Techniques

Bacteriocin Production and Semiselective Medium for Detection, Isolation, and Quantification of *Pseudomonas solanacearum* in Soil

Wen-Yen Chen and Eddie Echandi

Graduate research assistant and professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27650.
Paper 6821 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.
The use of trade names in this publication does not imply endorsement by the N. C. Agricultural Research Service of the product named nor criticism of similar ones not mentioned.
Accepted for publication 18 June 1981.

ABSTRACT

Chen, W.-Y., and Echandi, E. 1982. Bacteriocin production and semiselective medium for detection, isolation, and quantification of *Pseudomonas solanacearum* in soil. Phytopathology 72:310-313.

A bacteriocin technique was developed to detect, isolate, and quantify *Pseudomonas solanacearum* in soil. Tetrazolium chloride agar, incorporated with chloramphenicol (10 μg/ml) and pentachloronitrobenzene (37.5 μg/ml), was used as a basal medium. Wide-spectrum bacteriocin-sensitive strains 159, 209, and 217 of *P. solanacearum* were used as indicators. Plates containing the medium were dried, spread with soil suspension, overlaid with 1.5% water agar, and incubated for 24–28 hr before being overlaid with 0.7% water agar containing the bacteriocin indicator strain. Colonies of *P. solanacearum*, identified by clear inhibition zones, were isolated 24 hr later. This technique was sensitive to 4.2×10^3 colony-forming units per gram of oven-dried soil, and the efficiency of recovery of *P. solanacearum* from soil at 4.2×10^4 colony-forming units per gram of oven-dried soil ranged from 92.0 to 100%.

Survival and ecological studies of *Pseudomonas solanacearum* E. F. Smith have been hampered by the lack of a sensitive selective medium or technique to monitor bacterial populations in soils. Selective media for isolating and quantifying *P. solanacearum* in soils have been developed (4,5,9), but they are either ineffective with North Carolina soils or inhibitory to some strains of *P. solanacearum*. Serological tests also have been used to quantify *P. solanacearum* in soils (4), but these techniques do not discriminate between live and dead cells nor between virulent and avirulent ones. Therefore, a more efficient method to quantify the bacterium in soil is desirable.

Bacteriocins are nonreplicating, bactericidal substances, the majority of which contain proteins; they are produced by certain strains of bacteria and are active against other strains of the same or closely related species. Bacteriocin production has been used as a tool in typing and identifying species of bacteria. Because most strains of *P. solanacearum* produce bacteriocins (1), detection of *P. solanacearum* in soil might be possible by using bacteriocin-sensitive strains. This article describes a simple and sensitive semiselective medium for detection, isolation, and quantification of *P. solanacearum* in soil.

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0031-949X/82/03031004/$03.00/0

MATERIALS AND METHODS

Screening of antimicrobial chemicals. Antimicrobial chemicals employed in selective media for isolating pseudomonads (5,8,10) were screened for possible use in a selective medium for *P. solanacearum*. Chemicals screened and concentrations used were: bacitracin (50 μg/ml), chloramphenicol (10 μg/ml), penicillin G (1 μg/ml), tyrothricin (20 μg/ml), polymyxin B (100 μg/ml), pentachloronitrobenzene (PCNB) (37.5 μg/ml), cycloheximide (0.014 μg/ml), benomyl (50 μg/ml), and chlorothalonil (60 μg/ml). Fifty milligrams of each chemical was dissolved in and sterilized with 1 ml of 70% ethanol for 60 min and added in the appropriate amount to melted tetrazolium chloride (TZC) agar medium (6) at 45 C immediately before being dispensed into petri plates. Plates with medium were dried for 3 days at room temperature before use. These chemicals were screened against 11 common soilborne

TABLE 1. Source and host of strains[a] of *Pseudomonas solanacearum*

Source	Number of strains for host plant								
	Tobacco	Tomato	Potato	Peanut	Pepper	Eggplant	Banana	*Heliconia*	Plantain
Colombia	0	0	6	0	1	0	0	6	0
Costa Rica	0	1	0	0	1	0	16	16	1
Florida	1	0	0	0	0	0	0	0	0
Georgia	3	12	0	0	0	0	0	0	0
Honduras	0	0	0	0	0	0	5	0	0
Israel	0	0	1	0	0	0	0	0	0
North Carolina	22	10	2	3	3	3	0	0	0
South Carolna	1	1	0	0	0	1	0	0	0
South Rhodesia	2	0	0	0	0	0	0	0	0
Taiwan	1	1	0	0	0	0	0	0	0
Trinidad	0	1	0	0	0	0	0	0	0
Venezuela	0	0	0	0	0	0	1	0	0

[a]Bacterial strains were obtained from the collection maintained in the Department of Plant Pathology at North Carolina State University, Raleigh, NC.

microorganisms (eight bacterial species and three fungal species) in addition to *P. solanacearum* strains K-60 and 121. The microorganisms were isolated from TZC plates spread with diluted suspensions of soil collected at Oxford and Clayton, NC. One loop of a water suspension containing 10^7 colony-forming units (cfu) per milliliter of each one of the test bacteria was streaked, and a disk (5 mm in diameter) of each test fungus from a 48-hr plate culture was placed on the plate containing an antimicrobial chemical. These plates were incubated at 30 C and observations were made at 24 and 48 hr. The chemicals most inhibitory to undesirable bacteria and fungi and having no effect on *P. solanacearum* were selected and incorporated into TZC agar.

Detection of bacteriocin-sensitive strains. Strains of *P. solanacearum* sensitive to bacteriocins were selected by a modification of the procedure of Echandi (3). All strains in Table 1 were tested as bacteriocin indicators (bacteriocin-sensitive) against all strains as bacteriocin producers. Petri plates containing casamino acid-peptone-glucose (CPG) agar (2) were spot-seeded with 12 different test strains and incubated for 40 hr at 30 C. Colonies were then transferred from these plates to fresh CPG agar plates with a multipoint replicator with 4-mm diameter aluminum rods and were incubated 40 hr at 30 C. Plates were next inverted, exposed to the vapor of 3 ml of chloroform for 1 hr, and allowed to stand with the lids off for another hour. A 0.2-ml sample of the indicator strain (10^7 cfu/ml) was added to 4 ml of 0.7% melted water agar (45 C) and poured over the bottom layer of agar. Plates were incubated for an additional 24 hr at 30 C. Strains sensitive to bacteriocins from the greatest number of strains were chosen to overlay the new selective medium.

RESULTS

Screening of antimicrobial chemicals and selection of bacteriocin-sensitive strains. Chloramphenicol (10 μg/ml) and PCNB (37.5 μg/ml) were the most inhibitory antibacterial and antifungal agents, respectively; they inhibited 85% of bacteria and 100% of the fungi tested. All strains of *P. solanacearum* were inhibited by one or more bacteriocin-producing strains, but strains 159, 209, and 217 were the most sensitive. Strain 217 was inhibited by bacteriocins from all strains isolated from tobacco, eggplant, and potato and by 25 of the 26 strains isolated from tomato. Strain 159 was inhibited by bacteriocins from all strains isolated from banana. Strain 159 was inhibited by bacteriocins from 18 of 22 *Heliconia* strains. The remaining four *Heliconia* strains produced bacteriocins against strains 209 or 217 (Table 2). Zones of inhibition on strains 159, 209, and 217 were clear and measured 1–8 mm from the edge of the colony to the edge of the zone.

Development of the bacteriocin technique. TZC agar was selected as the basal medium, and chloramphenicol (10 μg/ml) and PCNB (37.5 μg/ml) were added to develop the selective medium, TZCCP. TZCCP reduced bacterial and fungal contaminants 75–95% more than TZC did. Growth of *P. solanacearum* on TZCCP plates required 24 hr more incubation at 30 C than on TZC agar before typical fluidal virulent and butyrous avirulent colonies appeared, but TZCCP did not affect the colony number or the size of the zones of inhibition produced by *P. solanacearum* on the plates.

TABLE 2. Sensitivity of strains 159, 209, and 217 to bacteriocins from 121 *Psuedomonas solanacearum* strains

Origin of strains	No. of strains tested	No. of strains producing bacteriocins against indicator strain		
		159	209	217
Tobacco	30[a]	19	27	29
Tomato	26	17	25	25
Potato	9	2	9	9
Peanut	3	0	3	3
Eggplant	4	3	4	4
Pepper	5	4	5	2
Banana	22	22	4	2
Heliconia	22	18	11	7

[a]Tobacco strains include 217 which is not sensitive to its own bacteriocin.

However, detection of *P. solanacearum* in soil with TZCCP medium was not satisfactory because bacteria and fungi occasionally contaminated the plates before the appearance of typical colonies of *P. solanacearum*. To prevent the spread and reduce the size of bacterial and fungal contaminants, 5 ml of 1.5% melted water agar (45 C) was spread over the surface of TZCCP plates with the soil suspension. The colonies of *P. solanacearum* that developed on TZCCP overlaid with water agar were too small to be recognized even after a 70-hr incubation at 30 C. We then postulated that the zones of inhibition that would develop by the use of a bacteriocin-sensitive strain overlay would facilitate the detection of these minute colonies. This was accomplished by incubating the plates at 30 C for 24–28 hr and adding an additional layer of 4 ml of melted 0.7% water agar (45 C) containing a bacteriocin-sensitive strain (0.2 ml, 10^7 cfu/ml). The zones of inhibition appeared after 24–36 hr of additional incubation.

Detecting and quantifying *P. solanacearum* in soil with the bacteriocin technique. *P. solanacearum* was detected and quantified in soil as follows. A virulent strain, K-60, and an avirulent strain, 121, were cultured separately in 200 ml of CPG broth on a rotary shaker (120 rpm) for 48 hr at 30 C. Bacteria were thoroughly mixed into a sandy loam and a clay soil to give concentrations of 10^2–10^6 cfu/g of oven-dried soil. Five grams from each mixture was collected 20 min after mixing and dispersed in 95 ml of sterile distilled water. Each sample was diluted 10-fold with sterile distilled water, and a 0.1-ml aliquot from each dilution was placed on the surface of dried TZCCP and TZC plates and spread uniformly with a bent glass rod. After 10 min, 5 ml of melted 1.5% water agar (45 C) was poured on the seeded plates, which were incubated 24–28 hr at 30 C. Finally, 4 ml of melted 0.7% water agar (45 C) was mixed with 0.2 ml of a water suspension (10^7 cfu/ml) of bacteriocin-sensitive strain 217 and poured over the agar surface of each plate. Plates were incubated an additional 24 hr at 30 C. Bacterial colonies with zones of inhibition and with characteristics

of *P. solanacearum* (Fig. 1) were recorded. When 4.2×10^4 cfu of *P. solanacearum* per gram of oven-dried soil was used, *P. solanacearum* colonies could be detected on both TZC and TZCCP; when the number dropped to 4.2×10^3 cfu/g of oven-dried soil, *P. solanacearum* colonies were detected on TZCCP only (Table 3).

Evaluation of the bacteriocin technique. Six different North Carolina tobacco fields, four from Momeyer, one from Oxford, and one from Kinston, with wilted tobacco plants were selected for assay. Ten soil samples from each field were collected at random from the top 15 cm, pooled in 4-kg polyethylene bags, and assayed the following day. Strain 217 was used as indicator strain, and TZCCP and TZC were used as test media. TZCCP suppressed other soil bacteria and fungi, and the bacteriocin technique successfully detected the population of *P. solanacearum* in soil (Table 4). The population of *P. solanacearum* in infested fields ranged from 10^5 to 10^7 cfu/g of oven-dried soil.

The effectiveness of the bacteriocin technique to isolate *P. solanacearum* was determined from the six infested soils. For every soil, 10 colonies were isolated randomly from the center of inhibition zones shown by indicator strain 217 on TZCCP plates and streaked on TZC plates. All 60 colonies sampled produced typical, fluidal colonies resembling *P. solanacearum* strain K-60 after 48 hr at 30 C incubation. A single colony from each plate was subcultured and retested by the bacteriocin technique. All colonies from the subcultures produced uniform zones of inhibition against the indicator strain 217. Infiltration (7) was used to test pathogenicity. All subcultures caused dark necrotic areas surrounded by a yellow halo on infiltrated areas of leaves of Hicks tobacco within 5 days. These symptoms were similar to those caused by strain K-60.

TABLE 3. Recovery of *Pseudomonas solanacearum* colony-forming units (cfu) from sandy loam and clay soils by bacteriocin technique, using TZCCP[a] and TZC[a] as media and strain 217 as bacteriocin indicator

Strain	Bacteria added (cfu/g of oven-dried soil)[b]	Bacteria recovered[c] (% cfu)			
		Sandy loam		Clay soil	
		TZC	TZCCP	TZC	TZCCP
K-60	4.2×10^3	0	72.3	0	51.2
	4.2×10^4	63.1	100	84.0	92.0
121	6.6×10^3	0	63.8	0	67.4
	6.6×10^4	34.6	86.6	77.9	90.0

[a] TZC = Tetrazolium chloride medium. TZCCP = TZC medium with chloramphenicol (10 μg/ml) and pentachloronitrobenzene (37.5 μg/ml).
[b] Soil samples with 10^3 and 10^4 cfu/g of oven-dried soil were diluted to 10^{-2} and 10^{-3}, respectively. Of the diluted sample, 0.1 ml was spread on the surface of the agar plate.
[c] Figures are averages from two plates. Zero readings indicate plates were overgrown by soil microorganisms.

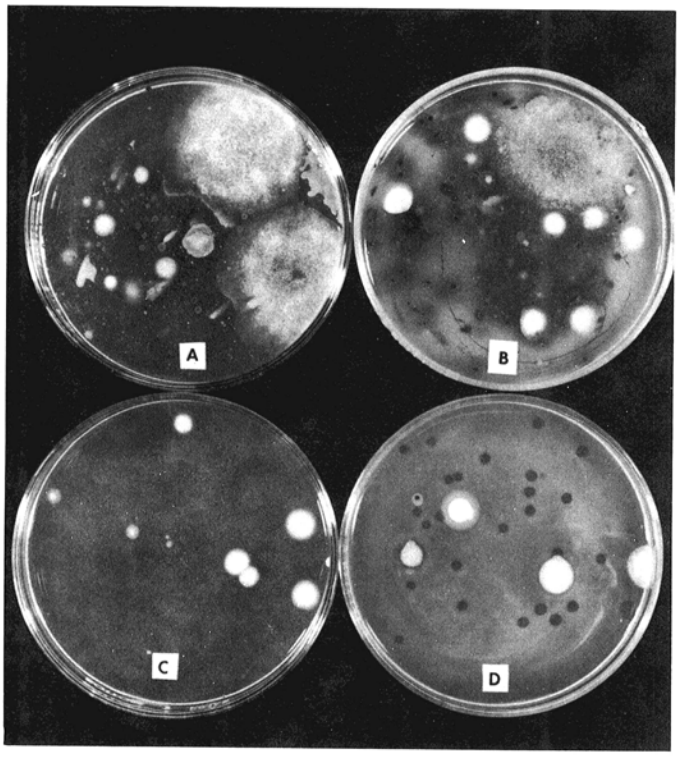

Fig. 1. Comparison of tetrazolium chloride (TZC) medium and TZC plus chloramphenicol and pentachloronitrobenzene (TZCCP) medium for growth of *P. solanacearum* and inhibition of soil microbes from Momeyer sandy soil. Plates A (TZC) and C (TZCCP) had no bacteriocin indicator strain added. Plates B (TZC) and D (TZCCP) had indicator strain 217 added. Plate D shows clear inhibition zones. Approximately 6.6×10^4 cells of strain 121 were added per gram of oven-dry soil.

DISCUSSION

Reduction of soil bacteria and fungi and selection of wide-spectrum bacteriocin-sensitive strains are two prerequisites for quantifying *P. solanacearum* in soil by the bacteriocin technique. TZCCP medium reduced growth of soil bacteria and fungi 75–95% more than TZC medium did and facilitated the counting and isolation of *P. solanacearum* from infested soil. However, in naturally infested soil, if the population of *P. solanacearum* is high and that of contaminant microbes is low, TZC medium may be satisfactory for the bacteriocin test. A soil sample might contain a *P. solanacearum* strain that does not inhibit the indicator strain selected. Therefore, use of two or more different indicator strains with a wide spectrum of bacteriocin sensitivity to *P. solanacearum*, eg, strains 159 and 209 or 159 and 217, is advisable.

Several factors influence the effectiveness of the bacteriocin technique for enumerating and isolating *P. solanacearum* from soil. The soil suspension should be diluted so that 30–120 *P. solanacearum* colonies develop per plate; more than 120 colonies per plate results in coalescent zones of inhibition. The length of the incubation period before adding the indicator strains affects the assay. If the period is too short, the zones of inhibition are not clear; if the period is too long, contaminating microbes tend to overgrow and interfere with the assay.

The concentration and the quantity of the layer of agar also affect the enumeration of *P. solanacearum*; 5 ml of 1.5% water agar was optimum in our test. The concentration of the indicator strain is also an important factor. The indicator strains must grow quickly and uniformly, and the inhibition zones must be visible 20–36 hr after the indicator strain is applied. Soil contaminants interfere with detection when suspensions of the indicator strains are too dilute and will delay the appearance of inhibition zones. Occasionally, zones of inhibition occur as a result of the action of soil-antagonistic bacteria; however, they are easily distinguished

TABLE 4. Populations[a] of *Psuedomonas solanacearum* and other bacteria in naturally infested soils, determined by the bacteriocin technique

Source of soil sample[b]	*P. solanacearum* detected by indicator strain 217 on		Other soil bacteria detected on	
	TZC[c]	TZCCP[c]	TZC	TZCCP
Momeyer Field				
1	1.4	1.7	20.9	6.1
2	1.1	1.4	17.9	1.2
3	0.9	1.1	27.6	5.8
4	1.0	1.2	11.0	2.6
Kinston	106.7	105.6	211.7	45.0
Oxford (tobacco plant rhizosphere)	···	155.7	···	217.6

[a] In values given $\times 10^5$ colony-forming units per gram of oven-dried soil.
[b] Ten soil samples collected at random from each naturally infested field.
[c] TZC = Tetrazolium chloride medium. TZCCP = TZC medium with chloramphenicol (10 μg/ml) and pentachloronitrobenzene (37.5 μg/ml).

from those of *P. solanacearum* by colony shape and color as well as size of the zone. Finally, this technique detects only bacteriocin-producing strains. Strains that do not produce bacteriocins may not be detected by this technique.

The bacteriocin technique may be useful in isolating *P. solanacearum*, estimating its population in soil and rhizosphere of host plants, and as a tool in monitoring populations of the bacterium in the field.

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