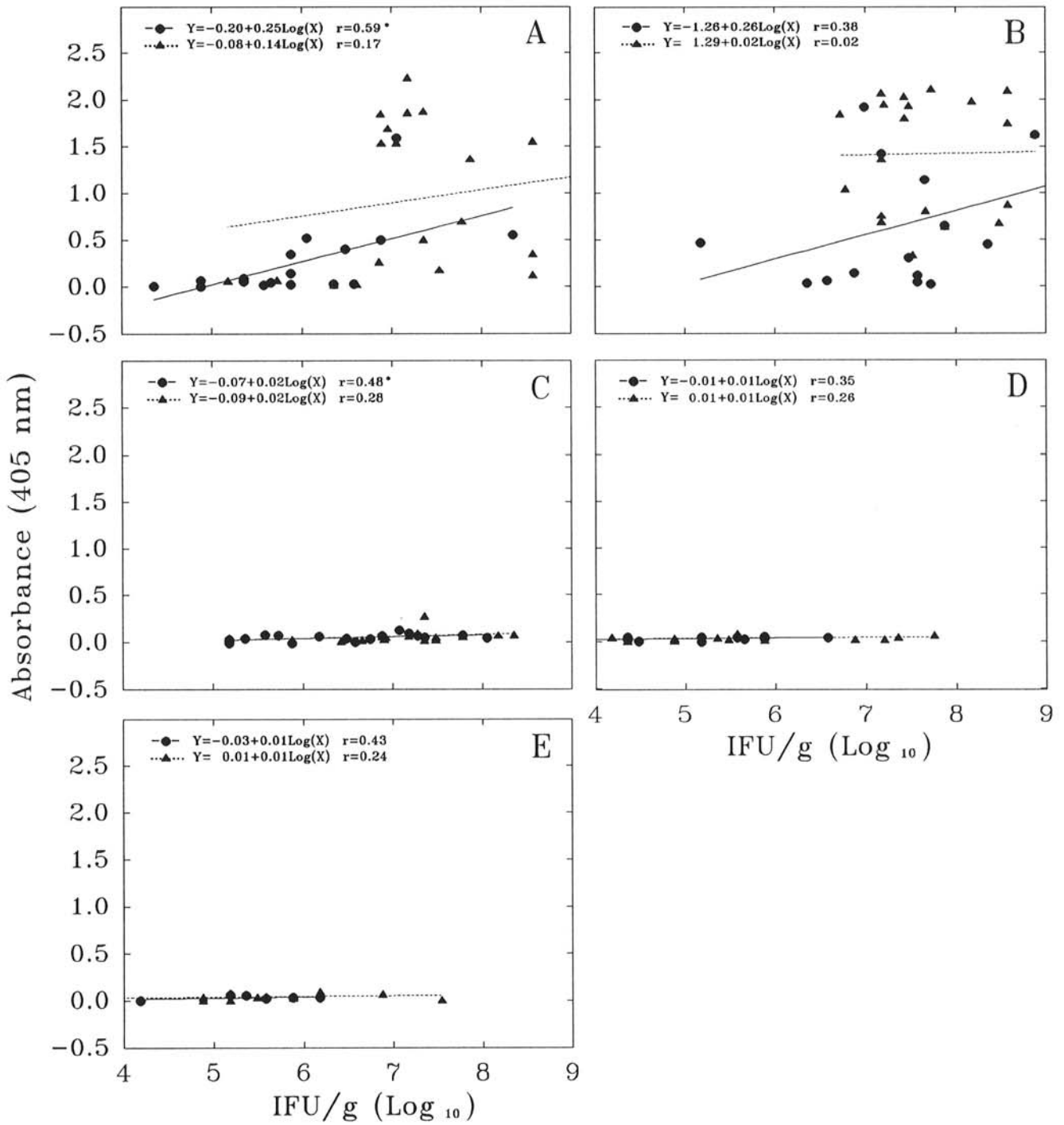

Fig. 3. Relationship of ELISA absorbance values to IFU/ml of *Clavibacter michiganensis* subsp. *sepedonicus* in infected stem samples of Norchip (-●-) and Russet Burbank (-▲-) potato plants in 1990. Each data point is the mean of four ELISA samples. **A,** Strain OFF; **B,** strain Wi2; **C,** strain INM-1; **D,** strain AK14; and **E,** strain SD1. * represents significance at $P = 0.05$.

DISCUSSION

ELISA performed on suspensions of nonmucoid strains of *C. m. sepedonicus* grown in vitro demonstrated that these strains were difficult to detect using this serological technique. Based on these data, we decided to determine if nonmucoid strains were detectable serologically in planta at some point during the growing season. The possibility existed that nonmucoid strains could revert back to a natural mucoid state in planta. If this were true, we hypothesized that ELISA could detect mucoid and nonmucoid strains equally well in infected potato stems. However, results reported here indicate that nonmucoid strains do not produce sufficient exopolysaccharide (EPS), or produce a different EPS, and are undetectable using antibodies made against EPS of mucoid strains. Intermediate strains such as ND9 and Wi2, however, are easily detected using ELISA. All available antibodies for use in ELISA, either from commercial or private sources, have been produced against mucoid strains of *C. m. sepedonicus*.

Stem populations of the *C. m. sepedonicus* strains used in this study were similar to those reported in other systems (5,10,11,16). Although populations of nonmucoid *C. m. sepedonicus* strains were frequently lower than mucoid or intermediate strains at a specific sampling date, high populations developed. ELISA is ineffective in detecting nonmucoid strains INM-1, AK14, and SD1 at any population density. In contrast, mucoid and intermediate strains could be detected effectively at populations as low as 10^5 IFU/g, which is in agreement with a previous study (16). Not only were ELISA absorbance values for the nonmucoid strains in planta much lower than absorbance values for mucoid and intermediate strains OFF, ND9, and Wi2, but absorbance values for nonmucoid strains did not increase with higher bacterial populations (Figs. 2 and 3). Most ELISA absorbance values for INM-1, AK14, and SD1 in 1989 and 1990 were in the negative range, despite high bacterial populations. ELISA values for these strains were negative at the end of our sampling period, indicating that antigen was not building up as the season progressed.

The ability of a test to identify a diseased individual is defined as sensitivity, and the ability to recognize noninfected individuals is specificity. These abilities are expressed as percentages. Sensitivity and specificity occur as a continuum of paired sets to characterize the performance of a test. A decision level must be chosen, often arbitrarily, which affects the rates of affected individuals with a "positive" result and also the noninfected individuals who, nonetheless, have a "positive" result (i.e., false positives). An IFAS test result of 1 IFU/hpf was used as the decision level for affected plant samples. IFAS and ELISA have been found to have the same level of sensitivity and specificity in detecting certain population sizes of *C. m. sepedonicus* (16). This information was used in comparing the ability of ELISA to detect mucoid, intermediate, and nonmucoid strains. Specificity of ELISA was similar for each strain of *C. m. sepedonicus* studied, meaning the ability of this test to detect noninfected plants is not affected by the bacterial strain. However, sensitivity of ELISA was 40% or less in detecting nonmucoid strains of *C. m. sepedonicus* in 1989 and 1990 compared to 81–95% for a mucoid or intermediate strain (Table 4). We consider a sensitivity of <40% as unacceptable for a serological technique to be useful in detecting plants infected with nonmucoid strains.

Efficiency is defined as the fraction of results that are correct, that is, true positives and true negatives divided by all results (24). Although efficiency by itself is inadequate for judging test performance, it helps us to understand the likelihood that a result is a true result. Efficiency should be regarded only as a rough estimate because borderline positives are treated the same as high positives. ELISA had an efficiency rate between 88–95% in detecting mucoid and intermediate strains in 1989 and 1990. ELISA performed with an efficiency of 47–82% in detecting stems infected with similar population sizes of nonmucoid strains in 1989 and 1990 (Table 4).

The monoclonal antibody used in our ELISA test to detect *C. m. sepedonicus* was produced against the EPS of this bacterium (14). Our study suggests that the inability of ELISA to detect nonmucoid strains is a function of a lack of antigen, not lack of a detectable population size. Nonmucoid strains appear to produce low levels of this antigen, or an antigen not specific for the ELISA antibodies. De Boer (7) has suggested that the difference in antigens between mucoid and nonmucoid strains was quantitative rather than qualitative. However, studies performed in our laboratory, using many of the strains used in the experiments reported here, indicate that the EPS of mucoid and nonmucoid strains differs qualitatively (P. J. Henningson and N. C. Gudmestad, *unpublished*). The EPS of mucoid strains has a higher molecular weight and different carbohydrate makeup than the EPS of nonmucoid strains. It has been hypothesized that antibody binding to polysaccharide antigens is based on recognition of an epitope consisting of six or seven sugar residues (19). Recent studies have demonstrated that a conformational epitope is responsible for antibody recognition of the exopolysaccharide layer (23). This epitope may be fully expressed only in high molecular weight forms of the saccharide. Further studies on exopolysaccharides of *C. m. sepedonicus* may shed light on the inability of the currently available ELISA antibodies to detect nonmucoid strains.

If an ELISA system is to be effective in detecting all *C. m. sepedonicus* strains, a diffusible antigen common to both mucoid and nonmucoid strains must be found. This problem has been cited previously by Bishop et al (1) when frustrated in the initial diagnosis of a nonmucoid strain. Their findings "underscores the hazard of reliance on a single antigen for diagnosis of *C. m. sepedonicus*" (1). De Boer (7) compared mucoid and nonmucoid strains in double diffusion tests and demonstrated that half of the plant samples with nonmucoid strains did not produce visible precipitin bands. Results reported here and elsewhere demonstrate the need to develop anti-*C. m. sepedonicus* antibodies against a diffusible antigen, as required by an ELISA system, which is common to mucoid and nonmucoid strains. The cell wall antigen specific monoclonal antibody 9A1 (13) used in IFAS is thus not useable in ELISA. IFAS may be the only existing serological technique capable of detecting mucoid and nonmucoid strains with equivalent sensitivity.

TABLE 4. Sensitivity, specificity, and efficiency calculated for ELISA[x] against selected populations of *Clavibacter michiganensis* subsp. *sepedonicus* in 1989 and 1990

	1989			1990		
Strain[y]	Sensitivity (%)	Specificity (%)	Efficiency (%)	Sensitivity (%)	Specificity (%)	Efficiency (%)
OFF	95	97	95	81	97	88
ND9	89	97	93	ND	ND	ND
Wi2	ND[z]	ND	ND	89	97	92
INM-1	36	97	67	3	97	47
AK14	24	96	82	0	97	61
SD-1	ND	ND	ND	0	97	61

[x] Chicken anti-*C. m. sepedonicus* polyclonal antibody coated ELISA plates, mouse 1H3 anti-*C. m. sepedonicus* monoclonal antibody used as the sandwich antibody.

[y] CsOFF = OFF; CsND009 = ND9; CsWi002 = Wi2; CsIdNM-1 = INM-1; CsAk014 = AK14; and CsSDHanke = SD1.

[z] ND = Not done. All strains were not included in each year of the study.

Occurrence of nonmucoid or nonfluidal strains of *C. m. sepedonicus* in nature is a phenomenon that has only recently been reported (1,7). We do not know whether the occurrence of nonmucoid strains of *C. m. sepedonicus* is a recent event or if these strains simply were not previously recognized. The possibility exists that the nonmucoid strains are phenotypic artifacts of culturing bacteria on artificial media. The reduced exopolysaccharide production by bacteria grown in an artificial medium for long periods of time is well documented (4,15).

At present, it is difficult to ascertain the magnitude of the ring rot disease problem caused by nonmucoid strains. However, the incidence of these strains occurring naturally may be quite high. In De Boer's (6) studies of 19 strains from several different locations, 10 were nonmucoid. In our laboratory we examined the morphology of 31 strains in our culture collection that were sent to us over a 2-yr period, and 10 were mucoid, 7 were intermediate, and 14 were nonmucoid when grown on NBY agar (N. C. Gudmestad and D. Baer, *unpublished*).

Interesting questions remain regarding the occurrence of nonmucoid strains of *C. m. sepedonicus* in natural infections and their importance to ring rot disease of potato. Recovery of some of the strains used in these studies was from plants that were sent to our laboratory for confirmatory diagnostic testing because of atypical symptomatology. Control of ring rot is directed primarily at the use of disease-free, certified seed potatoes. Certified seed potato fields are visually inspected for ring rot symptoms by qualified seed inspectors. However, these inspectors are trained to look for plants exhibiting wilting, interveinal chlorosis, marginal leaf necrosis, and bacterial exudate. We cannot help but speculate that the possibility exists that nonmucoid strains are becoming more prevalent because they cause less severe or atypical disease symptoms. Nonetheless, more information is needed to determine the prevalence of these strains and their importance to bacterial ring rot of potato.

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