THE LATEX AGGLUTINATION TEST AS A RAPID SEROLOGICAL ASSAY FOR *CORYNEBACTERIUM SEPEDONICUM*

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Abstract

The latex agglutination test was evaluated for detection of the potato bacterial ring rot pathogen, *Corynebacterium sepedonicum*. The bacterium was detected in infected potato stems and tubers tested and in inoculated eggplant stems. Test reliability was confirmed using blind tests. A minimum of 10^6 bacterial cells/ml was requisite for observable agglutination. Nonspecific reactions were not observed from healthy extracts of plant tissues. Nonspecific reactions with other bacteria maintained in pure culture were eliminated by addition of bovine serum albumin to sensitized latex preparations.

Resumen

La aglutinación de látex fue evaluada como prueba para la detección de *Corynebacterium sepedonicum*, agente etiológico de la podredumbre anular en la papa. Tallos y tubérculos de papas infectadas, así como tallos de berengena previamente inoculados se mostaron positivas a la prueba. Las muestras fueron previamente codificadas, para que el individuo que realizó la prueba desconociera su procedencia (prueba a ciegas). Para observar la aglutinación de látex fue necesario tener una cantidad mínima de 10^6 bacterias/ml. No se observaron reacciones inespecíficas contra extractos de tejidos vegetales sanos. Las reacciones inespecíficas contra cultivos puros, de otras bacterias fueron eliminadas al agregar albumina bovina a la solución amortiguadora con el látex sensibilizado.

Introduction

Bacterial ring rot of potato caused by *Corynebacterium sepedonicum* (Spieck. & Kotth.) Skapt. & Burkh. was discovered in North America in 1931 (1). The disease is now found throughout North America and is the major reason for rejection of seed potatoes entered for certification (8). Generally, bacterial ring rot rejections are due to the uniformly strict

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"zero" disease tolerance among all North American potato seed certification agencies and do not reflect high disease incidence within seed lots. Thus, when few or questionable specimens are the basis for diagnosis, considerable debate may ensue.

Recently, a committee of the Pathology Section of the Potato Association of America was established to review available bacterial ring rot diagnostic procedures. Standard criteria for diagnosis were recommended in a report which was endorsed at the 1979 Annual Winter Meeting in Denver, Colorado (7). In all cases, diagnostic criteria included evaluation of vine and/or tuber symptoms. For disputed cases, diagnoses based on symptoms were required to be supplemented by a Gram stain, a serological test, a pathogenicity test, or pathogen isolation and identification.

In general practice, bacterial ring rot diagnosis is based on symptoms and is aided by a confirming Gram stain test. However, the lack of specificity of this test also may be a source of problems (2, 9). Since *C. sepedonicum* has been shown to be distinct serologically from other *Corynebacterium* spp. (6), several serological tests have been suggested as specific diagnostic aids to supplement bacterial ring rot determinations (3, 4, 9).

The latex agglutination test has been shown to be a rapid, reliable, sensitive, and simple test for potato viruses S and X in Wisconsin (5). Since this procedure showed promise as a diagnostic assay for *C. sepedonicum*, this study was initiated to evaluate the application to a bacterial plant pathogen. A preliminary report has been presented (10).

Materials and Methods

Sources of bacterial cultures and production of *C. sepedonicum* antiserum have been reported (9). The latex agglutination test was performed as described previously (5). Polystyrene spheres (Sigma Chemical Co., St. Louis, MO) now are sold as a 10% rather than a 30% suspension, but dilution-series experiments demonstrated that a 1/15 (v/v) dilution of the 10% suspension in 0.85% NaCl, as with the 30% suspension, produced best results. After preliminary experiments, conjugation of antiserum and latex particles (sensitized latex) was modified by addition of bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) to the final resuspension buffer.

Test sensitivity was determined by evaluating *C. sepedonicum* dilution series against antiserum dilutions of sensitized latex. Cell concentrations were adjusted spectrophotometrically with $A_{600nm} = 1.0$ equivalent to 2×10^9 cells/ml.

Test antigens were prepared either from bacteria maintained in culture on nutrient dextrose agar (NDA: 0.3% beef extract, 1.0% peptone, 1.0% dextrose, and 1.8% agar) or from stem or tuber tissue of plants. From

culture, bacterial suspensions were adjusted spectrophotometrically to approximately $A_{600nm}=0.5$ for tests. Stem and tuber samples were prepared in 0.2 or 0.3 ml of 0.1 M Tris (hydroxymethyl) aminomethane-HCl, pH 7.4, plus 0.85% NaCl (TBS) containing 0.1% sodium azide. Potato, *Solanum tuberosum* L., tubers were cut perpendicularly to the stolon end and squeezed to detect vascular breakdown. Bacterial ooze and tissue (ca. 1 mm^3) from deteriorating regions of the vascular ring were collected with a sterile scalpel. Potato stems were cut transversely within 1-2 cm of seed piece attachment. Stems were squeezed, examined for a characteristic milky white bacterial ooze, and smears were made on glass microscope slides. The terminal 1-2 mm of stem sample was excised for serological tests. Eggplant, *Solanum melongena* L. cv. Black Beauty, stems were sampled as described previously (9).

For blind tests, tubers from known healthy and ring rot-infected potatoes were collected from plots maintained by F.E. Manzer. In January and February of 1979, potato stems were collected from field plants exhibiting bacterial ring rot symptoms in Florida. Stems were tested on site or in Madison, WI. The latter samples were sent by mail after being wrapped in moist paper towels and placed inside unsealed plastic bags.

Undiluted and 1:10 dilutions of samples were tested with at least two antiserum dilutions of sensitized latex in each assay. Agglutination reactions were rated on a scale of 0-5 (0=no agglutination, 5=complete agglutination) with values ≥ 2.0 scored positive.

Positive (*C. sepedonicum*) and negative (*C. insidiosum* or *C. michiganense*) controls were included in each test series. Control antigens were adjusted to $A_{600nm}=0.5$ in 0.85% NaCl containing 0.1% sodium azide and stored for 6-9 months at 4°C.

Results and Discussion

The latex agglutination test was highly effective for detection of *C. sepedonicum* in diseased plant tissue. Initial tests indicated that sensitized latex could be prepared and tests performed as described previously (5). In this study, twofold antiserum dilutions of 1:25-200 were used routinely for sensitized latex preparations. Approximately one thousand samples were assayed per ml of initial antiserum. Antiserum dilutions of sensitized latex that were best for tests varied slightly among preparations with antiserum dilutions of 1:50-200 generally best for the 2 months following preparation and 1:25-50 generally best thereafter. Reaction intensity did not diminish 8 months following preparation (the longest period tested) for the latter antiserum dilutions. When antigen-antibody ratios were optimal, agglutination within capillary tubes was visible almost immediately. Since weaker reactions were encountered, pipettes were rotated in routine tests for 15 min at 7 rpm before examination with the aid of a stereomicroscope (ca. 10x).

Test sensitivity varied from 10^6-10^7 cells/ml. Therefore, tests were approximately 2-10x more sensitive than droplet agglutination or double diffusion tests, but less sensitive than indirect fluorescent antibody stain tests or Gram stain tests (9). Agglutination values $\geq$2.0 always were observed for *C. sepedonicum* suspensions of $>10^7$ cells/ml.

Test specificity was evaluated by examining reactions of strains of *C. sepedonicum*, other phytopathogenic *Corynebacterium* spp., and other bacteria that cause potato diseases. When 12 strains of *C. sepedonicum* from diverse geographic regions of North America were tested, complete agglutination was observed with all strains. No agglutination was observed with cell suspensions of *Erwinia carotovora* var. *carotovora* (SR-206), *E. carotovora* var. *atroseptica* (SR-8), and *Pseudomonas solanacearum* (K60) or with vascular tissue from potato tubers injected with suspensions of *E. carotovora* var. *atroseptica* or *Clostridium* sp. No agglutination was observed with *C. fascians* or *C. insidiosum*, but when optimum antiserum dilutions for some sensitized latex preparations were used, low agglutination values (0.5-1.0) were observed with *C. flaccumfaciens*, *C. michiganense*, and *C. poinsettiae*. Addition of 0.004% BSA to sensitized latex preparations eliminated agglutination with *C. flaccumfaciens* and minimized agglutination (scores of 0-0.5) for the latter two species. Therefore, in subsequent tests, including those used for plant assays, 0.004% BSA was added to all preparations.

Except for *C. sepedonicum*, an agglutination value $\geq$2.0 was recorded with only one bacterium. When tested against sensitized latex without BSA, an agglutination value of 3.5 was obtained for an uncharacterized Gram (-) bacterium maintained in culture. A BSA titration showed that 0.4% BSA eliminated the nonspecific reaction and 0.04% BSA reduced agglutination to $<$2.0. The latter concentration also eliminated all nonspecific reactions with *C. michiganense* and *C. poinsettiae*. None of the BSA concentrations affected reaction intensity or sensitivity with *C. sepedonicum*. Therefore, if there is evidence of nonspecific reactions, it may be desirable to include 0.04-0.4% BSA in preparations to avoid nonspecific reactions. However, it should be noted that when plant tissues were assayed with sensitized latex preparations containing 0.004% BSA, nonspecific reactions were never observed.

Initial attempts to detect *C. sepedonicum* in confirmed ring rot-infected tubers were successful. The bacterium was detected from tissue suspended in 0.2 ml buffer and following a 1:10 sample dilution. If characteristic bacterial ring rot symptoms were present, agglutination values with 1:10 sample dilutions were greater than or equal to values for undiluted samples. We have continued to test both antigen dilutions because positive assays were obtained only with undiluted samples for some tubers with incipient or latent infections. Initial buffer volumes for samples were later increased to 0.3 ml because the larger volume permitted resampling and

assay sensitivity was not affected. When three healthy tubers each of cvs. Atlantic, LaChipper, Norgold Russet, Red LaSoda, and Russet Burbank were tested, no agglutination was observed both with and without 0.004% BSA in sensitized latex preparations.

Cooperative blind tests then were arranged. One investigator selected healthy and ring rot-infected cv. Katahdin tubers and prepared Gram-stained slides and buffered tissue samples for latex agglutination tests. A second investigator assayed the coded samples (Table 1). It is interesting to note that, although sensitivity data would suggest otherwise, more positive assays were obtained by latex agglutination tests than by Gram stain tests. Nonspecificity was not suggested for the latex tests because only tubers collected from known infected mother plants were positive.

TABLE 1. — *Detection of* Corynebacterium sepedonicum *in potato tubers by latex agglutination tests (LAT) in blind tests.*[1]

Mother plant	Daughter tuber symptoms	Test 1		Test 2		Mean AV[2]	
		Gram stain	LAT	Gram stain	LAT	Not diluted	Diluted 1:10
Infected	Yes	3/3[3]	3/3	6/6	6/6	3.7	3.5
Infected	No	3/4	4/4	5/8	7/8[4]	3.6	3.3
Healthy	No	0/3	0/3	0/6	0/6	0.2	0.0

[1]First investigator selected the 30 cv. Katahdin tubers of known history, prepared and inspected Gram stains, and placed 1 mm^3 vascular tissue into numbered vials with Tris buffered saline containing sodium azide. Second investigator tested vial contents by latex agglutination independent of first investigator.

[2]Agglutination values (AV) represent mean score on a scale of 0-5 at the optimum antiserum dilution (0=no agglutination; 5=complete agglutination).

[3]Numerator is number of plants positive and denominator is number of plants tested.

[4]Tuber assaying negative also was negative by Gram stain test.

In order to determine whether or not *C. sepedonicum* could be detected in decomposing tubers, tubers from the first blind test were wrapped in moist paper towels, sealed in individual plastic bags, and stored at 22°C for 4 days and 32°C for an additional 5 days. Following this treatment, tissue from the largely decomposed tubers was removed with sterile toothpicks from the estimated vicinity of the vascular ring. Results were the same as before except that the tuber, which initially assayed negative by the Gram stain test and positive by the latex agglutination test, tested negative.

Eggplant and potato stems also were tested. In a greenhouse test, 20 of 20 inoculated eggplants indexed positive by the latex agglutination test and all six uninoculated eggplants were negative. Visually, only 13 of the 20 inoculated eggplants were scored positive and all six uninoculated egg-

plants were scored negative. Positive assays were obtained for all 34 potato stems tested from potato plants showing symptoms in plantings near Homestead, Florida. Twenty-nine assays were made following mail shipment. Three samples were received with considerable tissue drying or decomposition, but positive assays were still obtained. All assays were confirmed by a Gram stain test. Samples included cvs. Katahdin, Oneida, Penn 71, Russet Burbank, and Superior. Stems of 20 apparently healthy cv. Katahdin plants, which were selected as control samples, assayed negative.

The data indicate that the latex agglutination test is a valuable diagnostic assay for bacterial ring rot. In addition to the test simplicity, results can be obtained within the same time that the Gram stain test can be performed and costly equipment (e.g., fluorescent microscope setup) is not required. In an attempt to provide a broader data base to evaluate test reliability further, a cooperative venture involving several laboratories has been initiated.

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