

Section 8

Culture media for *Ralstonia solanacearum* Isolation, identification and maintenance

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The bacterium *Pseudomonas solanacearum* E. F. Smith multiplies readily in its hosts, but it is slower growing in vitro than some other bacterial pathogens and many plant and soil saprophytic organisms. It is thus difficult for inexperienced researchers to isolate. In culture, its rate of mutation to an avirulent type can be very rapid, and only storage in water is practical. Specialized media are required to classify *P. solanacearum* into biovars (Bvs) and phenotypic strains of By 2. These methods are presented here, along with the information needed to interpret the use of their results, plus host range, to establish classification of this bacterium.

A. KELMAN'S TZC AGAR (Kelman, 1954): Useful for distinguishing *P. solanacearum* among other

bacteria during isolation, and for distinguishing virulent (wild type) colonies from avirulent mutant ones during purification of cultures.

TZC stock solution: Dissolve 1 g of 2, 3, 5 triphenyl tetrazolium chloride (TZC) in 100 ml of distilled water, place in a light-proof capped bottle, and autoclave for only 8 mm. or sterilize by filtration. Store refrigerated.

Basal medium:

Dextrose	10 g (or 2.5 g) *
Peptone	10g
Casamino acids (Difco)	1 g
Agar	18g
Water (distilled)	1000 ml

Modification by reducing the amount to 2.5 g results in a better growth rate, especially of the potato strain (biovar 2-A = race 3). Sucrose can be used as a substitute (French and Hebert, 1980).

This medium, modified by the exclusion of TZC, is useful for multiplication of inoculum free of formazan pigment (which is slightly bacteriostatic).

Preparation for plating: The basal medium can be autoclaved and stored, then melted as needed. To each liter of the melted, somewhat cooled agar basal medium, add 5 ml of the TZC solution to give a final concentration of 0.005% (aliquots of 200 ml are recommended for ease of handling; to these, 1 ml of TZC solution is added).

Plating and storing: Pour about 20 ml per petri plate. When the gel is set, store inverted. Keep 1-2 days before use to permit surface drying (longer storage may result in poor bacterial growth).

B. SOIL ISOLATION MEDIUM SMSA-E. To isolate from soil it is best to modify Kelman's TZC agar. A series of modifications have been proposed, and we present another slight modification of the last published semi-selective medium, South Africa (SMSA) by Englebrecht (1994), as developed by J. Elphinstone and so far unpublished.

Bactopeptone (Difco)	10 g
Glycerol	5 ml
Casamino acids (Difco)	1 g
Bacto agar (Difco)	15 g
Distilled water	1000 ml

Sterilize for 15 minutes at 121 °C.

Add to 250 ml of melted medium at a temperature of 50 °C:

1% Polyimyxin B sulphate (Sigma)	2.5 ml
(Final, conc., 100 ppm)	
1% Crystal violet	125 ul
(Final conc., 5 ppm)	
1% Tetrazolium salts (Sigma)	1.25 ml
(Final conc., 50 ppm)	
1% Bacitracin (Sigma)	625 ul
(Final conc., 25 ppm)	
0.1% Penicillin (Sigma)	1.25 ul
(Final conc. 0.5 ppm)	
1% Chloramphenicol (Sigma)	1.25 ul
(Final conc., 5 ppm)	

When additional inhibition of fungal contaminants or soil inhabitants is desired add:

1% Cycloheximide (Sigma)	2.5 ml
(Final conc., '100 ppm).	

C CARBOHYDRATE MEDIA FOR BIOVAR (By) DETERMINATION (Hayward, 1964,1976): The determination of Bvs of *P solanacearum* is based on the utilization of the disaccharides cellobiose, lactose and maltose, and the oxidation of the hexose alcohols dulcitol, mannitol and sorbitol. Because the costlier cellobiose and dulcitol are not essential to classify the presently known strains, they need not be used in the initial test of classification.

However, they should be for subsequent confirmation, especially prior to publication of results.

Basal medium:

Ammonium dihydrogen phosphate	
NH ₄ H ₂ PO ₄	1.0 g
Potassium chloride (KCl)	0.2 g
Magnesium sulphate (MgSO ₄ .7H ₂ O)	0.2 g
Peptone	1.0 g
Bromothymol blue	0.03 g
Agar	3.0 g

Distilled water

1000 ml

Adjust the final pH of the medium to 7.0-7.1 (an olivaceous green color) by drop wise addition of 40% w/v NaOH solution. Melt the agar by steaming or heating in a double boiler, with constant stirring (or weigh the agar per flask and add dry prior to dispensing the rest of the medium). Dispense 90 ml aliquots into flasks and autoclave at 121 C for 20 minutes.

Prepare 10% solutions of the carbohydrates in 10 ml amounts. Heating may be needed to dissolve these sugars. The hexose alcohols are relatively heat-stable and can be autoclaved at 110 °C for 20 minutes. The disaccharides are heat-labile and should be sterilized by filtration (Seitz or 0.22 micron Millipore membrane) into pre-sterilized test tubes or small flasks or by Tyndalization (steaming at 100 °C for 20 minutes on 3 successive days).

To the melted basal medium cooled to about 60 °C, add the carbohydrate solution.

Dispense 34 ml into previously sterilized test tubes (150 mm x 10 mini size or similar) using a sterile cotton stopped pipette.

Prepare about 4 ml of inoculum suspension in distilled water (O.D. = +0.1 at 600 nm) from 2-day-old cultures. With a sterile Pasteur pipette, add 0.1 ml to each tube. Use 2 replicates and a control with no carbohydrate. Incubate at 30°C and examine at 3, 7 and 14 days for change to acid pH (yellow color) from the top downward. Hexose alcohols usually take 3-5 days; disaccharides may take a few days longer.

Bv determination is based on the following:

- Bv 1 = utilization and oxidation tests negative.
- Bv2 = utilizes disaccharides-, does not oxidize the alcohols.
- By 3 = utilization and oxidation both positive.
- Bv4 = utilization of disaccharides negative-, oxidizes the hexose alcohols.
- By 5 = utilizes disaccharides-, oxidizes mannitol but not dulcitol or sorbitol (He et al., 1993).

Table 1 shows this information in detail. Bvs and races correlate in the following manner: Bvs 1, 3 and 4, if isolated from non musaceous hosts, are race 1; Dv 2 is race 3 (only the cool-temperature strains of Dv 2-A); musaceous host isolates that cause bacterial wilt or "moko disease" are race 2, Bvs 1 or 3 (Buddenhagen and Kelan, 1964); By 5 (isolated from mulberry) are race 4 (He et al. 1983) (see Table 2).

D. MEDIA FOR DIFFERENTIATION OF *P. solanacearum* By 2 phenotypes 2-A and 2-T (Hayward, 1994; Hayward et al., 1989, 1991). Using the same basal medium (90 ml) for Dv determination, add 10 ml of a 10% solution of either D (-) ribose or U (+) trehalose to determine whether oxidation results in production of

acid, which changes the medium from green to yellow. Likewise, add 10 ml of a solution of L F) tryptophan or L (±) tartrate to determine if these are utilized, resulting in a change in color from green to blue (alkalinization). Table 3 lists these tests along with the characteristics of pathogenicity to potato (French et al., 1993; Mann, 1992) and the degradation of pectates (additional tests that can be performed).

Table 1. Classification of *Ralstonia solanacearum* into biovars based on the ability to utilize disaccharides and oxidize hexose alcohols producing acid when positive (+).

Physiological tests	Biovars				
	1	2	3	4	5
Utilization of disaccharides					
Cellobiose	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Oxidation of alcohols					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

Table 2. Definition of races of *Ralstonia solanacearum* by host range, and biovars determined in each.

Race	Natural hosts	Biovars
1	Many Solanaceae, some diploid bananas, numerous other crops and weeds in many families	1, 3 or 4
2	Triploid bananas, certain heliconias	1 or 3
3	Potato, tomato, and rarely, a few other hosts	2
4	Mulberry	5

E. USE OF MICROTITER PLATES. A more efficient alternative to the conventional use of test tubes for these physiological tests for Bv and phenotype determination, is to utilize microtitration 96-well Linbro/Titertek polystyrene sterile plates with covers (Flow Laboratories, Mc Lean Virginia 22102 U.S.A.).

Table 3. Differentiation of *Ralstonia solanacearum* biovar 2 isolates into the metabolically less active Andean phenotype Bv 2-A/race 3 and the metabolically more active tropical lowlands Bv 2-T.

Test	Bv 2-A	Bv 2-T
Acid from D (-) ribose	-	+
Acid from D (+) trehalose	-	+
Utilization of L (-) tryptophan	-	+
Utilization of L (+) tartrate	-	+
Degradation of pectates	+	+++
Pathogenicity to potato [25°C] ¹	High	Low

¹/French et al., 1993; P. Aley and E. R. French, unpublished.

Note. This publication is also available in Spanish as a fascicle for a training course at the International Potato Center (CIP): *Medios de cultivo para el aislamiento, mantenimiento e identificación de Pseudomonas solanacearum. Manual de Capacitación 4: Enfermedades de la Papa: .1. Enfermedades Bacterianas. CIP, Lima-Perú. 5 pp.*

Their use economizes medium constituents and substrates, facilitates filling, handling and reading, results develop more rapidly and recognition of patterns is easier (Figures '1 and 2). Well capacities are about 0.35 ml, so only 0.15 to 0.20 ml of the prepared media is pipetted into each well, and one drop (30 to 40 microliter) of a bacterial suspension (0.0= ±0.05) is added with a Pasteur pipette. Use 3 replications. Cover plates with their lids, seal them with parafilm or plastic wrap, then place in a plastic bag and seal it. Bv determination reactions are completed in 3 to 6 days, but those for phenotypes of Bv 2 take '13 days for ribose (Hayward et al., 1989).

F. STORAGE IN WATER (Kelman and Person, 1961): Cultures are maintained in their wild-type form best when stored in distilled or deionized water (or tap water boiled to eliminate chlorine) in screw cap test tubes. Two loopfulls of bacteria from a composite of about six individual 2-day-old colonies grown on Kelmans TZC agar (or the same medium without TZC when an isolate is sensitive to the formazan pigment it produces from TZC) are transferred to 5 ml of sterile water. These should be streaked on Kelmans TZC agar every 6 months and purified if mutants are numerous.

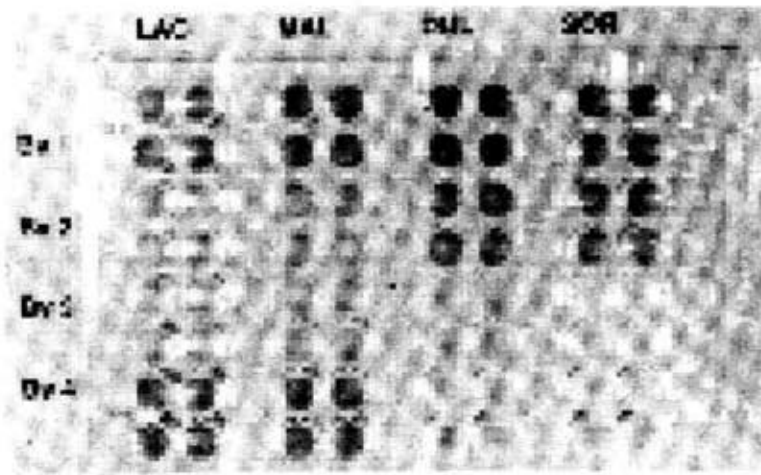


Figure 1. Results and pattern visualization, of physiological tests in microtitration plates, for the determination of biovars (Bvs) of *R. solanacearum* using one isolate of each Bv 1 through 4.

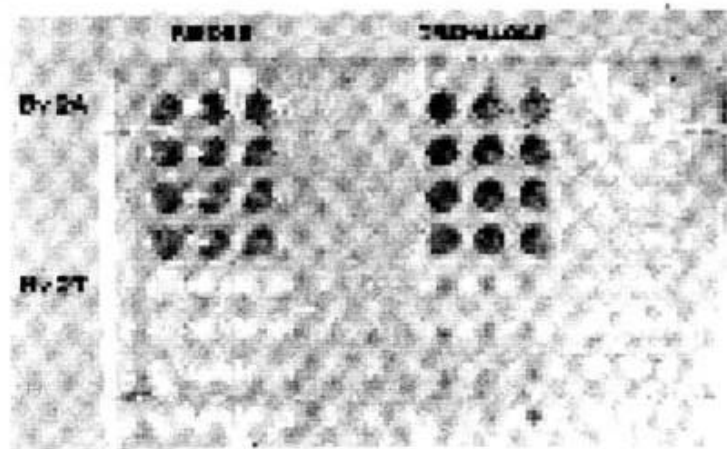


Figure 2 Test for differentiation of phenotypes A and T of biovar 2 of *R. solanacearum* as visualized in a microtitration plate.

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