EVALUATION OF IMMUNOFLUORESCENCE WITH MONOCLONAL ANTIBODIES FOR DETECTING LATENT BACTERIAL RING ROT INFECTIONS

S.H. De Boer and M.E. McNaughton[1]

Abstract

Corynebacterium sepedonicum was detected in symptomless potato stems and tubers with immunofluorescence using monoclonal antibodies specific for the bacterial ring rot pathogen. The concentration of bacterial cells in potato tissue preparations ranged from >500 cells/microscope field to 1 cell per preparation. Symptomless tubers containing ring rot bacteria planted in field plots yielded plants with ring rot symptoms, plants with latent ring rot infections, or plants with no detectable levels of ring rot bacteria. Tubers with the greatest number of bacteria were most likely to develop plants expressing ring rot symptoms, but even some seed tubers with a low number of bacteria developed into plants with symptoms. Some seed tubers with high levels of ring rot bacteria produced plants with only low numbers of *C. sepedonicum*.

Resumen

Se detectó *Corynebacterium sepedonicum* en tallos y tubérculos de papa que no mostraban síntomas de la enfermedad, mediante inmunofluorescencia que utilizaba anticuerpos monoclonales específicos para el patógeno de la pudrición anular. La concentración de células bacterianas en las preparaciones de tejidos de papa varió de >500 células/campo del microscopio a 1 célula por preparación. Los tubérculos sin síntomas, portando la bacteria de la pudrición anular, y sembrados en parcelas en el campo, desarrollaron plantas con síntomas de pudrición anular, plantas con infecciones latentes, o plantas sin niveles detectables de la bacteria. Los tubérculos con el mayor número de bacterias fueron los más propensos a desarrollar plantas mostrando síntomas de pudrición anular, pero aún así, algunos tubérculos-semillas con bajo número de bacterias produjeron plantas con síntomas. Algunos tubérculos-semillas con altos niveles del patógeno produjeron plantas con solo un bajo contenido de *C. sepedonicum*.

Introduction

The use of seed potatoes free of bacterial ring rot [*Corynebacterium sepedonicum* (Spieck. & Kotth.) Skapt. & Burkh.] was initially recommended

in 1938 to control the disease (22). Subsequently a zero tolerance level for ring rot in all classes of seed potatoes was accepted by all North American potato certification agencies (25). The accuracy of its implementation, limited by the number of plants and tubers that could be inspected, was well understood at the time (16). Nevertheless, the practical difficulty of a zero tolerance regulation has been an area of concern over the years (3, 15, 23). Dissatisfaction with zero tolerance was expressed as far back as 1941 (9) and is still a matter of misunderstanding and difficulty in the seed potato industry.

Failure of the zero tolerance regulation to eradicate ring rot from North America can be attributed at least in part to the presence of symptomless, latent infections. The term "latent infection" was first applied by Dykstra (10) in a subtitle to describe an experiment in which potatoes, inoculated with ring rot, produced no visibly infected plants or tubers that year, but in the following year the same tuber lots produced as many as 57% infected plants. Subsequently Nelson (19) showed that disease can develop from a very small number of bacteria inoculated into seed pieces, and that symptoms may not show until three years after inoculation. Little is known, however, regarding the frequency with which latent infections occur, the range of bacterial populations that may be involved under natural field conditions, and the fate of such natural latent infections during several growing seasons. The confusion surrounding latent infections is evident from the many conflicting anecdotal stories about ring rot outbreaks. Instances of sudden appearance of ring rot in seed lots that were disease-free for many years are contrasted with known instances of backyard gardeners, selecting symptomless tubers from their own ring rot infected crop, and subsequently producing healthy appearing crops for at least several years after the ring rot outbreak.

The detection of latent infections is fundamental to eradication of the ring rot disease. Several procedures to detect symptomless infections have been published recently. A bioassay test utilizing eggplants as a means of detecting symptomless infections has been described (13, 21, 28). Furthermore, serological techniques providing sensitive tests for detecting bacteria directly in plant tissue extracts have been published (6, 24, 26, 27). The availability of monoclonal antibodies with a high degree of specificity for *C. sepedonicum* provides increased potential for sensitive and specific diagnostic tests (5).

The application of sensitive detection procedures whereby the presence of symptomless infections may be determined, adds a new dimension to the zero tolerance regulation. The possibility exists that potatoes can be certified on the basis of a laboratory test rather than observation of plant or tuber symptoms. However, before such tests are implemented in a certification program careful evaluation of their limitations is required to preclude erroneous conclusions based on insufficient test data.

In this study we evaluated the immunofluorescence technique, utilizing monoclonal antibodies, for detecting latent ring rot infections one and two potato generations after inoculation with the bacterial pathogen.

Materials and Methods

Field Plots—In 1983, 60 tubers of each of the cultivars Bintje, Red Pontiac, and Russet Burbank were inoculated with *C. sepedonicum* strain CS 3. Tubers were cut in half prior to inoculation by vacuum infiltration (17) using a 1/100 dilution of a stock bacterial suspension at $O.D._{660}=0.5$ (ca. 10^7 cfu/ml). Inoculated tubers were planted in field plots and, at harvest, progeny tubers without external bacterial ring rot symptoms were selected for further testing. Tubers that did not show internal ring rot symptoms following cutting were subjected to immunofluorescence (IF) testing as described below.

In 1984 tubers were selected from the 1983 crop on the basis of the number of fluorescing cells observed in microscope slide preparations of tuber heel ends. Five tubers of each cultivar with >50, 5-50, <1-4, and 0 cells/average microscope field (mf), if available, were planted one meter apart in rows two meters apart. Each plant grown from an individual seed tuber was assessed for foliage symptoms during the growing season and tuber symptoms at harvest. The presence of ring rot bacteria was determined in four stems and four symptomless progeny tubers of each plant by IF. The stems from each plant were combined into one sample but the four progeny tubers were sampled separately. For 1985 field plots, tubers were selected from among the 1984 progeny on the same basis as in 1984. Stems and tubers were monitored for symptoms and presence of ring rot bacteria in the same way.

IF Procedure—Foliage samples were taken from stems at soil level and sap squeezed from the cut ends of stem segments with pliers. If stems were woody and difficult to squeeze, stem segments about 1 cm in length were placed in small plastic bags with 1.0 ml of distilled water and macerated by tapping the bag with a blunt object while holding the bag tightly closed. Tubers were sampled at the point of stolon attachment by removing a cone of tissue (ca. 0.5 g) with a sterile scalpel. The tuber tissue was macerated in small plastic bags with 1.0 ml of distilled water in the same manner as the woody stem tissue samples. Dilutions of 1/10, 1/100, and 1/1000 were made of macerated stem and tuber samples in distilled water and 20 μl applied to wells of multi-well slides. Initially the undiluted samples as well as the three dilutions were put on slides, but since the undiluted samples contained a great deal of tissue debris which adhered very poorly to the slides, washing off during subsequent staining, only the three dilutions were used in later samples. Preparations applied to the slides were dried at 50 C on a slide warmer, fixed in acetone for 10 min and dried again at 50 C.

For staining of slide preparations, monoclonal antibody was diluted in phosphate buffered saline (pH 7.2) plus 5% fetal calf serum as blocking agent and applied at 20 μl/well. For 1983 samples, monoclonal antibody (McAb) 2 was used but in the following years the more specific McAb 5 was used (5). McAb was incubated on slide preparations for 1 hr at 37 C in a humidified chamber before being washed off with a stream of distilled water. Slides were dried at 50 C and then stained with fluorescein conjugated anti-mouse antibodies (conjugated antibody from several different suppliers was used but did not show any differences in reaction when diluted appropriately). The antibody conjugate was also diluted in PBS plus blocking agent and incubated on the slide preparations for 1 hr at 37 C. Finally slides were rinsed with distilled water, dried at 50 C and coverslips mounted with PBS-glycerol (1:10) containing p-phenylenediamine to prevent fading of the fluorescence (11).

Slide preparations were observed with epi-illumination using a 100X neofluar oil objective on a Zeiss photomicroscope equipped with appropriate filters for fluorescein fluorescence. Total magnification was 1500X. Phase contrast illumination at a low light level was used in combination with epi-illumination in order to focus accurately on preparations that appeared to contain no fluorescing material. Each slide preparation was scanned for the presence of fluorescing cells and recorded as having on the average >50, 5-50, <1-4, and 0 cells/mf. The <1-4 cells/mf category included those preparations in which many microscope fields contained no fluorescing cells but one or more cells were found after extensive scanning of the entire preparation.

Since the concentration of positive cells did not consistently decrease according to sample dilution, data from wells with the greatest number of cells were used for each sample in the final analysis.

Results

Due to limitations in number of IF tests that could be done, the number of plants of each cultivar that could be tested was necessarily small. Hence the data in the tables are those combined for all plants of all three cultivars used in the experiments.

In 1983 immunofluorescent positive cells were present in preparations from potato tubers without distinct ring rot symptoms but with varying levels of internal vascular discoloration (Table 1). Some of the tubers with distinct vascular yellowing had no positive cells in IF, whereas other tubers with no visible vascular abnormalities had >50 positive cells/mf.

In 1984 more plants and more tubers developed symptoms of bacterial ring rot than in 1985 (Table 2). The weather was distinctly different during the two growing seasons. The 1984 growing season was generally cool and wet with a total of 48 rainy days providing 37.1 cm of precipitation (Table 3).

TABLE 1. — *Concentration of fluorescing cells observed in tuber heel end preparations from tubers with different levels of vascular discoloration and stained by the indirect immunofluorescence procedure using a monoclonal antibody to* C. sepedonicum.

Degree of Vascular discoloration	Number of Tubers			
	Average no. of cells/microscope field			
	>50	5-50	<1-4	0
Distinct	3	0	5	8
Slight	4	2	7	15
None	5	5	31	99

TABLE 2. — *Percent of plants, grown from seed tubers with different levels of* C. sepedonicum, *with foliage and tuber symptoms.*

	Year	C. sepedonicum populations[a] (cells/mf) in seed tubers			
		>50	5-50	<1-4	0
Foliage	1984	63	23	27	7
	1985	29	8	7	0
Tubers	1984	100	62	33	0
	1985	21	15	0	0

[a]Based on number of fluorescing cells per microscope field (mf) in preparations stained by the indirect immunofluorescence procedure.

TABLE 3. — *Mean monthly weather data at field plot location during potato growing season (May-August) of 1984 and 1985.*

1984	May	June	July	August
Temperature				
Mean of daily maximum	15.4	19.2	23.9	23.2
Mean of daily minimum	5.6	9.4	10.1	10.8
Total hours of sunshine	165.4	151.7	339.4	261.6
Precipitation				
Total amount in cm	22.4	7.3	2.3	5.1
No. of days with rain	24	14	3	7
1985				
Temperature				
Mean of daily maximum	17.9	20.7	27.8	24.4
Mean of daily minimum	6.8	8.5	11.4	10.2
Total hours of sunshine	207.2	257.6	393.5	283.6
Precipitation				
Total amount in cm	7.9	6.7	0.2	1.2
No. of days with rain	15	9	2	5

The 1985 growing season was warmer and considerably dryer than the 1984 season; 31 days of rain provided only 16 cm of precipitation. The 1985 plot was irrigated once in July.

The population of ring rot bacteria as estimated by IF varied in the seed tubers used to plant the 1984 and 1985 plots but all tubers used for seed had no visible ring rot symptoms. As expected those seed tubers with >50 cells/mf developed more plants and tubers with ring rot symptoms than those with 5-50 cells/mf or less (Table 2). Ring rot foliage symptoms, however, were often difficult to determine. Although initial symptoms sometimes appeared in the first week of August, most symptoms were not evident until the last week in August or the first week of September at which time lower leaves were senescing. Thus symptoms recorded in 7% of the plants grown from tubers in which no ring rot cells had been found probably were due to other causes, since only very low numbers of cells were found in those foliage samples (Table 4). In all other cases high populations of ring rot bacteria were found in stem samples from plants which had symptoms.

TABLE 4. — *Percent of plants, grown from seed tubers with different levels of* C. sepedonicum, *having various* C. sepedonicum *populations in stem samples*[a] *as determined by immunofluorescence.*

C. sepedonicum populations[b] in stems	Year	*C. sepedonicum* populations[b] (cells/mf) in seed tubers			
		>50	5-50	<1-4	0
>50 cells/mf	1984	88	62	27	0
	1985	71	31	0	0
5-50 cells/mf	1984	0	8	0	0
	1985	7	8	7	0
<1-4 cells/mf	1984	13	31	47	47
	1985	21	38	36	33
0 cells[c]	1984	0	0	27	53
	1985	0	23	57	67

[a]Samples consisted of four stems/plant taken at harvest. Inclusion of stems with symptoms, if present, in samples for immunofluorescence was erratic since symptoms were often masked by effects of senescence at time of sampling.
[b]Based on average number of fluorescing cells per microscope field (mf) in preparations stained by the indirect immunofluorescence procedure.
[c]No cells found in entire preparation.

Ring rot cells were detected in all foliage samples of plants grown from seed tubers with >50 cells/mf (Table 4). One half to one third of foliage samples from seed tubers in which no cells had been found also had at least some cells. The percent of foliage samples in which no cells could be found increased with decreasing cell population in the seed tuber.

The number of plants with progeny tubers containing >50 cells/mf decreased with decreasing cell populations in the seed tubers (Table 5). However, at least a third of the plants grown from all categories of seed tubers had progeny tubers with no detectable numbers of ring rot bacteria.

TABLE 5. — *Percent of plants*[a], *grown from seed tubers with different levels of* C. sepedonicum, *having various* C. sepedonicum *populations in progeny tubers as determined by immunofluorescence.*

C. sepedonicum populations[b] in progeny tubers	Year	C. sepedonicum populations[b] (cells/mf) in seed tubers			
		>50	5-50	<1-4	0
>50 cells/mf	1984	63	54	13	0
	1985	57	38	0	0
5-50 cells/mf	1984	38	46	13	0
	1985	50	31	0	0
<1-4 cells/mf	1984	75	77	60	53
	1985	64	77	69	67
0 cells[c]	1984	38	38	80	86
	1985	36	46	92	100

[a]Based on number of plants with one or more tubers having the indicated number of IF positive cells. Since individual plants had tubers that differed in numbers of IF positive cells, values in columns are not additive.
[b]Based on average number of fluorescing cells per microscope field (mf) in preparations stained by the indirect immunofluorescence procedure.
[c]No cells found in entire preparation.

For example, 38% of plants, grown from tubers with >50 cells/mf in 1984, produced tubers without detectable populations of ring rot bacteria (Table 5), although at least some other tubers from all these plants had symptoms (Table 2). The distribution of progeny tubers according to number of cells/mf did not differ greatly between symptomatic plants and symptomless plants containing a high number (>50 cells/mf) of ring rot bacteria (Table 6).

Discussion

The seasonal carryover of symptomless infections was confirmed by detection of ring rot bacteria in tubers from plants grown from symptomless tubers. Tubers without ring rot symptoms had a wide range of ring rot cell concentrations ranging from >500 cells/mf to 1 cell/preparation. As expected the symptomless infected tubers with the greatest number of bacteria were most likely to develop plants expressing ring rot symptoms. However, some seed tubers with low numbers of cells developed plants with symptoms. The

TABLE 6. — *Percent symptomless progeny tubers with different levels of C. sepedonicum harvested from symptomatic and symptomless, infected plants.*

| | | *C. sepedonicum* population (cells/mf)[a]of progeny tubers | | | |
	Year	>50	5-50	<1-4	0
Tubers from plants with at least some tuber symptoms	1984	23	14	2	13
	1985	31	25	44	0
Tubers from plants with no visible symptoms but at least one tuber with >50 cells/mf	1984	16	33	42	8
	1985	26	21	26	26

[a]Based on average number of fluorescing cells per miscroscope field (mf) in preparations stained by the indirect immunofluorescence procedure.

difference in number of plants that developed symptoms in 1984 and 1985 was probably due to differences in environmental conditions (1, 12, 15, 20).

Despite the difference in symptom expression during 1984 and 1985, the percent of plants with symptomless infected tubers differed little. In 1984 and 1985, 100 and 21% of plants grown from seed tubers with >50 cells/mf, respectively, developed at least some tubers with symptoms (Table 2), whereas 63 and 57% of the plants had some tubers with >50 cells/mf (Table 5). Moreover, the total percentage of progeny tubers with latent infections harvested from symptomatic and asymptomatic plants did not differ as much as would have been expected on the basis of symptom development (Table 6). The percent of tubers with >50 cells/mf was slightly higher in 1985 than in 1984 suggesting that development of plant symptoms rather than bacterial movement and multiplication was affected by the dry conditions in 1985.

It is difficult to obtain precise quantitative data from microscopic observations. Counting individual cells in a large number of microscope fields for each preparation yields good quantitative data (e.g. ref. 4) but is extremely tedious and can only be done for a small number of samples. We estimated cell numbers as the average number/microscope field for each preparation and this provided a rough delineation between tubers that had many ring rot bacteria, those that had an intermediate number, and those that had only a few.

With our procedures, a single cell in an entire preparation of a 1/10 sample dilution represented 1.5×10^3 cells/g of potato tuber tissue. Sensitivity is probably somewhat lower than even this number, since preparations can hardly be scanned in their entirety (our microscope slide preparations

consisted of about 2500 microscope fields) and cells may be lost during sample preparation. Sample processing to eliminate plant tissue and subsequent concentration of the bacterial fraction seem well warranted as has been recommended for sampling procedures in which large numbers of tuber samples are bulked together (7, 18).

Since the monoclonal antibody in this study was highly specific for *C. sepedonicum* (5) and there were never more than 5 cells/mf in plants and tubers grown from seed in which no bacteria were found (Tables 4 and 5) or in other progeny tubers of stem cutting derived plants (unpublished data), it appears that the presence of >5 fluorescing cells/mf is a reliable indication that ring rot bacteria are present in the plant. The question regarding the reliability of low numbers of fluorescing bacteria in IF preparations is more problematic, however. Preparations in which <1-4 cells/mf were recorded included mostly preparations in which <1 cell/mf was observed and often only one cell was found in the entire preparation after extensive searching. It cannot be established with certainty whether in such a case the single cell is evidence that ring rot bacteria were present in the tuber or plant from which the sample was taken. The possibility that a cell washed over from a highly infected sample, that it came as an airborne deposit from aerosols perhaps created elsewhere in the lab, that a cell adsorbed antibodies nonspecifically (e.g., such as a Protein A containing *Staphylococcus aureus* cell), or represented a cross-reacting bacterium not previously encountered, must all be considered. Further quantitative work is required to determine the confidence with which low numbers of fluorescing cells can be taken as evidence of latent ring rot infections.

Foliage and tuber samples of a large number of plants contained <1-4 cells/mf in our experiments. Even plants grown from seed tubers in which no cells had been found often contained low numbers of fluorescing bacteria. The possibility that these low positive readings do not necessarily indicate the presence of *C. sepedonicum* has been discussed but other explanations for their presence are possible. The limitations of the IF test may have resulted in ring rot bacteria not being detected in the seed tubers while actually being present, some contamination of tubers could have occurred during sampling despite all precautions to prevent it, and cross-contamination in the field during the growing season may have occurred. The literature on field spread of the bacterial ring rot disease is very limited (2, 10), and spread of latent infections in particular has not been studied. Transmission of ring rot via insects has been reported by several authors (8, 14) but other agents including rodents, birds, livestock, wind, rain and root contact are also theoretically possible.

Diagnosis of bacterial ring rot can readily be confirmed by the IF procedure (6) and its efficacy is enhanced by the use of monoclonal antibodies. In this paper we have shown that latent ring rot infections can be detected but the lower limits of detection have yet to be established. Caution

is warranted in interpretation of test results when only very few fluorescing bacteria are observed. If improved procedures for quantifying low bacterial population numbers can be developed, it may be possible to obtain adequate laboratory test data with statistically valid confidence intervals to replace the zero tolerance inspection criterion as a measure of ring rot risk in a consignment of seed potatoes.

Acknowledgment

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