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Comparison of a DNA Hybridization Probe and ELISA for the Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in Field-Grown Potatoes

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ABSTRACT

Drennan, J. L., Westra, A. A. G., Slack, S. A., Delserone, L. M., Collmer, A., Gudmestad, N. C., and Oleson, A. E. 1993. Comparison of a DNA hybridization probe and ELISA for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Plant Dis. 77:1243-1247.

Clavibacter michiganensis subsp. *sepedonicus*, the causal agent of bacterial ring rot, was detected in field-grown potatoes using a 1.078-kb repeated *C. m. sepedonicus* sequence as a probe in DNA hybridizations. Stem and petiole samples from susceptible and tolerant cultivars (Russet Burbank and Belrus, respectively), inoculated with 10 mM phosphate buffer (pH 7.2) or 10^2 or 10^9 cfu of either an aggressive or a less aggressive *C. m. sepedonicus* strain, were processed by directly blotting cut tissue sections on nylon membranes, macerating frozen tissues, and applying xylem fluid collected by centrifugation to nylon membranes (stems only). The efficiency of detection was significantly influenced by sampling date, plant part, inoculum dose, and cultivar. The probe was compared with an enzyme-linked immunosorbent assay (ELISA) and showed 95–100% agreement when underground Russet Burbank stems inoculated with 10^9 cfu of aggressive *C. m. sepedonicus* strain SS43 were directly blotted. Although overall detection rates with stem sections were higher for ELISA (18.4% with ELISA vs. 11.3% with direct blotting), a high rate of false positives (53.9%) occurred with petiole tissues at 90 days after planting when ELISA was used, whereas none occurred with DNA hybridizations.

Clavibacter michiganensis subsp. *sepedonicus* (Spieck and Kotth.) Skapt. and Burkh., the causal agent of bacterial ring rot of potato, has been the most serious pathogen to the North American seed potato industry for over 50 yr. Since 1940, zero tolerance limits have required that one infected plant or tuber causes the rejection of the entire lot for seed certification (6). Eradication has not been achieved, in part because the pathogen can persist at low levels without producing foliar or tuber symptoms (5). Furthermore, tolerant cultivars, such as Belrus, can maintain high populations of *C. m. sepedonicus* without symptom expression (8,10).

Current methods of detection include the Gram stain, the eggplant bioassay, and serological assays, such as the enzyme-linked immunosorbent assay (ELISA), latex agglutination, and immunofluorescent antibody technique (6). However, all these methods have drawbacks (5,6,16). The Gram stain is labor-intensive and requires the pathogen to be distinguished from secondary invaders. The eggplant bioassay is effective at detecting low levels of *C. m. sepedonicus* but is inappropriate for large-scale testing because of labor and space constraints. Although serological assays can easily be adapted for large-scale use, antisera occasionally cross-react with other bacteria (4,12).

DNA hybridization with an appropriate probe has the potential to be specific and sensitive and to permit large-scale testing. Mogen et al (14) characterized a 1.078-kb repeated sequence (RS) that shows promise for specific detection of *C. m. sepedonicus*. The multicopy RS is present in both the bacterial chromosome and pCS1, a 50.6-kb plasmid found in almost all *C. m. sepedonicus* strains (13). Specificity was confirmed with pure *C. m. sepedonicus* cultures, but evaluations were not performed on infected plant materials. Here we report the speci-

Accepted for publication 16 July 1993.

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Table 1. Strains, sources, and reactions of organisms tested with the RS probe

Organism Strain	Reaction	Origin[a]	Source
Clavibacter michiganensis subsp. *sepedonicus*			
SS13	+	California	D. Gross
SS14	+	Montana	D. Gross
SS20	+	Wisconsin	A. Kelman
SS28	+	New Brunswick	R. McKenzie
SS29	+	New Brunswick	R. McKenzie
SS32	+	Maine	D. Hammond
SS34	+	Alberta	R. Copeman
SS35	+	Alberta	R. Copeman
SS38	+	British Columbia	R. Copeman
SS42	+	Michigan	S. Slack
SS43	+	Maine	F. Manzer
SS44	+	Wisconsin	S. Slack
SS45	+	Wisconsin	S. Slack
SS46	+	Wisconsin	S. Slack
SS47	+	Wisconsin	S. Slack
SS48	+	Wisconsin	S. Slack
SS49	+	Wisconsin	S. Slack
SSNF50	+	Idaho	S. Slack

(*continued on next page*)

[a]Origins of *C. m. sependonicus* strains are listed to show geographic diversity.
[b]Strains isolated from sugar beet; all other strains were isolated from potato.
[c]Bacteria formerly classified in the genus *Corynebacterium*; these are not pathogenic on potato.

Table 1. (*continued from preceding page*)

Organism Strain	Reaction	Origin[a]	Source
SS69	+	North Dakota	N. Gudmestad
SS70[b]	+	North Dakota	N. Gudmestad
SS71	+	Maine	S. Slack
SS73	+	North Dakota	N. Gudmestad
SS75	+	North Dakota	N. Gudmestad
SS76[b]	+	North Dakota	N. Gudmestad
SSNF87	+	Idaho	S. Slack
SS93	+	Alaska	S. Slack
SS94	+	Alaska	S. Slack
SS95	+	Alaska	S. Slack
9850	+	...	ATCC
Botrytis cinerea			
85.067	−	...	P. Mullin
C. m. insidiosus[c]			
239	+	...	A. Vidaver
1109	+	...	A. Vidaver
226	−	...	D. Gross
10253	+	...	A. Oleson
33114	+	...	A. Oleson
M1B	−	...	D. Gross
P-2	−	...	A. Vidaver
Utah 1	+	...	N. Van Alfen
Utah 2	+	...	N. Van Alfen
Utah 3	+	...	N. Van Alfen
Utah 4	+	...	N. Van Alfen
C. m. michiganensis[c]			
3D21	−	...	D. Gross
8295	−	...	D. Gross
C. m. nebraskensis[c]			
SS9	−	...	D. Gross
Curtobacterium flaccumfaciens subsp. *flaccumfaciens*[c]			
SS1	−	...	D. Gross
SS2	−	...	D. Gross
C. f. poinsettiae[c]			
1	−	...	D. Gross
13	−	...	D. Gross
Erwinia carotovora subsp. *atroseptica*			
SR8	−	...	A. Kelman
E. c. carotovora			
F52	−	...	A. Collmer
Fusarium sambucinum			
84.048	−	...	P. Mullin
F. solani subsp. *phaseolicola*			
F28	−	...	G. Abawi
Helminthosporium solani			
SS3-5	−	...	R. Loria
Phytophthora infestans			
510	−	...	W. Fry
P. megasperma			
89.012	−	...	G. Bergstrom
Pseudomonas fluorescens			
84.004	−	...	P. Mullin
P. solanacearum			
90.086	−	...	H. D. Thurston
Rhizoctonia solani			
309	−	...	J. Aist
Rhodococcus fascians			
6887	−	...	D. Gross
SR319	−	...	A. Kelman
SR394	−	...	A. Kelman
Streptomyces scabies			
84-34	−	...	R. Loria
87-22	−	...	R. Loria
Verticillium albo-atrium			
002NY83	−	...	D. Kalb
V. dahliae			
86.056	−	...	P. Mullin
Unidentified bacterial species cross-reactive with ELISA			
A	−	...	N. Gudmestad
B	−	...	N. Gudmestad
F	−	...	N. Gudmestad
G	−	...	N. Gudmestad
L	−	...	N. Gudmestad
M	−	...	N. Gudmestad

ficity and sensitivity of the RS probe as well as the results of field-grown potato tissues prepared three ways and compared with a commercially available ELISA system. A preliminary report of this work has been published (7).

MATERIALS AND METHODS

Bacterial strains and culture. Strain sources are listed in Table 1. For general use, bacterial cultures were grown on nutrient broth-yeast extract (NBY) (17) medium for 3–5 days at ambient temperature (approximately 22 C) or maintained for extended periods on yeast extract-dextrose-$CaCO_3$ medium (1% yeast extract, 2% dextrose, 2% $CaCO_3$, 1.5% agar) at 4 C. Cultures were lyophilized in a 7% peptone and 7% sucrose solution for long-term storage.

Inoculum was prepared by growing cultures of SS13 and SS43 in NBY broth for 4–5 days, then centrifuging the cultures for 30 min at 10,000 *g*. Cell concentrations were adjusted in 10 mM phosphate buffer (pH 7.2) with a spectrophotometer at $A_{600nm} = 0.1$, equivalent to 10^8 cfu/ml (1).

Experimental design. Potato tuber seed pieces of susceptible cv. Russet Burbank and tolerant cv. Belrus were pierced twice with a pipette tip on opposite sides of the single eye, creating a small well underneath. Then, 10^2 or 10^9 cfu of *C. m. sepedonicus* aggressive strain SS43 or the less aggressive strain SS13 was inoculated in a 10-μl volume under the eye of each seed piece. Controls received 10 μl of 10 mM phosphate buffer (pH 7.2). Cultivar × strain × dose replicates consisted of 10 blocks of 15 plants that were repeated five times in a randomized complete block design and planted near Fargo, North Dakota.

Sample processing. Four plants from each block were destructively sampled at 30, 60, and 90 days after planting (DAP), and two stem and two petiole sections from each plant were selected, i.e., stem sections connected to the seed piece from below the soil line (BS) and at the crown region (S) and petioles from the lowest fully expanded leaf (BP) and midway up the plant (MP). Plant parts were processed by direct blotting, grinding, and centrifuging.

Directly blotted tissues consisted of 0.5 g of a cut stem or petiole section pressed against a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA) (Fig. 1). The samples were stored in a plastic bag and frozen at −80 C to be used as ground samples. Later, ground samples were prepared by crushing each stem or petiole section with a rubber mallet and adding 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) amended with 0.13% Na_2SO_3. A dot blot manifold (Bio-Rad) was used to apply 20 μl of this solution on a membrane.

A 100-μl amount of the ground sample was added to 400 μl of sample extract

buffer (0.4% BSA, 2% polyvinylpyrrolidone M_r 24,000–40,000, 0.13% Na_2SO_3, 0.02% sodium azide, pH 7.4, in PBST buffer [0.80% NaCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl, 0.05% Tween 20, pH 7.4]) and tested with double-sandwich ELISA (Agdia, Elkhart, IN) using the alkaline phosphatase enzyme system. Plates were read visually and with an ELISA reader (Bio-Tek Instruments, Winooski, VT) at 405 nm, with positive controls reaching an OD value of 2.0 before final readings were taken.

Centrifuged samples consisted of BS and S stem sections cut and fitted into 1.5-ml microfuge tubes. Petiole samples, which could not consistently produce 10 μl of sap during centrifugation, were not included. Tubes were centrifuged at 8,848 *g* for 25 min at ambient temperature. Then, 10 μl of the collected liquid was applied to a nylon membrane using a dot blot manifold.

Cells on membranes were lysed by placing membranes on blotting paper saturated with 0.4 N NaOH for 10 min, followed by two 5-min washes with 300 mM NaCl, 30 mM sodium citrate, pH 7.0. Membranes were air-dried for at least 90 min and then baked at 80 C for 30 min.

RS generation. A virtual full-length clone of the *C. m. sepedonicus* RS was prepared in the recombinant plasmid, pSR7F, which was transformed into *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, MD) and purified according to a modified procedure by Marko et al (11). The RS was isolated from pSR7F by restriction digestion with *Xba*I and *Eco*RI (Boehringer Mannheim, Indianapolis, IN) to yield the fragment: *Xba*I-(T7 promoter)-[RS]-*Eco*RI. Following horizontal electrophoresis in 0.6% SeaKem agarose (FMC, Rockland, ME) at 70 V for 90 min, the fragment was stained with 0.5 μg/ml of ethidium bromide for 20 min and separated from the agarose with the Bio-Rad Prep-a-Gene kit. The isolated RS was labeled with ^{32}P using a random primer kit (Boehringer Mannheim).

Hybridizations. Hybridization procedures were similar to those reported by Mogen and Oleson (13). Membranes were prehybridized for at least 90 min at 48 C. The RS was added to the prehybridization buffer (50% deionized formamide, 4× SSCP, 0.5% BLOTTO, 0.5 mg/ml of denatured salmon sperm DNA, 10× Denhardt's solution, and 1% SDS) and allowed to hybridize overnight at 48 C (13). Posthybridization washes (13) were done at 48 C, and membranes were exposed to Kodak X-Omat AR-5 film at −80 C for 2–4 days. Radioactivity was removed from the membranes by two 20-min washes in 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, and 0.5% SDS at 95 C.

RESULTS

Specificity and sensitivity. The RS probe hybridized with all 29 *C. m.*

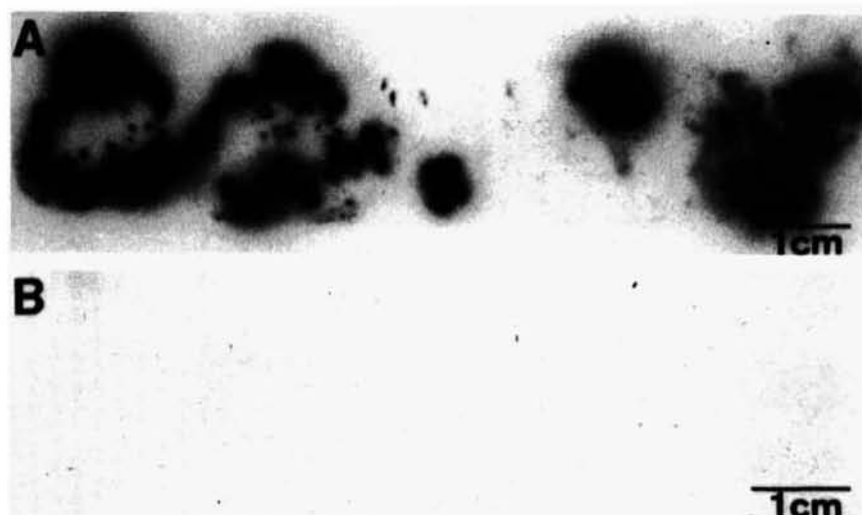

Fig. 1. Directly blotted samples processed 90 days after planting: **(A)** Five Russet Burbank stem samples inoculated with 10^9 cfu of *Clavibacter michiganensis* subsp. *sepedonicus* SS43 and **(B)** five buffer-inoculated Russet Burbank stems and petioles. *C. m. sepedonicus* may be present throughout the vascular ring, as seen in the two samples at left in A, or may be localized in selected areas in the xylem, as shown in the three samples at right.

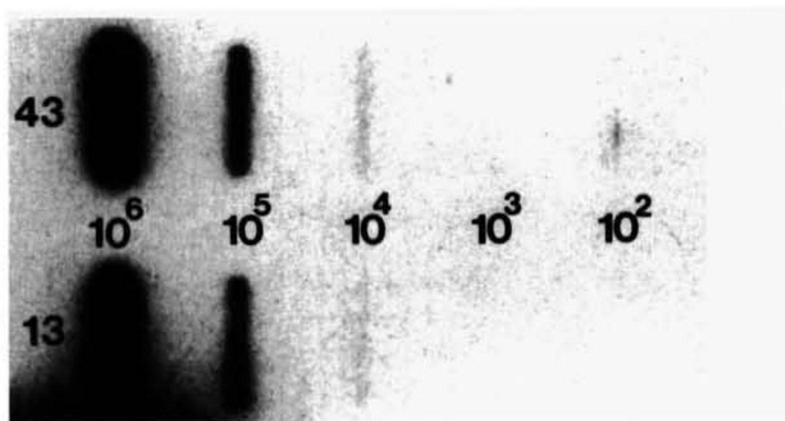

Fig. 2. Dilution series of *Clavibacter michiganensis* subsp. *sepedonicus* SS43 and SS13 at 10^6 to 10^2 cfu.

Table 2. Total number of plant samples inoculated with *Clavibacter michiganensis* subsp. *sepedonicus* that reacted positively at 30, 60, and 90 days after planting (DAP)

DAP	ELISA[a]				Directly blotted[b]				Ground[c]				Centrifuged[d]	
	BS[e]	S	BP	MP	BS	S	BP	MP	BS	S	BP	MP	BS	S
30	9	6	10	3	7[f]	1[f]	0[f]	0[f]	1[f]	0[f]	0[f]	0[f]	6[f]	0[f]
60	27	27	51	55	20[f]	18[f]	0	0	17[f]	15[f]	2	2	16[f]	15[f]
90	54	51	139	139	33[f]	28[f]	22	20	28	23	0	1	16	19

[a] Macerated tissue suspensions tested with a commercially available ELISA system (Agdia, Elkhart, IN).
[b] Freshly cut sample ends pressed against a nylon membrane and hybridized with the RS probe.
[c] Macerated tissue suspension put on a nylon membrane and hybridized with the RS probe.
[d] Sap from centrifuged stem sections put on a nylon membrane and hybridized with the RS probe.
[e] BS, S, BP, and MP refer to stem sections below soil line, stem sections at the crown region, lowest fully expanded petioles, and petioles from the middle of the plant, respectively. There was an average of 158 inoculated samples per plant part for each treatment.
[f] Treatments where the results from the RS probe and ELISA did not differ significantly.

sepedonicus strains tested (Table 1) and had a limit of detection determined by dilution series to be equivalent to 10^5 to 10^6 cfu (Fig. 2). No hybridizations occurred between the RS and endophytic or saprophytic bacteria isolated from healthy potato stems and tubers, including several unidentified bacterial strains known to cross-react with antisera prepared against *C. m. sepedonicus* (4), bacterial and fungal pathogens of potato, or most species closely related to *C. m. sepedonicus*. The exception was *C. m.* subsp. *insidiosus*. Cross-reactions with *C. m. insidiosus* were at first eliminated by raising the temperature from 42 to 48 C. However, later tests with an expanded number of *C. m. insidiosus* strains resulted in eight of 11 strains (72.7%) hybridizing with the RS at 48 C.

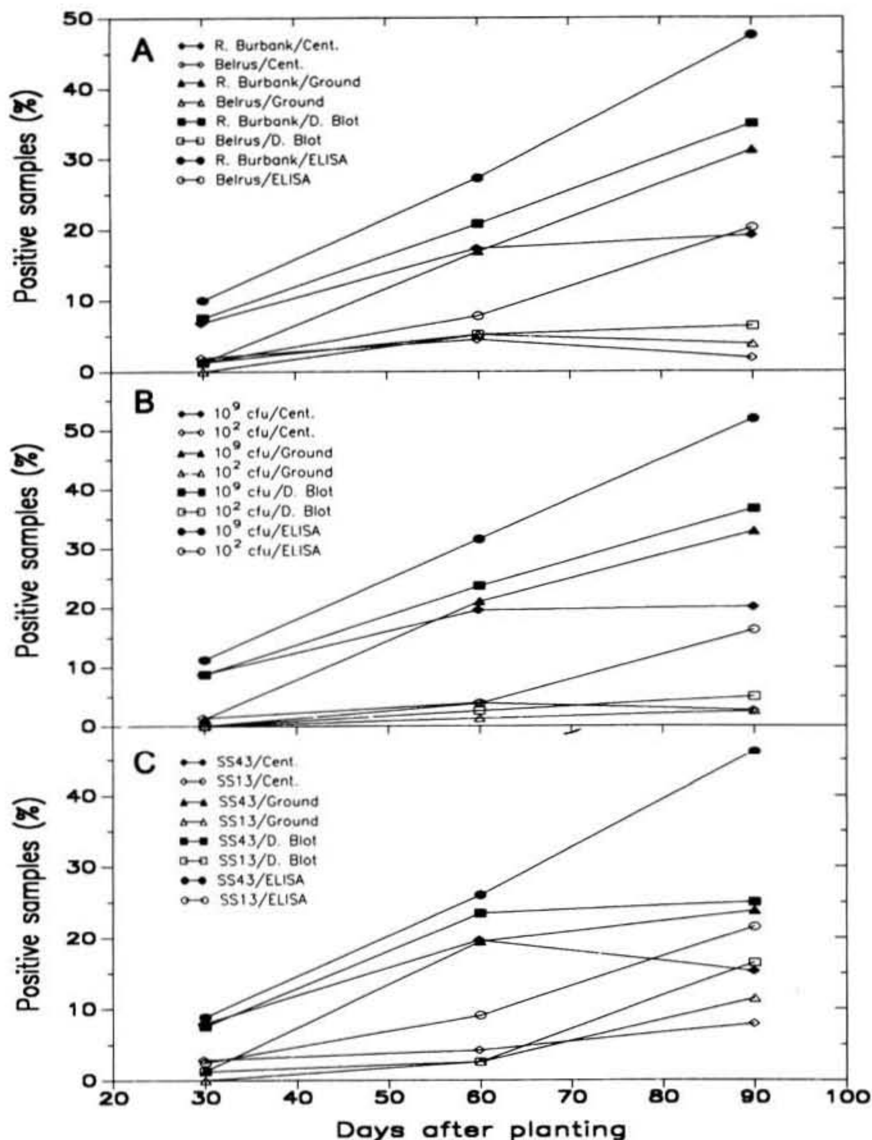

Fig. 3. Percentage of total inoculated stem sections from below the soil line giving a positive reaction according to **(A)** cultivar, **(B)** inoculum dosage, and **(C)** *Clavibacter michiganansis* subsp. *sepedonicus* strains. Samples were processed by centrifugation (Cent.), grinding (Ground), and direct blotting (D. Blot). An aggressive strain (SS43) and a less aggressive strain (SS13) of *C. m. sepedonicus* were used.

The RS was specific for *C. m. sepedonicus* under field conditions. No false positives occurred with any of the sample processing methods over the entire season, whereas ELISA had false-positive rates as high as 64.1 and 43.6% with BP and MP samples, respectively, at 90 DAP.

Detection of *C. m. sepedonicus* in field samples. Hybridization and ELISA results were compared on the basis of cultivar, dose, and strain using chi-square analyses. The efficiency of detection using the RS in hybridizations generally increased for all four plant parts as the growing season progressed, with stem sections having the highest percentage of positives (Table 2). The efficiency of detection was higher for Russet Burbank than for Belrus (Fig. 3A) when similar treatments were compared. The differences were not significant except at 90 DAP for ELISA BS sections ($P = 0.027$), directly blotted BS and S sections ($P = 0.021$ and 0.016, respectively), and ground BS and S sections ($P = 0.027$ and 0.035). Plants inoculated with 10^9 cfu of *C. m. sepedonicus* generally tested positive at a higher percentage than those inoculated with 10^2 cfu (Fig. 3B). Significant differences between inoculum doses, however, occurred only with 60 DAP BS ($P = 0.016$) and 90 DAP BS and S sections ($P = 0.002$ and 0.010) with ELISA and 90 DAP BS and S sections with directly blotted ($P = 0.003$ and 0.002) and ground ($P = 0.007$ and 0.033) methods. Similarly, a greater proportion of plants inoculated with strain SS43 tested positive with ELISA and the RS than those inoculated with strain SS13 (Fig. 3C), yet there were no significant differences throughout the entire season ($P \geq 0.434$).

When similar treatments were compared at 30 DAP, the sample processing

Table 3. Comparison of the effect of cultivar, inoculum dose, bacterial strain, and sampling date on the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato stems via ELISA and a DNA probe

	Belrus								Russet Burbank							
	SS13[b]				SS43[b]				SS13				SS43			
	10^2		10^9		10^2		10^9		10^2		10^9		10^2		10^9	
DAP[a]	ELISA[c]	D. blot	ELISA	D. blot	ELISA	D. blot	ELISA	D. blot	ELISA	D. blot	ELISA	D. blot	ELISA	D. blot	ELISA	D. blot
30	0.0[d]	0.0	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	0.0	0.0	35.0	30.0
60	0.0	0.0	5.3	5.3	5.0	0.0	22.2	16.7	0.0	0.0	31.6	5.3	10.5	10.5	65.0	65.0
90	5.0	10.0	21.1	10.5	15.0	5.0	40.0	0.0	10.0	0.0	50.0	45.0	35.0	5.0	95.0	90.0

[a] Days after planting.
[b] *C. m. sepedonicus* strains.
[c] Potato stems from below the soil line were tested by pressing a freshly cut end against a nylon membrane and then hybridizing it with the RS probe (D. blot). Stem sections were then macerated and tested with a commercially available ELISA system (Agdia, Elkhart, IN).
[d] Percentage of samples giving a positive reaction based on 18–20 plants per treatment.

methods for all stem and petiole sections were not significantly different from ELISA ($P \geq 0.463$), whereas after 60 DAP, only BS and S sections were not significantly different ($P \geq 0.340$) (Table 2). After 90 DAP, only directly blotted BS and S sections were not significantly different from ELISA ($P = 0.096$ and 0.068, respectively), whereas ground BS sections differed significantly ($P = 0.032$) (Table 2). Direct blotting of Russet Burbank BS samples inoculated at 10^9 cfu of *C. m. sepedonicus* strain SS43 provided results nearly equal to those of ELISA (Table 3). The three sample processing methods did not differ from one another significantly throughout the season ($P \geq 0.191$).

Thirteen discrepancies occurred at 60 and 90 DAP, in which ELISA results were scored as negative and hybridization results were scored as positive. The greatest number of discrepancies occurred with directly blotted tissues (nine occurrences), followed by ground and centrifuged samples (three and one occurrences, respectively). There were no biases due to cultivar, strain, or dose, but 69% of the discrepancies occurred with BS segments and 31% with S segments, representing a total of 1.5% of BS and 0.7% of S samples throughout the season.

DISCUSSION

The RS shows promise as a hybridization-based detection method for *C. m. sepedonicus*. It proved to be specific with field samples, since none of the 475 buffer-inoculated plant samples reacted positively. This is in contrast to the ELISA system, which was specific when stem segments were used but was not reliable when senescent petiole tissue was used. The RS hybridized with 72.7% of the *C. m. insidiosus* strains tested, but this should not be a problem, since this bacterium is only known to be viable in leguminous plants (2,3,18).

Interestingly, in all of the directly blotted S stem samples with discrepancies between ELISA and hybridization results, the BS segments also were positive. It is possible that populations of *C. m. sepedonicus* that do not yet produce enough antigen to react positively with ELISA would have enough DNA to hybridize with the RS probe (15). This might be the case prior to symptom expression or with latent infections.

Direct blotting of samples proved to be the most effective way to prepare tissues for hybridization with the RS probe. It had the highest detection efficiency for *C. m. sepedonicus* of the three sample preparation methods, and there were no significant differences with ELISA throughout the season when BS or S sections were used. Furthermore, it was also the least time-consuming method to perform, requiring only the cutting of tissues and application of the cut end to a nylon membrane. The ground method was second best in terms of detection efficiency. It was not significantly different from ELISA except at 90 DAP, where it differed only marginally when BS segments were used. For the most accurate results with the RS in hybridization assays, only stems from the soil line or below, collected as late in the season as possible, should be tested by the direct-blotting procedure.

Under optimal conditions, such as a susceptible cultivar infected with an aggressive *C. m. sepedonicus* strain at a high inoculum dose, the efficiency of detecting directly blotted samples with the RS was not statistically different from ELISA. However, under less than optimal conditions, ELISA was generally able to detect *C. m. sepedonicus* at a more favorable rate than the RS in hybridization assays. Tests are now in progress to evaluate the detection efficiency under less than optimal conditions using the polymerase chain reaction, which may provide greater test sensitivity (9).

ACKNOWLEDGMENTS

This study was supported in part by Cornell Hatch Project NYC-153406 and by USDA-ARS Potato Grant 58-1275-1-142.

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