A Selective Medium for Soil Isolation and Enumeration of Xanthomonas campestris

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ABSTRACT

A new medium, SX agar, based primarily on starch digestion was developed for the isolation of *Xanthomonas campestris*. Growth of the bacterium on SX agar was comparable to that on standard plating media; plating efficiencies ranged from 13-63 percent for different isolates. For soil isolations, SX agar was superior to standard plating media and the selective medium, D-5. Because many soil microorganisms grew poorly and failed to hydrolyze starch, it was possible to assay several natural soils at dilutions as low as 1:10. The recovery of *X. campestris* when added to natural soils was over 10%. By using SX agar, it was possible to determine the relative populations of the pathogen in soil samples taken from a naturally infested field. SX agar also appeared useful for the isolation of eight other *Xanthomonas* spp.

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Black rot of crucifers, caused by *Xanthomonas campestris*, is commonly found on cultivated cruciferous plants throughout the world (2, 5, 13) and is endemic in cabbage in the southeastern United States. Seed and plant refuse are important in black rot epiphytotics (4, 9, 13, 14); however, the apparent elimination of these sources of inoculum has not controlled the disease. This suggests that other factors such as wild cruciferous hosts or soil may be important reservoirs of inoculum. It has been assumed from the use of bait plants (3) that *X. campestris* does not survive in soil, even though it was later demonstrated that soil inoculum was potentially important in black rot of rutabagas (9). There are, however, no quantitative data available on the survival of *X. campestris* in soil. A selective medium (D-5) for the isolation of *X. campestris* is available (8), but we found that *X. campestris* only grew from mass streaks. This study was initiated to develop a plating medium for determining soil populations of *X. campestris*. An added benefit of this study was the indication that several other xanthomonads could be isolated from soil by the medium. A preliminary report has been published (10).

MATERIALS AND METHODS. —*Organisms.* —The bacterial strains used in this study are listed in Table 1.

Carbon sources.—The dilution plate method was used to determine a satisfactory carbon source for selective growth of *X. campestris*. In a preliminary test, 0.2 g of several complex carbon compounds were added to a basal medium containing 0.5 g ammonium chloride, 0.2 g potassium diphosphate, 1.5 g Bacto agar, and 100 ml distilled water. Triplicate plates were inoculated with

approximately 1×10^2 viable cells of *X. campestris* isolate BBs. After 7 days of incubation at 30 C, colony diam and numbers were recorded.

Medium.—The final selective medium (SX agar) based on Starr's minimal medium (12), contained the following ingredients: soluble-potato starch (Baker), 10 g; beef extract (Difco), 1 g; ammonium chloride, 5 g; potassium diphosphate, 2 g; methyl violet B (National Aniline and Chemical Co.), 1 ml of a 1% solution in 20% ethanol; methyl green (Coleman and Bell Co.), 2 ml of a 1% solution; cycloheximide (UpJohn Co.), 250 mg; agar, 15 g; and distilled water, 1,000 ml. All ingredients were added prior to autoclaving, and the pH of 6.8 left unadjusted. All plates were stored and incubated in the dark.

Comparative plate counts.—Early log phase cells were prepared by growing a fresh culture in medium 523 [a basal salts + glucose broth (8)] at 27 C on a rotary incubator shaker to a turbidity reading of 40-50 Klett units (optical density of 0.08-0.1) as measured with a Klett-Summerson photoelectric colorimeter (green filter). This corresponded to a viable cell count of approximately 1×10^{10} cells/ml. The actual number of viable cells in each inoculum was determined by pipetting 0.1 ml from 10^{-7} and 10^{-8} dilutions onto the surface of triplicate plates of Difco nutrient agar (NA). The inoculum was spread evenly with a L-shaped rod using a turn table and the plates incubated 3-6 days at 30 C prior to counting. For comparative counts, all test media were inoculated with 0.1 ml of the same dilution series. Plating efficiencies were expressed as the percentage of colonies growing on SX in comparison to NA agar.

Soil isolations.—*X. campestris* was isolated from soil by adding 1 g soil (wet weight) to 9 ml water, mixing the suspension for 3-5 sec with a Vortex mixer, and immediately pipetting 0.1 ml onto the appropriate media. Greater dilutions were made depending upon the population of *X. campestris*. A modified method of Schroth et al. (11) was used to determine the capacity of SX agar to reveal relative soil populations of *X.*

TABLE 1. *Xanthomonas* strains employed in this study

Organism	Source
X. campestris BBs	R. G. Grogan, Davis, Cal.
X. campestris B-5[a]	Author, Pine Mountain, Ga.
X. campestris B-18[a]	Author, Sanford, Fla.
X. campestris B-24[a]	L. Moore, Corvallis, Ore.
X. dieffenbachiae B-400[a]	J. F. Knauss, Apopka, Fla.
X. corylina B-408[a]	L. Moore, Corvallis, Ore.
X. translucens XT	W. C. Schnathorst, Davis, Cal.
X. pelargonii XP-2	W. C. Schnathorst, Davis, Cal.
X. phascoli XP-27	W. C. Schnathorst, Davis, Cal.
X. malvacearum R1	W. C. Schnathorst, Davis, Cal.
X. malvacearum R4	R. N. Goodman, Columbia, Mo.
X. maculifoliigardiniae 069-3031	J. Miller, Gainesville, Fla.
X. fragariae 070-1277	J. Miller, Gainesville, Fla.
X. begoniae 068-1380	J. Miller, Gainesville, Fla.
X. poinsettiacola 067-769	J. Miller, Gainesville, Fla.
X. nigramaculans Zinnia	J. Miller, Gainesville, Fla.
X. vesicatoria Capsicum	J. Miller, Gainesville, Fla.
X. vesicatoria Lycopersicon 069-663	J. Miller, Gainesville, Fla.
X. juglandis XJ	D. Hildebrand, Berkeley, Cal.
X. incanae XI-Y	D. Hildebrand, Berkeley, Cal.

[a]Author's (N.S.) designation.

TABLE 2. Comparative plate counts of four *Xanthomonas campestris* isolates on selective and nonselective media

Media[b]	Mean no. colonies per plate at 10^{-7} dilution[a]			
	BBs	B-5	B-18	B-24
NA	64.3 ± 10.0[c] x	69.6 ± 4.5	317.6 ± 32.2	95.5 ± 9.1 x
YDC	14.6 ± 4.3 z	42.4 ± 10.0 x	110.2 ± 14.3 x	76.2 ± 14.7 xyz
PDP	50.1 ± 4.0 x	32.2 ± 8.0 x	184.0 ± 13.7 y	80.1 ± 5.0 xy
PDA	0.0	0.0	0.0	0.0
523	28.0 ± 17.8 yz	23.8 ± 7.5 x	184.4 ± 18.3 y	89.0 ± 8.2 xy
D-5	0.0	0.0	0.0	0.0
SX	25.3 ± 1.0 y	8.6 ± 5.8	100.4 ± 16.4 x	59.2 ± 12.2 z

[a]Bacteria were grown in 523 broth to a turbidity of 45 to 55 Klett units (differing with each isolate), diluted serially to 10^{-7} and 0.1 ml assayed. Counts are from five plates for B-5, B-18, and B-24 and three plates for BBs. Counts between media only are comparable.

[b]Abbreviations: NA, nutrient agar; YDC, yeast extract-dextrose-$CaCO_3$; PDP, potato-dextrose-peptone; PDA, Difco potato-dextrose agar; 523, D-5 Kado et al. (8); SX, this paper.

[c]90% confidence limits; numbers followed by the same letter are not significantly different, $P = 0.05$.

campestris. One-half ml of an appropriate dilution of a 50 Klett suspension of the pathogen was added to 4.5-g samples of field-collected soil. Each sample was mixed, two 1-g samples were removed, diluted appropriately, and assayed as previously stated.

RESULTS.—Preliminary screening of a number of complex carbon compounds, including several known to be utilized by *X. campestris* (1, 5), showed that *X. campestris* isolate BBs utilized most of the compounds tested. Only D-xylose, lactose, cellobiose, and DL-serine failed to support growth of *X. campestris*. Good growth occurred on pectin, sucrose, starch, D-galactose, D-maltose, DL-leucine, glycine, DL-tyrosine, and D-sorbitol. Since no single compound favored growth of the pathogen, starch was selected for the final medium because its utilization can be easily detected and it is assimilated by few other soil microorganisms (1). Two basic dyes, methyl violet B and methyl green, were added to improve colony differentiation and give the medium greater selectivity. Although we did not determine which soil bacteria were eliminated by the dyes, Fung and Miller (6), have shown that many gram-positive bacteria do not grow in the presence of 10 μg/ml of methyl violet B or methyl green. Beef extract was added because the growth rate of *X. campestris* was somewhat improved without increasing that of the other soil flora. Although the development of the medium was based on a single culture (BBs), several fresh *X. campestris* isolates from diseased kohlrabi, kale, broccoli, rutabaga, collards, and cabbage were subsequently screened by the streak plate technique. All isolates grew as single colonies, digested starch, and had similar colony morphologies.

Most *X. campestris* colonies were visible after 3 days of incubation at 30 C as small translucent colonies surrounded by a clear, 4- to 5-mm-diam zone of starch digestion. By 5 days, the colonies were 3 mm in diam, mucoid, glistening, convex, circular with entire margins, and translucent with bluish-purple centers; the starch digestion zone had a diam of 8-10 mm. The growth rates of the bacterium isolated from plant or soil environments appeared to vary somewhat. For example, most colonies were visible after 3 days of incubation, but some were not visible until after 4 days of incubation. After 5 days, colony diam ranged from 3-5 mm. For this reason, soil plate counts were taken after incubations of 3 and 5 days. Three-day readings were important only if significant numbers of slow-growing, starch-negative colonies were present. When such colonies occurred within the zone of starch digestion surrounding colonies of *X. campestris* the pathogen would often be indistinguishable or overgrown on the fifth day. Fungi did not normally interfere with the later counts. *X. campestris* colonies typically were 7 mm in diam after 7 days of incubation. After becoming familiar with *X. campestris* colonies, we could differentiate them from soil bacterial colonies with an accuracy of 80-100%. Questionable colonies were easily screened by streaking them onto yeast extract-dextrose-$CaCO_3$ (YDC) agar (15) and observing the characteristic yellow, mucoid colonies. All isolates tentatively identified as *X. campestris* on SX and YDC agars were pathogenic when tested on cabbage.

Table 2 shows the results of comparative plate counts of four *X. campestris* isolates on nonselective media; NA, YDC, potato-dextrose peptone (PDP), Difco potato-

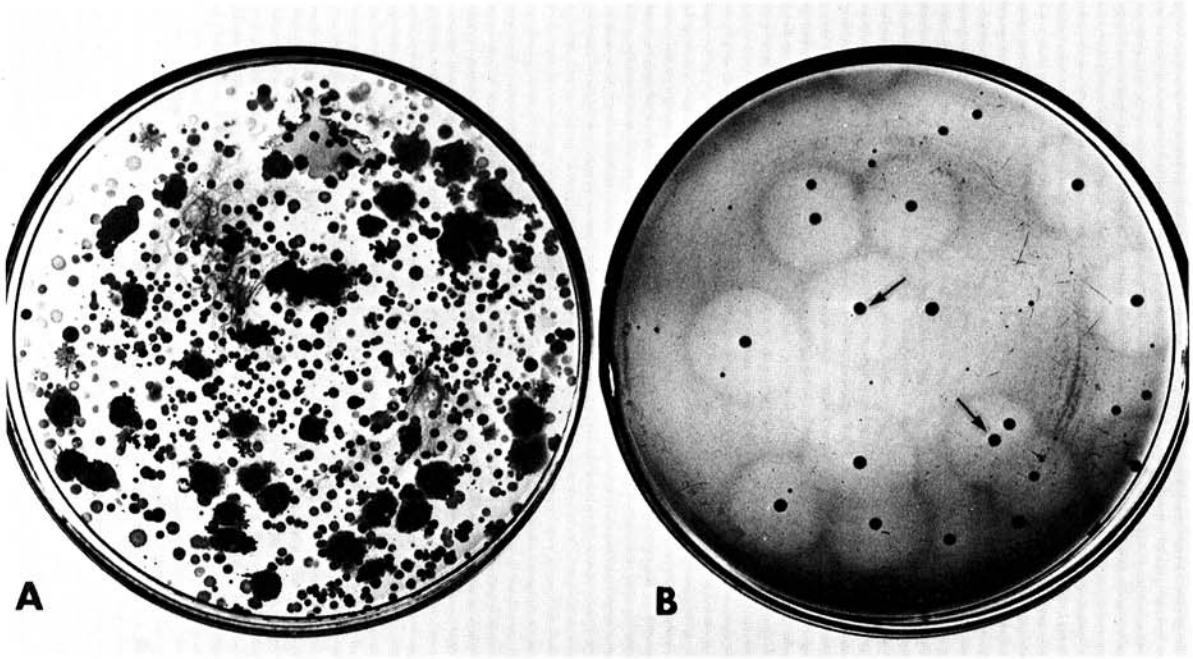

Fig. 1-(A, B). Comparison between **A)** nutrient agar and **B)** SX agar for the selective isolation of *Xanthomonas campestris* from a natural Lloyd clay loam soil. To one gram wet wt. soil was added 1 ml sterile distilled water which contained 1.7×10^5 viable *X. campestris* cells. After adding 8 ml water, the suspension was mixed using a Vortex mixer, diluted 1:10, and 0.1 ml assayed. All the colonies on SX agar surrounded by a large, clear halo were identified as *X. campestris*. Two such colonies are marked with arrows.

dextrose agar (PDA), medium 523 (8) and the selective media, D-5 and SX. Growth of all four isolates occurred on all media except Difco PDA and the selective medium D-5; on these media *X. campestris* grew only when seeded with a mass of cells. Numbers of colonies per plate tended to be greatest on NA; numbers were significantly greater (5%) with two of the four isolates tested. Colony counts of each isolate were, in most cases, significantly less on SX agar than on YDC, PDP, or 523 agars; however, counts on SX agar were considered comparable to those on the above nonselective media (Table 2). Variation did occur in the ability of different isolates to grow on SX agar; the plating efficiences of isolates BBs, B-5, B-18, and B-24 were 39, 12, 32, and 61 percent, respectively.

For isolation and population determinations of *X. campestris* in field soil, SX agar was superior to any of the other media tested. A typical comparison of the numbers of *X. campestris* and background colonies found in natural soil diluted 1:100 and assayed on NA and SX agar is shown in Fig. 1. Although the soil was infested with 1.7×10^5 viable cells, no *X. campestris* colonies were detected on NA due to the presence of over 600 colonies of soil bacteria, including many large, spreading ones. Fewer than 25 soil bacterial colonies (all starch-negative) were present on SX agar, and the 13 starch-positive, mucoid *X. campestris* colonies (arrows, Fig. 1-A) were easily differentiated. Results similar to those on NA were

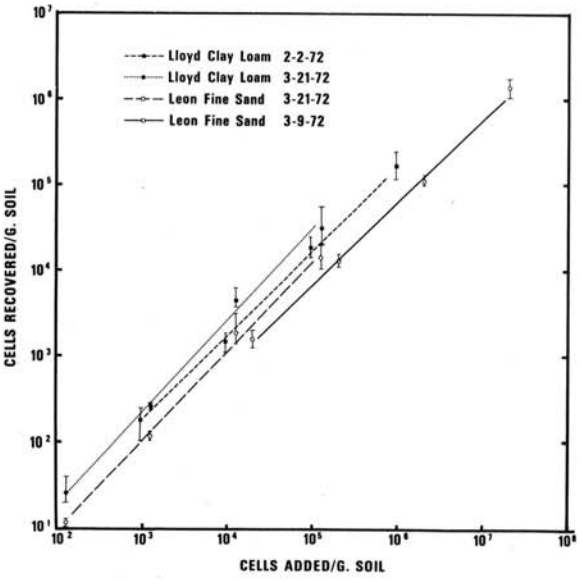

Fig. 2. Logarithmic plot of the percentage recovery of *Xanthomonas campestris* isolate BBs added to 4.5-g samples of a natural Lloyd clay loam (Griffin, Ga.) and Leon fine sand (Sanford, Fla.). Each point represents the mean of two, 1-g samples assayed in triplicate on SX agar plates.

TABLE 3. Growth and plating efficiency of pure cultures of several *Xanthomonas* spp. on SX agar

Species	Colony diameter, (mm)[a]	Colonies per plate at 10^{-7} dilution		Plating efficiency[b]
		NA	SX	
X. campestris BBs	1.5	151	100	38
		177	12	
		156	51	
X. campestris B-24	1.5	528	334	63
		463	311	
		704	328	
X. corylina	2.0	164	67	31
		203	26	
		170	83	
X. nigramaculans	2.0	82	48	37
		104	18	
		91	36	
X. maculifoliigardiniae	3.0	771	848	54
		736	358	
		900	100	
X. incanae	1.0	168	50	42
		182	90	
		200	90	
X. begoniae	3.0	447	95	26
		426	119	
		477	148	
X. fragariae	4.0	307	70	23
		306	96	
		245	33	
X. vesicatoria 069-663	4.0	80	17	40
		147	9	
		57	90	
X. dieffenbachiae	3.0	109	132	109
		117	104	
		101	122	

[a] After 4 days of incubation at 30 C.

[b] Plating efficiency = [(Mean no. colonies on SX agar)/(Mean no. colonies on Na)] × 100.

obtained with YDC and the selective medium D-5; *X. campestris* was not detectable in soils diluted 1:100 because of too many background colonies.

Many soils diluted only 1:10 were successfully assayed on SX agar because a sufficient number of soil bacteria were suppressed. For example, counts of soil bacterial colonies on SX agar ranged from 200 to 700 per plate (mean of 400 to 450) with sandy soils from southern Georgia and 100 to 400 colonies per plate (mean 225 to 250) with clay soils from central and northern Georgia when diluted only 1:10. Furthermore, 80 to 90% of the colonies of soil bacteria on SX agar remained less than 2 mm in diam and only about 10% hydrolyzed starch. An added advantage of SX agar, was the elimination of many of the widely distributed (7), yellow-pigmented bacteria which could have been easily confused with xanthomonads on the other media. For example, several soil bacteria including *Pseudomonas cepacia*, *Flavobacterium aquatile*, *Flavobacterium peregrinum*, and several *Cytophaga* spp. produce yellow colonies on NA, but do not utilize starch (1). Fig. 2 is a logarithmic plot of the percentage recovery of *X. campestris* from two natural soils, a Lloyd clay loam from Griffin, Ga., and a Leon fine sand from Sanford, Fla., and illustrates the isolation of the pathogen from soil containing as few as 100 viable cells/g soil.

That SX agar was capable of determing relative populations of *X. campestris* in naturally infested soil was shown by assaying three soil samples collected on February 28, 1972, from a black rot variety test plot at Sanford, Florida. The pathogen was found at concns of 1.5×10^4 and 1.0×10^3 cells/g soil in two of the samples.

The possibility of using SX agar for isolating several other *Xanthomonas* spp. known to digest starch (1) was also determined. Each xanthomonad listed in Table 1 was checked for growth on SX agar. The following species grew in mass streak only: *Xanthomonas phaseoli*, *Xanthomonas poinsettiacola*, *Xanthomonas malvacearum*, *Xanthomonas juglandis*, *Xanthomonas translucens*, *Xanthomonas pelargonii*, and *Xanthomonas vesicatoria* pathotype Capsicum. Eight other species, however, grew well enough to determine plating efficiencies by the dilution plate technique. Plating efficiencies ranged from 23% for *X. fragariae* to 109% for *X. dieffenbachiae* (Table 3). Furthermore, most species grew more rapidly on SX agar than did *X. campestris* (Table 3).

DISCUSSION.—Data on population dynamics of *X. campestris* in natural soils has not been available due to the lack of a selective medium for isolating the pathogen. A selective medium, D-5, was recently made available; however, we found that D-5 not only failed to support the growth of *X. campestris* but also failed to limit adequately the growth of soil bacteria. Our medium, SX agar, was not perfect. SX agar detected only about 10 percent of the population of *X. campestris* added to soil. Furthermore, variation occurred in the growth of several pure culture isolates on SX agar; plating efficiencies ranged from 13-63%. Still, the medium was superior to any other tested for soil isolations of the pathogen. Because *X. campestris* was easily distinguished by its colony morphology and starch digestion from the relatively few colonies of soil bacteria which grew on SX agar, many soils were successfully assayed on SX agar at 1:10 dilutions. On the other hand, when the same soils were assayed on general plating media or the selective medium D-5, they had to be diluted 100-fold more in order to reduce the number of background colonies. Using SX agar, relative inoculum levels of the pathogen in natural soil were determined. Recoveries of *X. campestris* from soils infested with between 10^2 and 10^8 cells/g soil were linear and the pathogen was detected in soil taken from a naturally infested field.

Besides allowing the quantitative isolation of *X. campestris* from natural soils, SX agar should be useful in the presumptive identification of starch-digesting xanthomonads. By using the medium in diagnostic laboratories, one could eliminate the many false-positive yellow-pigmented bacteria which so commonly appear on general plating media.

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