EXTRACTION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* FROM COMPOSITE SAMPLES OF POTATO TUBERS

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Abstract

The presence of *Clavibacter michiganensis* subsp. *sepedonicus* was detected by immunofluorescence and ELISA in diluent in which cores from 200 potato tubers had been incubated on a rotary shaker. By this procedure the ring rot pathogen was detected in composite samples from naturally infected tubers and tuber samples "spiked" with infected tissue or a pure culture of the pathogen. Populations of saprophytic bacteria increased about 100-fold in the diluent during 18 h of incubation but did not interfere with serological detection of *C. m. sepedonicus*. Some of the results suggest that *C. m. sepedonicus* cells multiply during incubation of tuber samples. Extraction of ring rot bacteria from potato tuber tissue by incubation on a rotary shaker requires little work and minimizes the amount of plant tissue in the extract compared to the standard extraction procedure involving maceration and differential centrifugation.

Compendio

Se detectó la presencia de *Clavibacter michiganensis* subsp. *sepedonicus* por inmunofluorescencia y ELISA, en una dilución en la cual las partes centrales de 200 tubérculos de papa habían sido incubadas sobre un agitador giratorio. Siguiendo este procedimiento se detectó al agente de la pudrición en anillo en una mezcla de muestras de tubérculos infectados naturalmente y de muestras de tubérculos "pinchados" con tejido infectado o con un cultivo puro del patógeno. Las poblaciones de bacterias soprofíticas se incrementaron en la dilución aproximadamente en 100 veces durante 18 horas de incubación, pero no interfirieron con la detección serológica de *C. michiganensis* subsp. *sepedonicus*. Algunos de los resultados sugieren que las células de *C. m. sepedonicus* se multiplican durante la incubación de las muestras de tubérculos. La extracción de las bacterias de la pudrición en anillo de los tejidos de los tubérculos de papa por incubación sobre un agitador giratorio requiere poco trabajo y minimiza la cantidad de tejido vegetal en el extracto en comparación con el procedimiento estándar de extracción que incluye maceración y centrifugación diferencial.

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Introduction

During the last two decades bacterial ring rot of potatoes *(Clavibacter michiganensis* subsp. *sepedonicus)* has been an increasing problem in several countries particularly due to concerns regarding international trade of seed potatoes. In countries where the disease does not occur, it is feared that once the disease is introduced it will be very difficult to eliminate the pathogen. Since the disease is spread from tuber- to-tuber by handling of plant material during planting, harvesting, and storage operations it is very important that potatoes used for seed are free from the pathogen. Therefore, many countries and organizations now initiate seed potato production from meristematic tissue or stem cuttings to produce disease-free nuclear plant material (11, 12).

Indexing of nuclear plant material and seed lots for bacterial ring rot cannot be based solely on visual inspection because the disease may be latent and fail to express disease symptoms (16). Isolation of the causal bacterium is impractical because the pathogen is a slow-growing Gram-positive bacterium that is readily overgrown by fast-growing saprophytic microorganisms associated with plant material (15). A bioassay using eggplants to selectively increase *C. m. sepedonicus* populations has also been described (10, 13) but its sensitivity is variable and is influenced by the environmental conditions under which the plants are grown.

Immunofluorescence is commonly used for detecting bacterial ring rot in seed potato lots (1, 3). A protocol for ring rot testing of composite potato samples based on immunofluorescence has been introduced as part of an official European Community procedure (1). Problems with serological cross-reactions in immunofluorescence using polyclonal antisera (2) have been substantially reduced but not eliminated by development of specific monoclonal antibodies (5). Monoclonal antibodies have also given good results in the enzyme linked immunosorbent assay (ELISA) (7).

Prior to applying immunofluorescence for testing potato samples, bacteria must be at least partially separated from the plant tissue. The most commonly used method for extraction of bacteria from the plant material is maceration of the tissue followed by differential centrifugation (1). Incubation of tissue pieces in distilled water or other diluent on a rotary shaker has also been reported as effective in releasing C. *m. sepedonicus* from infected potato tissue (8). In this study we evaluated the effectiveness of incubating potato tissue on a shaker to prepare samples for serological testing.

Materials and Methods

Bacterial Strains—Strain R14 of *C. michiganensis* subsp. *sepedonicus* isolated in 1991 from a potato tuber (cv. Atlantic) was used in the laboratory

experiments. Another bacterium (strain 16 C) which was antagonistic to *C. m. sepedonicus* and identified on the basis of its fatty acid profile as *Arthrobacter* sp. was also used in some of the experiments (9). Bacterial cultures were stored at -80 C in 10% glycerol and grown on YGM medium (3) at 23 C.

Serological Procedures—For immunofluorescence, undiluted and three 10-fold dilutions of samples were acetone-fixed to multiwell microscope slides. The indirect immunofluorescence staining procedure with monoclonal antibody 9A1 as described by De Boer and McNaughton (5) was used. Bacterial concentrations were estimated from visual or automated counts of fluorescing cells and recorded as number of immunofluorescing units (IFU). Visual counts were generally made when the number of bacteria were fewer than 10/field and then 15 randomly selected microscopic fields were counted at the optimum dilution. Automatic counts were made on an equal number of fields using a Zeiss Vidas image analysis system in a similar manner as reported previously (4).

In addition to immunofluorescence the ELISA test was used for some of the samples. The procedure used was previously described by DeBoer *et al.* (7).

Extraction Procedures—Extractions were carried out on composite samples of 200 tuber cores with the epidermis left in place (Fig. 1). Cores were washed and placed in a 500 ml Erlenmeyer flask with 200 ml of distilled water as diluent. Flasks were placed on a rotary shaker (50 rpm) in an incubator at 23 C or at room temperature. Diluent fluid was removed and tested directly in serological tests or concentrated 100-fold by centrifugation (20 minutes at 5000 g) and resuspension prior to testing.

For the maceration procedure, washed composite tuber core samples were homogenized in a Waring blender with 100 ml of distilled water for 30 sec and filtered through cheese cloth (Fig. 1). The filtrate was centrifuged at 190 g for 10 minutes. The supernatant fluid was used for the ELISA test, but for immunofluorescence the supernatant was centrifuged at 4000 g for 30 min and resuspended in 0.5 ml of distilled water.

Tuber Experiments—To investigate whether *C. m. sepedonicus* multiplies in potato tuber samples during the incubation period, 1 ml of suspension of *C. m. sepedonicus* at about 10^9 cfu/ml was added to 100 grams of potato pieces in 100 ml of distilled water. In one experiment 10^6 cfu/ml (final conc) of strain 16 C, which is antagonistic to *C. m. sepedonicus*, was added to an identical sample in addition to the *C. m. sepedonicus* inoculum. Distilled water containing *C. m. sepedonicus*, but no potato tissue, was used as control. Preparations were incubated on the rotary shaker as indicated above and sampled at 0, 8, 12 and 24 h to determine bacterial populations using the quantitative immunofluorescence procedure.

The influence of tuber-associated saprophytic bacteria in the diluent fluid on *C. m. sepedonicus* was tested in an experiment with flasks of 100 g of

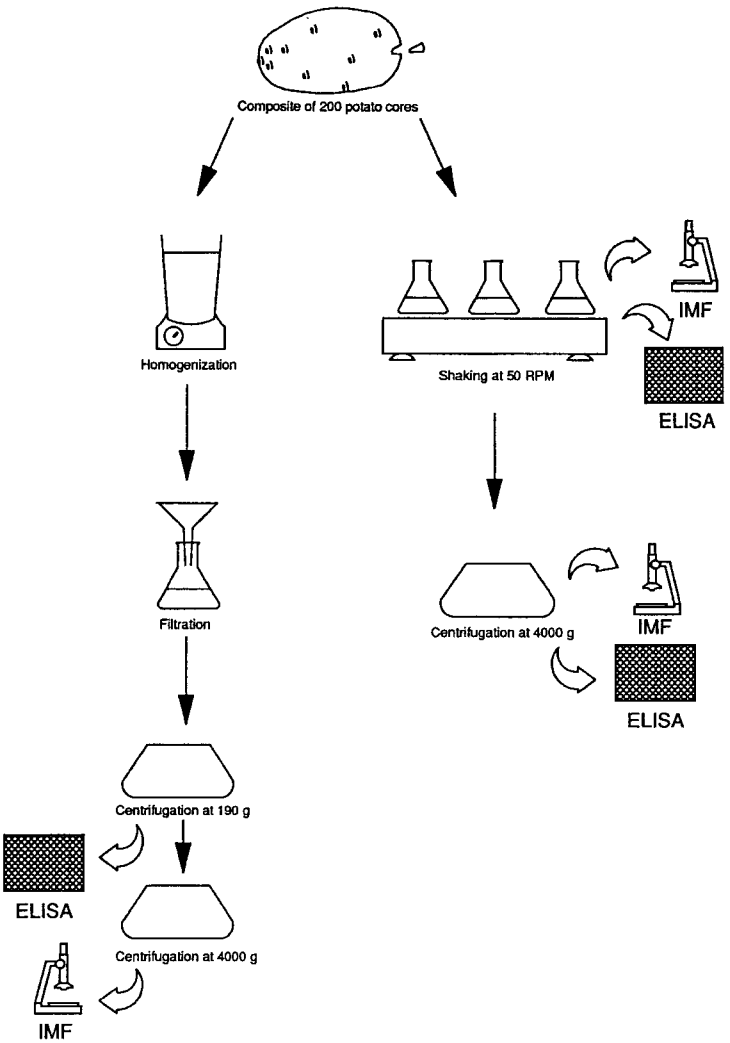

FIG. 1. Schematic representation of the maceration (left sequence) and incubation (right sequence) procedures for extracting *C. m. sepedonicus* from potato tuber tissue for serological analysis.

potato tuber cores incubated with 100 ml of distilled water and *C. m. sepedonicus* from pure culture. Duplicate flasks were inoculated to obtain final concentrations of 10^4, 10^6, and 10^8 cfu/ml of *C. m. sepedonicus*. After

incubation for 3 and 18 h, samples were removed to determine density of saprophytic bacteria by standard plate count procedures on nutrient agar medium (Difco) and indexed for *C. m. sepedonicus* by immunofluorescence and ELISA.

Immunofluorescence and ELISA determinations were also made on extracts in which 199 healthy cores were mixed with one infected core to determine the effect of length of the incubation period. Samples were tested after 4, 24, 48, 72 and 96 hours of incubation. In another experiment the incubation and maceration procedures were compared. Negative controls for maceration and extraction were performed.

Results and Discussion

The initial density of *C. m. sepedonicus* detected in the diluent of composite tuber samples inoculated with the pathogen was about 5-fold less than in diluent without plant tissue but to which an equal concentration of *C. m. sepedonicus* had been added, probably because the bacteria adsorbed to the plant tissue (Fig. 2). In distilled water the population of *C. m. sepedonicus* detected by immunofluorescence decreased from 2.4×10^6 to 1.1×10^6 IFU/ml after 8 h of incubation.

In contrast to changes in bacterial density in distilled water, in composite tuber samples the number of bacteria detected by immunofluorescence increased during the period of 8-24 hours of incubation. The population of *C. m. sepedonicus* increased from 1.1×10^5 to 2.1×10^6 IFU/ml during 24 hours (Fig. 2). This increase could be due either to the release of cells initially adsorbed to the plant tissue or multiplication of the bacterium. The population of *C. m. sepedonicus* ceased to increase in the presence of the antagonistic *Arthrobacter* sp., which could indicate that saprophytic bacteria do not inhibit the tests and it is a possibility that the increase of *C.m. sepedonicus* can be due to multiplication. For the sample in which *C. m. sepedonicus* and the arthrobacter were co-inoculated, the level of *C. m. sepedonicus* detected, increased from 5.7×10^4 to 9.5×10^5 IFU/ml after the initial decrease during the first 8 h of incubation. However, after 12 h of incubation no further increase occurred, which suggested that multiplication of *C. m. sepedonicus* was inhibited by the antagonist.

In Denmark extraction of *C. m. sepedonicus* from composite tuber samples by incubation of the tissue in water on a rotary shaker has been done for several years (8). It has been argued (14), however, that when the incubation method is used, saprophytic bacteria will multiply faster than slower growing *C. m. sepedonicus* cells, and that the latter will be rapidly overgrown and difficult to detect. In our study with *C. m. sepedonicus*-inoculated samples, an inhibitory effect of the antagonistic *Arthrobacter* sp. plus bacteria from the natural microbial flora in and on the tubers, only became evident after 12 h (Fig. 2).

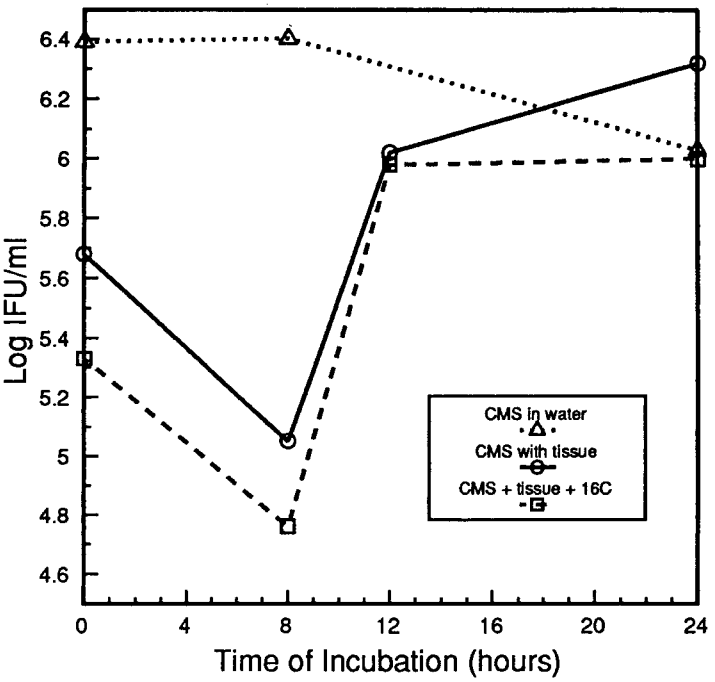

FIG. 2. Changes over time in densities of *C. m. sepedonicus* (CMS) detected by immunofluorescence when added to distilled water and composite samples of potato tuber tissue, alone and in the presence of an antagonistic *Arthrobacter* sp. (strain 16C).

In the procedure for extracting ring rot bacteria from composite tuber samples in Denmark, samples are incubated for about 18 hours during which the population of saprophytic bacteria in the diluent fluid would be expected to increase. After an initial 3 hr incubation period to allow release of bacteria from the tubers, the saprophytic population increased about 100-fold during the next 15 hrs (Table 1). Both immunofluorescence and ELISA readings for *C. m. sepedonicus* were higher at 18 h compared to the initial readings at 3 h despite the extensive multiplication of saprophytic bacteria (Table 1). It appears, therefore, that naturally contaminating bacteria including those antagonistic to *C. m. sepedonicus* that are also extracted and multiply during the incubation procedure do not hinder the detection of the ring rot pathogen. For the negative controls no IFU were found.

Composite tuber samples "spiked" with tuber tissue from different sources which were ostensibly infected with bacterial ring rot indicated that there was no advantage to incubating the samples for more than 24 h (Table

TABLE 1.—*Density of saprophytic bacteria in diluent fluid of composite samples of potato tuber tissue after 3 and 18 h of incubation on a rotary shaker, and corresponding immunofluorescence (IF) and ELISA readings for* C. m. sepedonicus *(Cms).*

Sample	Conc of Cms added per ml of diluent	IF reading at 1/100 dilution (IFU/field)		ELISA reading (absorbance at 405 nm)		Saprophyte density (cfu/ml)	
		3 h	18 h	3 h	18 h	3 h	18 h
1	10^4	ND[a]	ND	0.015	0.122	4.0×10^5	3.1×10^7
2	10^4	ND	ND	0.155	0.203	4.0×10^6	1.0×10^8
3	10^6	1.1	5.6	0.099	0.167	6.4×10^5	6.5×10^7
4	10^6	1.1	5.5	0.031	0.156	3.0×10^5	1.4×10^7
5	10^8	60.5	311.2	0.609	1.478	8.0×10^5	1.6×10^7
6	10^8	53.9	237.2	0.563	1.488	3.0×10^5	3.8×10^7

[a]Not done

TABLE 2.—*Immunofluorescence (IF) and ELISA values for composite potato tuber samples to which tissue from ostensibly ring rot infected tubers was added, after incubation for 4-96 h on a rotary shaker.*

Sample	Test	Time (hours)				
		4	24	48	72	96
I	IF[a]	6.80	9.93	12.60	13.00	9.13
	ELISA[b]	0.172	0.215	0.113	0.325	0.387
II	IF	7.41	25.93	19.13	25.27	20.33
	ELISA	0.123	0.448	0.282	0.522	0.663
III	IF	1.41	3.80	ND	1.17	1.85
	ELISA	0.129	0.093	0.048	0.230	0.237
IV	IF	0.90	2.93	ND	0.35	1.45
	ELISA	0.023	0.076	0.050	0.201	0.211

[a]Given as IFU/microscope field x dilution factor.
[b]Given as absorbance at 405 nm.

2). An initial increase in immunofluorescence and ELISA readings occurred from 4 to 24 h in two positive samples but incubation for up to 96 h did not provide better results (samples I and II, Table 2). The initial increase was probably due to release of bacteria from tissue and their multiplication.

TABLE 3.—*Comparison of immunofluorescence counts and ELISA values for composite tuber samples extracted by incubation on a rotary shaker and maceration/centrifugation procedure. Fluid from the incubated samples were tested directly and concentrated 100-fold by centrifugation.*

Sample	Immunofluorescence[a]		ELISA[b]	
	Direct	Concentrated	Direct	Concentrated
Extraction by incubation on shaker for 4 h				
A	177	7240	0.599	0.596
B	39	8063	0.864	0.744
C	97	3187	0.482	0.548
D	107	4686	0.697	0.634
Extraction by incubation on shaker for 24 h				
A	186	5414	0.512	0.304
B	440	8993	0.726	ND[c]
C	171	1984	0.465	0.397
D	340	3846	0.685	0.563
Extraction by maceration and differential centrifugation				
E	ND	4556	0.399	ND
F	ND	131	0.140	ND
G	ND	73	0.413	ND
H	ND	1447	0.257	ND

[a]Given as IFU/microscope field X dilution factor.
[b]Given as absorbance at 405 nm.
[c]Not done.

Subsequent changes in cell densities may reflect growth and death of bacteria as affected by competitive saprophytic microorganisms. The greater increase in ELISA values compared to counts of fluorescing cells may have been due to accumulation of antigen in the sample even when the number of intact cells decreased. The ELISA test is based on the extracellular polysaccharide whereas the immunofluorescence test is based on a somatic antigen and requires visualization of intact cells (6, 7). Samples III and IV (Table 2) were borderline positive in immunofluorescence but would have been considered negative in ELISA after 24 hours of incubation (Table 2). After 72 hours they gave a positive response in ELISA but such long incubation periods are not practical for routine testing.

Composite samples containing tissue from naturally infected symptomatic tubers gave positive immunofluorescence and ELISA readings after as little as 4 h (Table 3). Concentration of the diluent 100-fold after incubation significantly increased counts of fluorescing bacteria (Table 3). However, as expected, ELISA readings were not increased by centrifugation

because of the soluble nature of the antigen. Nevertheless, the soluble C. *m.sepedonicus* antigen was released into the diluent so that results of ELISA on diluent fluid were as good as those on the filtrate from macerated samples (Table 3).

Counts of fluorescing cells released from plant tissue by incubation in water and subsequently concentrated by centrifugation were generally higher than counts obtained with macerated samples (Table 3). Moreover, an important advantage of the incubation procedure over the maceration procedure is that preparations for immunofluorescence contain far less plant tissue than those prepared by maceration. Whereas undiluted and 1/10 dilutions of macerated samples often cannot be used for estimating cell numbers because cells are obscured and sometimes poorly stained because of the large amount of plant debris in the bacterial fraction, preparations from the incubation procedure are almost devoid of plant debris. This may also be an advantage when DNA amplification procedures are to be used since plant components may inhibit the polymerase chain reaction. A second advantage of the incubation procedure is that it does not require the laborious and time-consuming filtration and centrifugation steps required to separate bacteria from the bulk of the potato tissue when it is macerated. Furthermore, this procedure may be useful for indexing stem tissue which very often is woody and difficult to macerate in a blender.

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