A Selective Medium for the Isolation and Quantification of Pseudomonas solanacearum from Soil

W. C. Nesmith and S. F. Jenkins, Jr.

Assistant professor, Department of Plant Pathology, Kansas State University, Manhattan, KS 66506; and professor, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27607.

Portion of a Ph.D. thesis submitted by the senior author to the Graduate School, North Carolina State University, Raleigh, NC.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

Journal Series Paper 5399 of the North Carolina Agricultural Experiment Station, Raleigh, NC.

Accepted for publication 14 August 1978.

ABSTRACT

NESMITH, W. C., and S. F. JENKINS, JR. 1979. A selective medium for the isolation and quantification of Pseudomonas solanacearum from soil. Phytopathology 69:182-185.

A new selective medium was developed for monitoring populations of *Pseudomonas solanacearum* in naturally and artificially infested soils. The basal medium was derived by modification of a standard triphenyl tetrazolium chloride medium (TTC). The final selective medium is prepared by adding antimicrobial compounds at the time of use. The basal medium could be stored for 60 days at room temperature and the final selective medium for 10 days at 4 C without noticeable alteration of its efficiency. Fluidal and butyrous colonies of most strains of *P. solanacearum* tested on the selective medium were similar in appearance to those observed on TTC medium. With the selective medium, *P. solanacearum* could be detected easily when as few as 100 colony forming units were added per gram of oven dry soil, but meaningful quantification was not possible with less than 10^3 cells/g. Recovery of *P. solanacearum* was dependent on soil moisture, soil type, and bacterial strain. Most strains grew well on the selective medium and were recovered easily from soil, but some were partially or completely inhibited. Growth of other soilborne organisms seldom was observed at soil dilutions greater than 10^{-3}.

Knowledge of the ecology of *Pseudomonas solanacearum* E. F. Smith in soil is lacking because adequate techniques are not available to monitor the pathogen in soil (17). Survival of *P. solanacearum* in soil has been determined indirectly with the use of indicator plants to measure inoculum densities in soil (3,10,17). Serological techniques were used to detect 2.5×10^4 cells/ml soil (8), but live cells were not distinguished from dead cells, or virulent from avirulent ones. Media have been developed for isolation and quantification of *P. solanacearum* from soil (9,14), but none has gained wide acceptance. Potato dextrose agar with crystal violet (PDACV) was used to recover 10^4 cells of *P. solanacearum* per milliliter of soil suspensions (14). A selective medium was developed that reduced the background popuation by 90% but recovery of *P. solanacearum* below 10^5 cells/g oven dry soil was highly variable (9). Similar levels of the pathogen in soil also were detected with 2,3,5-triphenyl tetrazolium chloride (TTC) medium (15) and Drigalski's medium (18). A major limitation of these media is that low population densities of the pathogen are not detectable. None of these media was satisfactory in preliminary tests for selective isolation of *P. solanacearum* from North Carolina soil (13).

To study the ecology and survival of the bacterium in suppressive and compatible North Carolina soils, a technique was required to monitor low inoculum densities of *P. solanacearum*. An evaluation of available selective media and the development of a new selective medium for *P. solanacearum* in soil are described.

MATERIALS AND METHODS

In order to find suitable existing media for monitoring growth and recovery of *P. solanacearum* from North Carolina soil, TTC medium was compared with Okabe's PDACV (14) and the series of media described by Karganilla and Buddenhagen (9): Buddenhagen and Berger medium (BBM), experimental basal medium (EBM), and final modified basal medium (MBM), the final three media amended with recommended antimicrobial compounds. The principle of selective exclusion (19) was used to develop new media. Antimicrobial agents that inhibited soil microbes but exerted little effect on *P. solanacearum* were used to amend TTC medium, which is known to support excellent growth of the pathogen.

Strain K-60 (11) of *P. solanacearum* was used throughout these experiments, unless otherwise indicated, because it is similar to natural strains in North Carolina. Cultures were maintained in sterile water at room temperature (17). Single fluidal colonies were selected from TTC medium (11) that had been inoculated with cells from the stock cultures and incubated at 30 C for 48 hr. Bacterial suspensions containing 5×10^6 cells/ml (1) were made from the selected colonies and placed in sterile deionized water. Viable cell counts were made prior to each assay by plating on TTC medium.

Sensitivity of *P. solanacearum* to antimicrobial compounds was evaluated with paper disks impregnated with various materials. Disks dipped in solutions containing 10, 50, 100, or 500 μg/ml of the test compounds were placed on plates of basal TTC medium (without tetrazolium chloride) previously seeded with 10^8 cells of *P. solanacearum*. Compounds found to be noninhibitory to *P. solanacearum* by the paper disk assay were added individually to TTC to compare colony appearance and per cent recovery with unamended TTC. The same concentration of antimicrobial compounds was used in both assays. Each plate was seeded with 50 cells.

Initial assays used to screen available media consisted of plating 0.1 ml of bacterial or soil suspensions containing *P. solanacearum* on various media and incubating plates at 30 C for 48 hr. Tenfold soil dilutions from 10^{-1} to 10^{-7} were made and 5 min prior to plating 1 ml of a bacterial suspension (5,000 viable cells/ml) was added to 9 ml of each soil dilution. Controls contained 9 ml of sterile water plus 1 ml of the bacterial suspension. Later assays used to screen the preliminary and the final selective medium consisted of infesting 70 soil lots from 12 soils at inoculum densities from 50 to 5×10^7 cells/g oven dry soil. These soils included a variety of soil textures from clays to sands selected from the following piedmont and coastal plains series: Arapahoe, Appling, Cecil, Creedmoor, Goldsboro, Norfolk, Helenes, Portsmouth, Worsham, Wilkes, White Stores, and Vance. Five minutes after infestation, a 10-g soil sample was diluted ($\log_{10}$ dilution series) and 0.1-ml aliquants were

00031-949X/79/000030$03.00/0

plated on the media being evaluated. Field soils from nurseries, home gardens, and commercial plantings also were monitored for natural infestations.

RESULTS

Evaluation of available media. Recovery of *P. solanacearum* (isolate K-60) from artificially infested soil solutions varied with the different media used (Table 1). Colonies which could be differentiated most easily from other soil microbes developed on TTC medium. Abundant growth occurred on PDACV, BBM (with glucose), and EBM (with sucrose). No growth was observed on MBM or SM containing mannitol as the carbon source. The antimicrobial compounds used by Karganilla and Buddenhagen (9) were highly inhibitory to K-60; colonies were smaller, less fluidal, similar to the butyrous avirulent mutant, fewer in number, and were difficult to distinguish from those of other soil bacteria. The background population of soil organisms was reduced to 10^5 cells/g oven dry soil and *P. solanacearum* was not detected below this concentration. Recovery of K-60 improved when TTC medium was amended with the same antimicrobial compounds but the colonies of isolate K-60 were atypical. The PDACV medium was not selective in these tests and was not considered further. Crystal-violet pectate medium (4) could be used to isolate the pathogen from soil, but other pseudomonads caused similar shallow pits in the surface thus limiting its use.

Development of new medium. Our final selective medium (FSM) was prepared as follows. Stock solutions of each chemical were prepared with deionized water. The final concentration of each salt per liter of medium was: phosphate buffer (K_2HPO_4, 1.18 g and KH_2PO_4, 0.44 g); $(NH_4)_2SO_4$, 1.32 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 1.5 mg; $ZnSO_4 \cdot 7H_2O$, 1.6 mg; ferric citrate ($FeC_6H_5O_7 \cdot 5H_2O$) 3.0 mg plus citric acid, 1.9 mg. The final concentrations of each antimicrobial compound was: penicillin G (1 ppm), tyrothricin (20 ppm), chloromycetin [Chloramphenicol] (5 ppm), 2, 3, 5 triphenyl tetrazolium chloride (500 ppm), polymyxin B (100 ppm), all from Sigma Chemical Co., St. Louis, MO 63178; vancomycin (10 ppm) and bacitracin (50 ppm), both from Eli Lilly and Co., Indianapolis, IN 46206; benomyl (Benlate 50 W, 500 ppm) and chloroneb (Tersan SP 65 W, 100 ppm), both from E. I. duPont de Nemours, Wilmington, DE 19898; cycloheximide (Actidione, 50 ppm) from Nutritional Biochemicals, Cleveland, OH 48128; pentachloronitrobenzene (Terraclor 75 W 30 ppm) from Olin Corp., Little Rock, AK 77203; pimaricin (20 ppm) from Gist-Brocades NV, Industrial Products Divisions, Delft, Holland; and dichloran (Botran 75 W, 100 ppm) from the Upjohn Co., Kalamazoo, MI 49001. Each compound was dissolved in and sterilized with 1 ml 70% ethanol for 30 min, then added to sterile deionized water and stored in separate, stoppered serum bottles at 4 C. The tetrazolium chloride was autoclaved for 7 min at 1.05 kg-force/cm^2. Stocks of chemicals and antimicrobial compounds were not used after 90 days. The basal medium (BM) was made by adding appropriate amounts of each stock salt solution (in the order listed above) to an Erlenmeyer flask (precipitate may form) followed by 1 L of boiling deionized water. Dextrose (4 g), peptone (10 g), yeast extract (1 g), casamino acids (1 g), and agar (18 g) were added slowly while stirring until all ingredients were dissolved. The BM was dispensed into 200-ml bottles, capped, and autoclaved for 15 min at 1.05 kg-force/cm^2. The BM could be stored for 60 days without noticeable effects. The selective medium was assembled by melting the BM, cooled to 45 C, and the antimicrobial compounds were added aseptically one at a time. The medium was dispensed into petri plates, dried for 24 hr at 30 C, and 0.1 ml of a dilute soil suspension was placed on the surface of the dry medium and spread uniformly with a glass rod. Plates were incubated for 48 hr at 32 to 34 C in an inverted position. The FSM could be stored about 10 days at 4 C without noticeable alteration of its efficiency.

Evaluation of the selective medium. Colonies of *P. solanacearum* on the final selective medium (FSM) were similar in appearance to those observed on TTC. Fluidal colonies were raised, irregularly round, and mucoid with swirled red formazan patterns. Butyrous colonies were smaller, round, with a clear to white halo surrounding a dark red center. These characteristics were easier to observe by viewing the colonies from the bottom under oblique lighting. Fluidal colonies were identified easily but those of the butyrous, avirulent type were difficult to distinguish (particularly at low dilutions) from other soil pseudomonads. In a pathogenicity test, 100% of the virulent colony types sampled (30 colonies) were virulent to Rutgers tomato and Broadleaf Hicks tobacco and were similar to isolate K-60 according to Hayward's biochemical tests (7). Conversely, only 58% of the 30 butyrous colonies selected were confirmed as *P. solanacearum* following serological tests, a tobacco hypersensitivity test (12), and Hayward's biochemical tests. Butyrous colonies, especially from poorly drained soils, were difficult to identify at dilutions below 10^{-4} because other soil pseudomonads were abundant.

When added at concentrations greater than 10^3 colonies/g oven dry soil, isolate K-60 was recovered easily from the 12 soils tested with FSM. Recovery (when 2.5×10^5 colonies/g oven dry soil were added) from different soil types and different soil moisture levels (flooded, −0.33, −0.50, −1.0, −5.0, and −15 bars soil water potential) was 68–100% with a mean of 88.7% in 1,200 samples. Percentage of recovery was highest from soils with water potentials ranging from − 0.5 to −5 bars and from lighter textured soils, that are low in clay and organic matter. Recovery of *P. solanacearum* above 10^4 cells/g of oven dry soil ranged from 51–92% and averaged 84% compared with 59% recovery (range 12–78%) at 10^3. At concentrations of 10^2 cells/g oven dry soil or lower, *P. solanacearum* was detectable but not quantifiable because of high variation.

Thirty-two strains representing all four biotypes (7) and races (3)

TABLE 1. Comparison of different media for the selective isolation of *Pseudomonas solanacearum* strain K-60 from soil

Media[a]	Growth of *P. solanacearum*	Percentage recovery at dilutions[b]				Soil microbe inhibition
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	
TTC	Excellent	0	0	12	96	Poor
PDACV	Good	0	0	28	100	Poor
BBM	Good	0	0	6	86	None
EBM	Good	0	0	16	94	None
MEM	None	0	0	0	0	Poor
BBM + K&BAC	Atypical	0	8	42	86	Good
EBM + K&BAC	Atypical	0	22	60	90	Good
SM	None	0	0	0	0	Excellent
TTC + K&BAC	Atypical	0	46	74	92	Excellent

[a]Abbreviations: TTC = tetrazolium chloride medium (11), Okabe's PDACV = potato dextrose agar with crystal violet (14), Karganilla and Buddenhagen's series (9): BBM = Buddenhagen and Berger basal medium, EBM = experimental basal medium, MBM = final modified basal medium, and SM = MBM amended with antimicrobial compounds. K&BAC = antimicrobial compounds used by Karganilla and Buddenhagen (9).

[b]Dilutions were made from soils infested with 50×10^7, 10^6, 10^5, or 10^4 cells of *P. solanacearum*/g 5 min prior to plating 0.1 ml on poured agar plates of each medium. All plates were incubated 48 hr at 30 C.

of *P. solanacearum* produced fluidal type colonies on the BM and FSM. Some strains, however, were either completely or partially inhibited on the FSM, with only butyrous-type colonies developing, although the strains grew well on the BM. These strains were Thurston's T-238 (race 3, biotype 2, from potato in Colombia), Buddenhagen's B-257 (race 3, biotype 2, from *Solanum torvum* in Colombia), Zehr's DA-1 (race 1, biotype 3, from eggplant in The Philippine Islands), Nesmith's N-1 (race 1, biotype 1, from potato in North Carolina), Nesmith's N-11 (race 1, biotype 1, from soil in North Carolina), and Hayward's H-017 (race 3, biotype 2, from Australia). This atypical growth did not correlate with race or biotype. Most strains were recovered readily at concentrations of 10^5 cells/g oven dry soil or greater; but several were more difficult to recover than K-60 at 10^4 cells/g oven dry soil or lower. Recognition of *P. solanacearum* from other soil bacteria was not difficult if pure cultures of tested strains grown on FSM as controls were available for frequent referral.

When naturally-infested soils from nurseries and home gardens were assayed with FSM, 5.6×10^8 cells/g oven dry soil were detected in soil samples collected near diseased plants. With the FSM, the pathogen could be isolated easily from infected tobacco, tomato, eggplant, and Datura stems, and tap roots, except where soft rot had developed. The pathogen was difficult to detect in rhizoplane soil of these plants because other Gram-negative soil bacteria present at $\geqslant 10^4$ cells/g oven dry soil overgrew the assay plates within 24 hr.

Direct plating of soil or diseased tissue on FSM often was not successful because numerous colonies of antagonistic bacteria covered the plates. Direct plating was possible when the soil had dried enough to allow the soil to be sprinkled uniformly over the plate.

DISCUSSION

We know very little about the ecology of *P. solanacearum* because techniques to study the pathogen in soil free of its host have developed slowly (2,3,5,10,16). The selective media previously described (4,9,14) for detection and isolation of *P. solanacearum* were not effective with the North Carolina soils tested.

The principle of selective exclusion was used in developing FSM (19). This was accomplished by choosing a basal medium (BM) that encouraged growth of the pathogen and supplementing it with antimicrobial compounds to inhibit other organisms. Penicillin G, tetrazolium chloride, and vancomycin were used as the principal inhibitors of Gram-positive bacteria; bacitracin, tyrothricin, and chloromycetin were useful as broad spectrum antibiotics. Many Gram-negative soil-borne bacteria are also sensitive to tetrazolium chloride and this compound (at 4,000 ppm) has been used as the major bacterial inhibitor in a selective medium for *P. solanacearum* (6). However, isolate K-60 and many North Carolina strains were sensitive to TTC concentrations higher than 500 ppm during our preliminary tests. The use of yeast extract in the FSM and an elemental stock solution favored the growth of bacteria, especially *P. solanacearum*, and the colonies could be recognized within 36 to 48 hr, instead of the 72 hr required previously. Attempts were made to make the medium synthetic by selecting carbon and nitrogen sources to replace peptone, but none was found that did not make identification of *P. solanacearum* more difficult.

The major groups of soil microbes that grew on the FSM were the Gram-negative pseudomonads and actinomycetes. Strains from both groups may be antagonistic to *P. solanacearum*. The C:N ratio was changed by reducing dextrose to 4 g/L, thus reducing the numbers and growth rates of actinomycetes on dilution plates. All attempts to reduce bacteria by increasing the concentration of broad spectrum antibiotics also reduced recovery of the pathogen. The use of dry plates and incubation at a higher temperature (32–34 C) reduced the residual bacterial population.

Usefulness of FSM in the selective isolation of *P. solanacearum* from soil was dependent upon isolate, soil, and microflora involved. All strains we tested grew on BM but some were inhibited on FSM. Because soils are heterogeneous and possess a diverse microflora, the FSM should be tested under local conditions before undertaking large scale experiments.

The FSM may have wide application in the study of *P. solanacearum* in soil. Low populations of a variety of strains of the pathogen can be detected and counted directly from different soils. A major advantage of FSM is that colonies of the pathogen are similar to those observed on TTC (11), a medium which is used world-wide. It would also appear that FSM would be helpful in studying inoculum density, population dynamics, survival, and saprophytic behavior of *P. solanacearum* in soil. An obvious disadvantage of this medium is the large number of antimicrobial compounds and salts that it contains. However, results indicated that all compounds were required for isolation from the agricultural soils tested. In soil with less microbial activity, it may be possible to eliminate some compounds. Results were more consistent and *P. solanacearum* was more easily recognized when all salts were used than when none of them were present.

LITERATURE CITED

1. BREED, R. S., and J. D. BREW. 1916. Counting bacteria by means of the microscope. N. Y. Agric. Exp. Stn. Bull. 49. 31 p.
2. BUDDENHAGEN, I. W. 1967. The relation of plant-pathogenic bacteria to the soil. Pages 269-289 *in* K. F. Baker and W. C. Snyder, eds. Ecology of Soil-Borne Plant Pathogens. Univ. Calif. Press, Berkeley. 571 p.
3. BUDDENHAGEN, I. W., and A. KELMAN. 1964. Biological and physiological aspects of bacterial wilt caused by Pseudomonas solanacearum. Annu. Rev. Phytopathol. 2:203-230.
4. CUPPELS, D., and A. KELMAN. 1974. Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. Phytopathology 64:468-475.
5. GRAY, T. R. G., and S. T. WILLIAMS. 1971. Soil Micro-organisms. Oliver and Boyd. Edinburgh, Scotland. 240 p.
6. HARRIS, D. C. 1976. Media for estimating a strain of Pseudomonas solanacearum in Kenyan soils by the dilution plate technique. Pages 148-150 *in* L. Sequeria and A. Kelman, eds. Proceedings 1st International Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt Caused by Pseudomonas solanacearum. 18–23 July 1976, Raleigh, NC. 166 p.
7. HAYWARD, A. C. 1976. Systematics and relationships of Pseudomonas solanacearum. Pages 6-21 *in* L. Sequeria and A. Kelman, eds. Proceedings 1st International Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt Caused by Pseudomonas solanacearum. 18–23 July 1976, Raleigh, NC. 166 p.
8. JENKINS, S. F., JR., D. J. MORTON, and P. D. DUKES. 1967. Comparison of techniques for detection of Pseudomonas solanacearum in artificially infested soils. Phytopathology 57:25-27.
9. KARGANILLA, A. D., and I. W. BUDDENHAGEN. 1972. Development of a selective medium for Pseudomonas solanacearum. Phytopathology 62:1373-1376.
10. KELMAN, A. 1953. The bacterial wilt caused by Pseudomonas solanacearum. N. C. Agric. Exp. Stn. Bull. 99:1-194.
11. KELMAN, A. 1954. The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium. Phytopathology 44:693-695.
12. KLEMENT, Z. 1963. Methods for the rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 188:479-480.
13. NESMITH, W. C., and S. F. JENKINS, JR. 1976. Selective medium for isolation of Pseudomonas solanacearum from soil. Page 121 *in* L. Sequeria and A. Kelman, eds. Proceedings 1st International Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt Caused by Pseudomonas solanacearum. 166 p.
14. OKABE, N. 1969. Population changes of Pseudomonas solanacearum and soil microorganisms in artificially infested natural field soils. Bull. Fac. Agric. Shizuake Univ. 19:1-29.
15. RAMOS, H. 1976. Comparison of survival of two Pseudomonas solanacearum strains in soil columns under constant perfusion and in field plots devoid of host cover. Pages 123-131 *in* L. Sequeira and A. Kelman, eds. Proceedings 1st International Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt Caused by Pseudomonas solanacearum. 18–23 July 1976, Raleigh, NC. 166 p.
16. SCHUSTER, M. L., and D. F. COYNE. 1975. Survival factors of plant pathogenic bacteria. Neb. Agric. Res. Bull. 268:1-53.
17. SEQUEIRA, L., and A. KELMAN, eds. 1976. Proceedings 1st International Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt Caused by Pseudomonas solanacearum.

Raleigh, NC. 18–23 July 1976. 166 p.
18. TANAKA, Y. N., and N. NODA. 1973. Studies on the factors affecting survival of Pseudomonas solanacearum, the causal agent of tobacco wilt disease. Bull. Okayama Tob. Ext. Stn. 32:81-93.
19. TSAO, P. H. 1970. Selective media for isolation of pathogenic fungi. Annu. Rev. Phytopathol. 8:157-186.