The Use of Fuller's Formamide Method in the Serological Identification of *Pseudomonas solanacearum*

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INTRODUCTION

Pseudomonas solanacearum (E.F.S.) is the cause of bacterial wilt of tomatoes, tobacco, bananas, and many other economically important crops. It is thus one of the most injurious plant pathogens in the world. Rapid identification and maintenance of virulence of the organism has been facilitated by the work of Kelman (*1*, *2*)[2] and Kelman and Jensen (*3*). Their techniques have greatly helped in determining the presence of pathogenic strains and in host-range studies.

However, to our knowledge, serological tests with *Ps. solanacearum* have been limited to the work of Moraes (*4*) who by means of agglutination tests confirmed the identity of atypical isolates from potato. These atypical isolates failed to produce the brown discoloration of vascular tissue which is usually produced by this bacterium.

In our search for a serological method that would facilitate identification of the organism in a matter of hours we have made use of Fuller's formamide method (*5*) for the extraction of polysaccharides from bacteria. This method has been employed in extracting polysaccharides from *Staphylococcus* (*6*, *7*), *Brucella* (*8*), and *Shigella* (*9*), and was used successfully in serological identification of the organisms belonging to these genera. The present report deals with the use of Fuller's method in the rapid identification of *Ps. solanacearum*.

MATERIALS AND METHODS

CULTURES[3]

Fifty-nine cultures of *Ps. solanacearum* were employed in this study. These were isolated originally from different plant sources as follows:

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[2] Italic numbers in parentheses refer to Literature Cited, p. 152–3.

[3] We are indebted to Dr. A. Kelman, North Carolina State College, Raleigh, N.C., for supplying us with the majority of cultures used in this study. We are also thankful to Dr. Masao Goto, Shizuoka University, Shizuoka-ken, Japan, and to Dr. Ivan W. Buddenhagen, Vining C. Dunlap Laboratories, Department of Plant Pathology, Tela RR. Co., La Lima, Honduras, for supplying cultures.

Tomato, 15; tobacco, 14; potato, 7; eggplant, 5; *Heliconia* spp., 4; banana, 2; plantain, 1; *Piper auritum*, 2; *Physalis* spp., 2; dahlia, 2; peanut, 1; *Zinnia* spp., 1; *Solanum nigrum*, 1; sesame, 1; and *Eupatorium odoratum*, 1.

Distribution of the cultures as to countries of origin follows: United States, 24; Costa Rica, 13; Japan, 9; Puerto Rico, 4; Colombia, 2; and 1 culture from each of the following countries: Panama, Israel, Cyprus, Kenya, Trinidad (B.W.I.), Northern Rhodesia, and Southern Rhodesia. Most of the cultures were of recent isolation (1958–60). A few cultures, however, had been kept in the laboratory stock for periods up to 6 years. All were maintained under mineral oil in tryptone-glucose yeast agar (TGA) slants and also as distilled water suspensions (*10*). Transfers were made twice a year on TGA slants.

EXTRACTS

A loopful from a water suspension of each culture was removed and streaked onto two slants of TGA. After 3 days' incubation at 28–30° C. the bacterial growth was washed with 2-ml. sterile saline, and centrifuged at 3,000 r.p.m. for 20 minutes in an angle-head to sediment the bacteria. After decanting the supernatant, the wet bacterial sediment was processed by Fuller's formamide method (*5*). This method consists in extracting the bacteria with formamide at 150° C. for 15 minutes. After cooling the extract, acid alcohol is added to precipitate bacterial debris and broken-down proteins. The polysaccharide constituents will remain in solution and are then precipitated by the addition of acetone. The acetone precipitate is then dissolved in distilled water and neutralized with 2-percent Na_2CO_3, using phenol red as indicator. This is the final extract used for testing and will have a pink color (pH 7.2–7.4). Some undissolved material usually remains, but this may be removed by centrifuging lightly without altering the serological reactivity of the extract.

Sources of all 59 *Ps. solanacearum* cultures extracted as above described, appear in table 1.

To determine whether the culture medium and method of incubation used for growing the organisms might have an effect on the reactivity of the extracts, 12 cultures from various original plant hosts and countries of origin were selected and seeded in the synthetic liquid medium of Hussain and Kelman (*11*) for 48 hours, and also on slants of nutrient agar for a 3-day period. Bacterial sediments obtained by centrifugation of the growth in liquid culture medium, as well as from centrifuged washings of the growth in nutrient agar were extracted with formamide as described before. The extracts gave exactly the same results as those obtained from the cultures after growth on TGA medium.

TABLE 1.—*Results of ring tests with formamide extracts from 59* **Pseudomonas solanacearum** *cultures from different areas and antisera against selected cultures*

Culture[1]	Host[2]	Geographic area[3]	Antiserum against culture[4]									Serum controls[5]		
			ATCC-10692	PR-65	K-95	NCPPB 332	K-217	B-PG 237	B-312R	B-139B	B-100D	Gu-1, *Ps. tabaci*	EC-153, *E. carotovora*	Normal rabbit serum
K-25	Tomato	Georgia	+	+	+	+	+	+	+	+	++	–	–	–
K-63	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
K-72	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
K-77	do.	North Carolina	+	+	+	+	+	+	+	+	+	–	–	–
K-60R	do.	do.	+	+	+	++	+	+	+	+	+	–	–	–
ATCC-10692	do.	do.	+	+	+	+	+	+	++	+	++	–	–	–
ATCC-11696	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
PR-65	do.	Puerto Rico	+	+	+	++	+	+	+	+	+	–	–	–
PR-69	do.	do.	+	+	–	+	+	+	+	+	+	–	–	–
PR-80	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
PR-81	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
B-SN196T	do.	Costa Rica	+	+	+	+	+	+	+	+	+	–	–	–
H-Knagg's Nursery	do.	Trinidad	+	+	+	+	+	+	++	+	+	–	–	–
J-1	do.	Japan	+	+	+	+	+	+	+	+	+	–	–	–
J-2	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
J-3	Tobacco	do.	+	+	+	+	+	+	+	+	+	–	–	–
J-4	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
J-5	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
J-6	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
ATCC-9910	do.	North Carolina	+	+	+	+	+	+	+	+	+	–	–	–
K-46	do.	do.	+	+	+	+	+	+	++	+	++	–	–	–
K-95	do.	do.	+	+	+	+	+	+	+	+	+	–	–	–
K-113	do.	do.	+	+	+	+	+	+	+	+	++	–	–	–

K-146	Tobacco	North Carolina	+	+	+	+	+	+	+	+	++	−	−	−
K-106	do.	Georgia	+	+	+	+	+	+	+	+	+	−	−	−
K-109	do.	do.	+	+	+	+	+	+	+	+	+	−	−	−
K-105	do.	Florida	+	+	+	+	+	+	+	+	+	−	−	−
K-140	do.	Panama	+	+	+	+	+	+	+	+	++	−	−	−
K-178	do.	Northern Rhodesia	+	+	+	+	+	+	+	+	+	−	−	−
K-3	Potato	North Carolina	+	+	+	+	+	+	+	+	+	−	−	−
K-51	do.	do.	+	+	+	+	+	+	+	+	+	−	−	−
K-56	do.	Israel	+	+	+	++	+	+	+	+	+	−	−	−
K-118	do.	Cyprus	+	+	+	+	+	+	+	+	+	−	−	−
K-197	do.	Kenya	+	+	−	+	+	+	+	+	+	−	−	−
K-240	do.	Colombia	+	+	0	+	+	+	+	+	+	−	−	−
NCPPB-332	do.	Southern Rhodesia	+	+	+	+	+	+	+	+	+	−	−	−
J-8	Eggplant	Japan	+	+	+	+	+	+	+	+	+	−	−	−
J-9	do.	do.	+	+	+	+	+	+	+	+	+	−	−	−
K-82	do.	North Carolina	+	+	+	+	+	+	+	+	+	−	−	−
K-112	do.	do.	+	+	+	+	+	+	+	+	+	−	−	−
K-101	do.	South Carolina	+	+	+	+	+	+	+	+	+	−	−	−
K-199	*Heliconia*, spp.	Costa Rica	+	+	+	++	+	+	+	+	++	−	−	−
K-218	do.	do.	+	+	+	++	+	+	+	+	+	−	−	−
B-312R	do.	do.	+	+	+	++	+	+	++	+	+	−	−	−
B-H249C	do.	Colombia	+	+	+	+	+	+	+	+	+	−	−	−
B-100D	Banana	Costa Rica	+	+	+	++	+	+	++	+	++	−	−	−
B-139B	do.	do.	+	+	+	+	+	+	+	+	+	−	−	−
K-151	Plantain	do.	+	+	+	+	+	+	+	+	++	−	−	−
K-215	*Piper auritum*	do.	+	+	+	++	+	+	+	+	++	−	−	−
K-221	do.	do.	+	+	+	++	+	+	+	+	++	−	−	−
K-217	*Physalis*, spp.	do.	+	+	+	+	+	+	+	+	++	−	−	−
B-PG237	do.	do.	+	+	+	0	+	+	+	+	+	−	−	−
K-156	Dahlia	North Carolina	+	+	+	+	+	+	+	+	+	−	−	−
K-227	do.	do.	+	+	+	++	+	+	+	+	+	−	−	−

TABLE 1.—*Continued*

Culture[1]	Host[2]	Geographic area[3]	Antiserum against culture[4]									Serum controls[5]		
			ATCC-10692	PR-65	K-95	NCPPB 332	K-217	B-PG 237	B-312R	B-139B	B-100D	Gu-1, *Ps. tabaci*	EC-153, *E. carotovora*	Normal rabbit serum
K-30	Peanut	North Carolina	+	+	+	+	+	+	++	+	++	−	−	−
K-183	*Zinnia*, spp.	do.	+	+	+	++	+	+	+	+	+	−	−	−
K-153	*Solanum nigrum*	Costa Rica	+	+	+	+	+	+	+	+	++	−	−	−
K-201	*Eupatorium odoratum*	do.	+	+	+	+	+	+	+	+	+	−	−	−
J-7	Sesame		+	+	+	+	+	+	+	+	+	−	−	−
Other bacterial species:														
Pseudomonas tabaci, Gu-1[6]	Tobacco	Puerto Rico	−	−	−	−	−	−	−	−	−	+	−	−
Erwinia carotovora EC-153[7]	Bell pepper	Mexico	−	−	−	−	−	−	−	−	−	−	++	−

[1] Culture designation—Preceded by letters: K = received from Dr. Arthur Kelman, North Carolina State College, Raleigh, N.C.; ATCC = from American Type Culture Collection, Washington, D.C.; PR = Puerto Rico isolates; B = from Dr. I. W. Buddenhagen, Tela RR. Co., La Lima, Honduras; J = from Dr. Masao Goto, Shizuoka University, Shizuoka-ken, Japan; NCPPB = from National Collection of Plant Pathogenic Bacteria, Harpenden, Herts., England; and H = from Hankey Culture Collection, Trinidad, B.W.I.

[2] Host from which culture was isolated originally.

[3] Area where culture was isolated originally.

[4] + = thin ring of precipitate, formed after about 15 minutes incubation. ++ = thick ring of precipitate, formed usually within 2 to 5 minutes of incubation. − = minus sign indicates a negative reaction. 0 = indicates test was not performed.

[5] Serum controls included antiserum prepared against 2 different bacterial species (*Pseudomonas tabaci* strain Gu-1, isolated from tobacco in 1954 in Puerto Rico, and *Erwinia carotovora*, strain EC-153, received in 1957 from Dr. M. P. Starr, University of California, Davis, Cal.). Normal rabbit serum was also included as a control.

[6] Extract from *Pseudomonas tabaci* culture, used as control.

[7] Extract from *Erwinia carotovora* culture, used as control.

ANTISERA

Nine cultures were selected for preparing antisera. In selecting these cultures several factors such as original host, geographic area, date of isolation, and available data on host range were considered. Following are the culture designations preceded by the name of the host from which the organism was isolated: Tomato—American Type Culture Collection No. 10692 (ATCC-10692), originally Kelman's 3-1A isolate from North Carolina in 1949 and P.R.-65, a 1958 isolate from Puerto Rico; *Physalis* spp.—K-217, a strain pathogenic on Physalis and B-PG-237, a nonpathogenic mutant from a Physalis isolate (*14*); banana—B-139B, banana "fast wilt" strain and B-100D, a banana-distortion strain, both isolated in Costa Rica in 1959 (*14*); *Heliconia* spp.,—B-312R, an atypical strain isolated in Costa Rica in 1959; tobacco—K-95, isolated in North Carolina in 1955; and potato—NCPPB-332 from Southern Rhodesia in 1954.

For the preparation of antisera, cultures were grown in 200 ml. of liquid medium in a rotary shaker for 48 hours according to the method of Hussain and Kelman (*11*). Formalin was then added directly to the liquid cultures to a final concentration of 0.3 percent. Cultures were left at room temperature for 24 hours and then stored as stock suspensions in the refrigerator. For the immunization of rabbits, dilutions of the formolized suspensions were made in saline to a final concentration of approximately 10^7 to 10^8 organisms per milliliter, as determined by McFarland's nephelometric method (*12*). Rabbits were injected intravenously twice weekly on 2 successive days with 1 ml. and 2 ml., respectively, of the bacterial suspensions for a period of 4 weeks. The animals were then bled from the ear vein, the blood was allowed to clot, and the antiserum was obtained by centrifugation. The serum was then distributed aseptically in sterile glass vials and frozen. Twofold dilutions of antiserum were made in normal rabbit serum and the highest dilution which gave a positive ring test with formamide extract of the *Ps. solanacearum* culture used for immunization was taken as the precipitin titer of the serum.

Antisera against a strain of *Pseudomonas tabaci* (Gu-1) (*13*) isolated from tobacco in Puerto Rico in 1954, and against the EC-153 strain of *Erwinia carotovora* received from Dr. M. P. Starr of the University of California, Davis, Calif., in 1957, were similarly prepared to serve as antibacterial serum controls in the tests.

METHOD OF TESTING

Undiluted antiserum against each of the above-mentioned cultures was placed in a number of 6 x 50 mm. tubes by means of thin glass (capillary) pipettes. Approximately one-third of the tube was filled with serum, care-

fully avoiding the formation of air bubbles. Bacterial extracts prepared as described above were then carefully overlaid on the serum so that a clear interface formed between the two liquids. In this test the sharpness of the interface is very clearly outlined because the slightly alkaline extracts (pH 7.2 to 7.4) have a pink color derived from the phenol red indicator they contain, while the serum underneath is an amber to straw color.

After the extracts were overlaid on the serum the tubes were placed in holes of convenient size that had been made previously in a wooden board so that the tubes would fit snugly. The tubes were allowed to stand at room temperature for a maximum of 1 hour. The serum-extract interface was observed at intervals of 2, 5, 10, 15, 30, and 60 minutes. A thick, well-defined white ring of precipitate appearing usually within 2 to 5 minutes was recorded as ++ (strongly positive). A thin, but still well-defined ring of precipitate was recorded as + (positive); this usually took more than 15 minutes to appear. When no precipitate appeared within the 60-minute period of observation reactions were recorded as negative (—).

TESTS WITH PLANT EXUDATES

In order to evaluate the practical application of the above-described ring test directly on diseased plants, formamide extracts were made of exudate obtained from stem pieces of tomato (*Lycopersicon esculentum* Mill. var. Marglobe and Rutgers) from plants which had been infected previously, by the stem-puncture technique (*3*), with virulent cultures of PR-65, Knagg's Nursery, and ATCC-10692. Exudate was collected from several stem pieces of plants separately inoculated with each strain until about 0.3 to 0.5 ml. of exudate was pooled in a conical 12-ml. centrifuge tube. This exudate was then processed by the formamide method and the extracts thus obtained were tested serologically as described above. A limited number of tests was carried out in the same manner with field-infected tomato plants. Untreated sap from healthy tomato plants was collected and tested serologically as a control.

RESULTS

Results of ring tests performed with antisera against 9 cultures of *Ps. solanacearum* are shown in table 1. It can be seen that all formamide extracts from 59 cultures of this organism reacted with all 9 antisera. There were only 2 negative reactions in a total of 529 tests performed with the *Ps. solanacearum* cultures and antisera. This means that 99.7 percent of the tests were positive and only 0.3 percent negative. Further, it may be seen that not a single extract from *Ps. solanacearum* cultures reacted with antiserum to *Ps. tabaci* (Gu-1 strain), with antiserum to strain EC-153 of *E. carotovora*, or with normal rabbit serum. Thus the formamide test seems

to be a quick, reliable method for identification of *Ps. solanacearum*. On the other hand the test does not differentiate between strains of *Ps. solanacearum* that may be separated on the basis of host range and cultural methods (*14*, *15*, *16*).

Furthermore all formamide extracts made with exudates collected from diseased tomato plants, naturally or artifically infected, as described under Methods, gave a positive ring test. Antisera to strains PR-65, Knagg's Nursery, and ATCC-10692 were used in the tests with exudates. The extracts from exudates did not react with antiserum to either *Ps. tabaci*, *E. carotovora*, or normal rabbit serum. Control tests with sap from healthy tomato plants were negative with all sera.

Preliminary tests with three antisera against a single *Ps. solanacearum* culture had shown that sera of sufficiently high precipitin titer should be used for the formamide tests. Comparative tests with the three antisera demonstrated that a serum with a titer of $1/4$ or less gave a delayed positive (+) or sometimes a negative reaction. Therefore, an antiserum had to have a titer of $1/4$ to $1/32$ against the organism used for its production in rabbits, before use in tests with all the 59 extracts from different *Ps. solanacearum* cultures.

Finally, it should be pointed out that, although none of the *Ps. solanacearum* extracts reacted with antiserum to either *Ps. tabaci* or *E. carotovora*, an extract from each of these species reacted only with its own antiserum. This suggests use of the test herein described with other species of *Pseudomonas* and *Erwinia* in attempts to identify species belonging to these genera.

SUMMARY

1. Fifty-nine extracts from cultures of *Pseudomonas solanacearum* from various countries and plant sources were tested serologically by Fuller's formamide method (ring test) with 9 antisera for different strains of the species. More than 99 percent of the tests were positive.

2. Exudate from stem pieces of tomato plants either naturally or artificially infected with *Ps. solanacearum* and showing symptoms of bacterial wilt was collected. Formamide extracts from the exudates gave a positive reaction with antisera for *Ps. solanacearum*.

3. None of the extracts in 1 or 2 above reacted with antiserum for two other bacterial species or with normal rabbit serum.

4. Use of the test in rapid serological identification of *Ps. solanacearum* as well as other phytopathogenic bacteria is suggested.

RESUMEN

1. Cincuenta y nueve extractos de cultivos de *Pseudomonas solanacearum* obtenidos de diversas plantas hospedadoras en varios países, se probaron

serológicamente usando el método de formamida de Fuller (prueba del precipitado en forma de anillo). Para estas pruebas se usaron 9 antisueros preparados contra igual número de estirpes del *Ps. solanacearum*. Más del 99 por ciento de las pruebas fueron positivas.

2. Se recogieron exudados de pedazos del tallo de plantas de tomate, que habían sido infectadas artificialmente por inoculación con el *Ps. solanacearum* y de plantas de tomate traídas del campo con marchitez bacteriana. Extractos formamídicos de estos exudados dieron una reacción serológica positiva con antisueros contra el *Ps. solanacearum*.

3. Ninguno de los extractos mencionados arriba en 1 y 2 reaccionó con antisuero para otras dos especies bacterianas, ni con suero de conejos normales.

4. Se sugiere el uso de la prueba mencionada en la identificación serológica rápida del *Ps. solanacearum* y también de otras especies de bacterias fitopatógenas.

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