# An Improved Semiselective Medium for Recovery of Xanthomonas campestris pv. phaseoli

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#### ABSTRACT

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An improved solid semiselective medium (M-SSM) for isolation of Xanthomonas campestris pv. phaseoli was developed by the addition of starch, dyes, and agar to a liquid semiselective medium. The solid medium was equal or superior to two other semiselective media for isolation of X. c. phaseoli from diseased bean (Phaseolus vulgaris) tissues. The medium was easy to prepare, and it remained stable in cold storage over a 45-day period. Each of 19 strains of the pathogen grew on the medium; the colonies appeared earlier, and grew larger, than those of nontarget bacteria. Colonies of the pathogen were easily distinguished from other bacteria, and most nontarget bacteria were effectively suppressed.

Xanthomonas campestris pv. phaseoli (Smith) Dye and the brown pigmented strain, known as X. c. var. fuscans, cause serious problems in many bean (Phaseolus vulgaris L.)-growing areas of the world (15). Both bacteria are now recognized as X. c. phaseoli (1) and are hereafter referred to collectively by that name. Internally infected seed is the main source of primary inoculum for common blight disease (2,14).

In order to design effective disease control measures, it is necessary to know where plant-pathogenic bacteria survive between growing seasons and to detect low populations of X. c. phaseoli. Although numerous semiselective media have been developed for X. campestris (3,4,5,8,9,11, 12), only a few appear suitable for isolating X. c. phaseoli (6,7,13). In the present study, the performance of available solid semiselective media for recovery of X. c. phaseoli from various sources is compared with that of a liquid semiselective medium (13) for selective isolation of this pathogen.

### MATERIALS AND METHODS

Bacterial strains. A total of 19 strains of X. c. phaseoli were used in this study, most of which had been isolated from bean seed supplied by the Michigan Department of Agriculture and from diseased bean leaves collected from

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different growing areas in Michigan. Two strains, Sc-4A and LB-2, were supplied by A. K. Vidaver, Department of Plant Pathology, University of Nebraska, Lincoln, NE. Pseudomonas syringae pv. phaseolicola (Burkholder) Young et al strains Pp-P23 and P-P8 were isolated from diseased bean leaves from Kenya and Tanzania, respectively. Cultures of Corvnebacterium michiganense (Smith) Jensen, C. fascians (Tilford) Dowson, Agrobacterium tumefaciens (Smith and Townsend) Conn, Erwinia amylovora (Burrill) Winslow et al, and Bacillus megaterium de Bary were obtained from stocks maintained in our laboratory.

Stock cultures were maintained in 0.01 M phosphate buffer (pH 7.2) containing 40% glycerol at 5-7 C; others were preserved by routine lyophilization. Stock cultures were transferred to fresh yeast extract-calcium carbonate agar (YCA) (10 g yeast extract, 2.5 g calcium carbonate, and 15 g agar [Difco, Detroit, MI] per liter of distilled water) plates and incubated at  $27 \pm 1$  C to recover growing cultures. Cultures were routinely checked for colony characteristics and pathogenicity.

Pathogenicity tests. Colonies from 24-hr-old cultures grown on YCA were suspended in the above phosphate buffer and adjusted to 1.5-2.6 × 10<sup>8</sup> cfu/ml (optical density 0.2 at 620 nm). A 10-mm-diam area on the first trifoliolate leaf on 14-day-old greenhouse-grown Seafarer bean plants was water-soaked with a 3-cc hypodermic syringe without the needle. Four plants were inoculated with each culture. Plants were incubated in a greenhouse at 23-30 C, and disease was evaluated after 14 days.

Test media. Media MXP (6), SSM (13), and DSX (7) were prepared as reported. Bacto agar (Difco), 15 g/L, was

added to a liquid SSM (13). DSX, a medium that included soluble starch for isolation of X. c. campestris and X. c. phaseoli, was also tested (7). Stock solutions of antibiotics were prepared every 2 wk and, after filter sterilization, were stored at 5-7 C. Yeast extract-calcium carbonate agar was used as a standard for comparison purposes.

Soluble starch, basic dyes, and agar were added to SSM to give a semiselective solid medium on which individual colony growth could be observed. The concentration of gentamicin sulfate was increased from 0.05 to  $0.5 \mu \text{g} \cdot \text{ml}^{-1}$  to reduce the number of background organisms. For preparation of M-SSM medium, 1.0 g yeast extract, 8.0 g soluble potato starch, 15.0 g agar, 6.0 µl methyl green (1% aqueous solution), 3.0  $\mu$ l methyl violet 2B (1% solution in 20% ethanol), and 970 ml of 0.01 M phosphate buffer, pH 7.2, were mixed and autoclaved. After these ingredients were cooled to about 50 C in a water bath, the following antibiotics were added aseptically to give a final concentration in  $\mu g \cdot ml^{-1}$ : cycloheximide 25.0; nitrofurantoin, 2.0; nalidixic acid, 1.0; and gentamicin sulfate, 0.5.

Pure culture studies. Bacterial suspensions were serially diluted with sterile 0.01 M phosphate buffer (pH 7.2), and aliquots were spread on triplicate plates of test media. The time required for colonies to develop and colony shape, color, and size on the test media were noted. After 5-day incubation at 27 C, presumptive colonies of X. c. phaseoli were counted. Plating efficiencies were based on counts from YCA plates. The test was repeated three times, and the values were averaged.

Recovery from artificially inoculated bean tissue. Bean plants (cv. Charlevoix) were grown in the greenhouse in 10-cm-diam clay pots containing a 3:1 (v/v) mixture of soil and vermiculite. Plants were watered twice a day with tap water; air temperatures fluctuated between 24 and 30 C. Bacterial suspensions were prepared and adjusted turbidimetrically to contain  $1-2 \times 10^7$  cfu/ml. The first fully expanded trifoliolate leaf on 14-day-old plants was spray-inoculated with bacterial suspensions to runoff with an atomizer operated at 82.7 kPa. The in-

oculated plants were maintained in the greenhouse, and common blight symptoms were observed 8-10 days later. Two leaf disks, 10 mm in diameter, were excised from the asymptomatic areas and ground in 1.0 ml of buffer. After 1 hr, suspensions were serially diluted, and aliquots were plated on test media in triplicate.

Flowers of artificially inoculated bean plants, line I-84100, were also sampled. Plants at the two-trifoliolate leaf stage were spray-inoculated with approximately  $2 \times 10^7$  cfu/ml of X. c. phaseoli. At flowering (16 days after inoculation), five open blossoms were removed and comminuted in a mortar with buffer. After 1 hr, dilutions were prepared, and

**Table 1.** Plating efficiency of various strains of *Xanthomonas campestris* pv. phaseoli (Xp) and its fuscous strains (Xpf) on three semiselective media

Strain		Plating efficiency <sup>x</sup>			
	Medium <sup>y</sup>				
	MXP	SSM	M-SSM		
MI-1 $(Xpf)$	0.0 a <sup>z</sup>	79 b	73 b		
MI-2(Xp)	59 c	89 a	74 b		
MI-3(Xp)	88 a	91 a	97 a		
MI-4(Xp)	89 a	86 a	93 a		
MI-5(Xp)	96 a	87 a	92 a		
MI-6(Xpf)	15 b	63 a	54 a		
MI-7(Xp)	0.0 a	90 b	80 ь		
MI-8(Xp)	50 b	93 a	95 a		
MI-9(Xp)	87 ь	111 a	91 b		
MI-10(Xp)	78 a	88 a	76 a		
MI-11(Xp)	2 b	72 a	94 a		
MI-12(Xp)	48 b	75 a	93 a		
MI-13(Xp)	66 b	103 a	88 a		
MI-14(Xp)	3 a	63 b	54 b		
MI-15(Xpf)	0.0 a	106 b	125 b		
MI-16(Xpf)	77 a	88 a	84 a		
MI-17(Xp)	91 b	100 a	101 a		
Sc-4A(Xp)	81 a	88 a	85 a		
LB 2 (Xp)	80 ab	93 a	75 b		

<sup>\*</sup>Bacterial suspensions obtained from log-phase yeast extract-calcium carbonate agar cultures were adjusted to optical density 0.1 at 620 nm; tenfold serial dilutions were made in 0.01 M phosphate buffer (pH 7.2), and 0.1-ml portions were plated on test media and incubated at 27 C. Colonies were counted after 5 days. Figures in each row are from the same dilution.

<sup>x</sup> Cfu on semiselective medium/cfu on yeast extract-calcium carbonate agar × 100. Values are means of three experiments, each with three replicate plates for each medium.

For each isolate, numbers followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

**Table 2.** Colony diameters of *Xanthomonas campestris* pv. phaseoli (Xp) and fuscous strains (Xpf) on four test media 5 days after plating

Strain	Mean colony diameter (mm) <sup>x</sup>					
	Medium <sup>y</sup>					
	MXP	SSM	M-SSM	YCA		
MI-1 ( $Xpf$ )	0.0 d <sup>z</sup>	1.9 c	6.9 a	2.8 b		
MI-2(Xp)	6.3 b	2.9 с	7.1 a	3.0 с		
MI-3(Xp)	5.5 a	3.4 c	5.4 a	4.0 b		
MI-4(Xp)	4.7 b	2.0 d	6.1 a	3.8 c		
MI-7(Xp)	0.0 d	1.9 с	6.8 a	2.3 b		
MI-8(Xp)	6.0 a	3.6 c	5.0 b	4.0 c		
MI-9(Xp)	2.5 b	1.9 с	6.4 a	2.5 b		
MI-10(Xp)	3.8 b	1.8 c	7.5 a	2.0 с		
MI-12(Xp)	1.3 d	1.9 c	6.3 a	2.6 b		
MI-13(Xp)	1.6 c	1.9 c	6.1 a	2.7 b		
MI-15(Xpf)	0.0 c	1.9 b	6.0 a	2.1 b		
MI-16(Xpf)	1.6 c	1.7 c	3.1 b	3.9 a		
MI-17(Xp)	2.8 b	1.7 d	5.2 a	2.5 с		

<sup>&</sup>lt;sup>x</sup> Values are means of three experiments, each with three replicate plates. Ten colonies were measured on each plate. Plates containing 20–40 colonies were used.

0.1-ml aliquots were spread on each of triplicate plates. Data were log-transformed before analysis.

Recovery from naturally infected bean tissues. Naturally infected bean leaves were collected from commercial farms near Saginaw and Bay City, Michigan, and from the Crop and Soil Sciences and Botany and Plant Pathology research farms at East Lansing during the 1985 and 1986 growing seasons. Samples were stored at 5 C until used. Isolations were made as described above. The experiment was done four times. Presumptive colonies of X. c. phaseoli were transferred to YCA and later tested for pathogenicity.

Diseased bean leaves collected from commercial fields in Michigan were airdried and ground to a fine powder in a sterile mortar. The powder was stored in sterile glass test tubes at 23  $\pm$  1 C in the dark. After 9 mo, 0.1 g of the powder was added to 10 ml of sterile buffer, and the mixture was agitated in a vortex mixer and then shaken for 1 hr on a reciprocal shaker at 75 strokes per minute. Next, the mixture was allowed to sediment for 30 min; then the liquid phase was serially diluted in buffer, and portions were plated on the test media in triplicate. Growth of X. c. phaseoli, as well as that of other bacteria, was recorded. The experiment was done three times

Bean dust obtained from sieved bean seed from Puerto Rico was added to sterile buffer in a sterile test tube (1 g of dust per 10 ml of buffer) and mixed with a vortex mixer. Aliquots of dilutions (0.1 ml) were spread on the media in triplicate, and plates were incubated as described above. The experiment was repeated once.

The relative extent of contamination by bacteria and fungi was noted subjectively. It was not possible to count colonies because of frequently spreading growth.

Effect of media age. The test media were prepared and stored in closed plastic bags at 23 C for 4, 15, 30, and 45 days. Aliquots (0.1 g) of tissue powder from naturally diseased bean leaves were suspended as described above, and 0.1 ml aliquots of dilutions were spread on the test media in triplicate. Colony counts were analyzed statistically with the MSTAT program version 4.0 (Department of Crop and Soil Science, Michigan State University, East Lansing, MI) as a split-plot design with medium type as whole-plot factor and medium age as a subplot factor. Significant differences between means were estimated with Duncan's multiple range test.

#### RESULTS

All X. c. phaseoli isolates were pathogenic on bean plants. Of the four media, M-SSM and SSM were easier to prepare

y Media MXP (6) and liquid SSM (13) were prepared as reported. Difco Bacto agar (15 g/L) was added to liquid SSM. Soluble starch, basic dyes, antibiotics, and agar were added to liquid SSM to give M-SSM.

<sup>&</sup>lt;sup>y</sup> Media MXP (6) and liquid SSM (13) were prepared as reported. Difco Bacto agar (15 g/L) was added to liquid SSM. Soluble starch, basic dyes, antibiotics, and agar were added to liquid SSM to give M-SSM. YCA = yeast extract-calcium carbonate agar.

<sup>&</sup>lt;sup>2</sup> For each isolate, numbers followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

than MXP and DSX, in terms of time required.

Growth of X. c. phaseoli on semiselective media. The DSX medium was discontinued after initial evaluations, since recovery of X. c. phaseoli was very low and the plates required storage for at least 9 days before use (7). Colonies of the pathogen were visible on YCA in 3-5 days and grew to a diameter of 2-4 mm within 5 days. On solid SSM, small colonies of X. c. phaseoli were observed within 2-3 days and were 1.7-3.6 mm within 4-5 days. On MXP, colonies were visible after 3 days, when zones of starch hydrolysis were observed. Starch clearing differentiated X. c. phaseoli from other bacteria. After 5 days, colonies ranged from 1.3 to 6.3 mm in diameter. With DSX, colonies were visible within 4-5 days and had a diameter of about 3 mm in 6 days. Zones of starch hydrolysis developed but were smaller than those on other media.

With M-SSM, colonies appeared within 36-48 hr at 27 C and grew to a maximum diameter of 3.0-7.5 mm within 5 days. Zones of starch hydrolysis were easily seen around each colony under indirect light. The fuscous strains did not produce a brown pigment. Excessive production of slime occurred if glucose was added to M-SSM (0.5-1.0 g). Increased concentrations of soluble potato starch, up to 10 g, did not improve

Table 3. Recovery on four test media of various isolates of *Xanthomonas campestris* pv. *phaseoli* from artificially inoculated bean leaves<sup>w</sup>

Strain	X. c. phaseoli recovered (%)x				
	MXP	SSM	M-SSM		
MI-1	113 ab <sup>z</sup>	99 b	134 a		
MI-4	103 a	113 a	110 a		
MI-8	103 a	87 ь	107 b		
MI-9	79 ь	95 a	96 a		
MI-10	107 ab	108 ab	115 a		
MI-11	84 b	98 a	91 ab		
MI-13	87 a	95 a	99 a		
MI-17	92 a	113 a	95 a		

Bean plants (cv. Charlevoix) were sprayinoculated when the first trifoliolate leaf was fully expanded. Two 10-mm-diam discs were removed from diseased tissue 14 days after inoculation when disease symptoms were fully developed. Suspensions of comminuted leaf tissue were serially diluted and plated on test media. Plates were incubated at 27 C, and colony counts were made after 5 days.

recovery, and so 8.0 g was used. With M-SSM and MXP, colonies of X. c. phaseoli were yellow, mucoid, circular, and convex with entire margins and smooth surfaces; with SSM, colonies were light yellow and nonmucoid; with DSX, they were yellow and irregular in shape.

Plating efficiency of the test media. Pure cultures of 19 strains of X. c. phaseoli grew on SSM and M-SSM, whereas two fuscous strains (MI-1 and MI-15) and one strain (MI-7) of X. c. phaseoli did not grow on MXP (Table 1). Plating efficiencies on MXP, SSM, and M-SSM ranged from 0 to 96, 63 to 111, and 54 to 125% with means of 53, 88, and 85%, respectively. Colonies of X. c. phaseoli were similar in appearance on MXP and M-SSM. Approximately 92% of 13 strains produced larger colonies on M-SSM than on the other media (Table 2). Colonies of the fuscous strain were generally smaller in size on MXP (and, in one case, on M-SSM) than those of the strains that do not produce the brown pigment. For most strains, their colony size on SSM was not significantly different from that on YCA.

None of the other bacterial species tested grew on MXP. The six strains of P. s. phaseolicola also did not grow on M-SSM. P. s. syringae grew on SSM and M-SSM, but the number of colonyforming units was only 21 and 5%, respectively, of that observed on YCA. C. fascians (Cf-1) and E. amylovora also grew, at very reduced numbers, on SSM and M-SSM. B. megaterium grew on SSM only. In all cases, growth of these organisms was slower on the semiselective media than on YCA, and no zones of starch hydrolysis were observed. Moreover, colonies of the nontarget bacteria tested were smaller on the selective media than on YCA The nontarget bacteria took 3-4 days longer to develop than X. c. phaseoli, were white and flat, and thus were easily distinguished from X. c. phaseoli.

Recovery from artificially inoculated bean tissue. Generally, similar populations of X. c. phaseoli were recovered

on the different media (Table 3). Nontarget microbes did not interfere with recovery of X. c. phaseoli from bean leaves with any of the semiselective media; even with YCA, contamination was minimal. With bean blossoms, recovery of X. c. phaseoli was 83% on MXP, 104% on SSM, and 96% on MSSM compared to that on YCA. Colony counts were significantly lower (P=0.05) on MXP than on the other media.

Recovery of X. c. phaseoli from naturally diseased bean leaves was similar among the three media within each of the four experiments (Table 4). All presumed colonies were pathogenic on bean. Growth of nontarget bacteria was observed only on YCA. X. c. phaseoli was readily recovered from naturally infected bean leaf powder stored at 23 C for 9 mo (Table 5). In several instances, colony counts on MXP were significantly lower (P = 0.05) than those on other media. However, MXP prevented growth of saprophytic populations of both bacteria and fungi; M-SSM was nearly as effective, and SSM supported the highest levels of nontarget bacteria. On YCA, the development of rapidly spreading nontarget bacteria, in some cases, prevented recovery of common blight bacteria. Colonies of X. c. phaseoli consistently appeared on M-SSM within 48 hr, whereas on other media at least 72 hr was required.

Recovery of X. c. phaseoli from bean seed dust was highest on SSM (390%), followed by M-SSM (368%) and MXP (333%), relative to that on YCA. Colonies of the pathogen from bean seed dust were difficult to detect. Reduction of other microbial growth was greatest on MXP and least on SSM. In one experiment, recovery of X. c. phaseoli on YCA was hindered by high populations of saprophytic bacteria. Colonies of the pathogen were easily identified on MXP and M-SSM, based on presence of starch hydrolysis zones, growth rate relative to that of contaminants, colony morphology, and color. Random samples of presumptive X. c. phaseoli from all media were pathogenic to bean.

Table 4. Recovery on three semiselective media of Xanthomonas campestris pv. phaseoli from naturally infected bean leaves<sup>w</sup>

	Test number				
	1	2	3	4	
Medium <sup>x</sup>		Mean			
MXP	72 c <sup>z</sup>	104 a	85 a	67 b	82 a
SSM	103 a	115 a	91 a	95 a	101 a
M-SSM	88 b	95 a	80 a	89 a	88 a

<sup>\*</sup>Suspensions prepared from comminuted leaves were diluted, and 0.1-ml portions were plated on each medium in triplicate. Plates were incubated at 27 C for 5 days.

<sup>\*</sup>Cfu on semiselective medium/cfu on yeast extract-calcium carbonate agar × 100. Values are means of four experiments, each with three replicate plates.

y Media MXP (6) and liquid SSM (13) were prepared as reported. Difco Bacto agar (15 g/L) was added to liquid SSM. Soluble starch, basic dyes, antibiotics, and agar were added to liquid SSM to give M-SSM.

For each strain, means followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

<sup>&</sup>lt;sup>x</sup> Media MXP (6) and liquid SSM (13) were prepared as reported. Difco Bacto agar (15 g/L) was added to liquid SSM. Soluble starch, basic dyes, antibiotics, and agar were added to liquid SSM to give M-SSM.

 $<sup>^{</sup>y}$  Cfu on semiselective medium/cfu on yeast extract-calcium carbonate agar  $\times$  100.

<sup>&</sup>lt;sup>2</sup> Within each experiment, numbers followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

Table 5. Recovery on four test media of Xanthomonas campestris pv. phaseoli from infected dry bean tissue stored for 9 mo at 23 C<sup>w</sup>

	Test number <sup>x</sup>				
Medium <sup>y</sup>	1	2	3	4	
	Cfu per plate				
MXP	88 c	51 b	57 c	121 b	79 с
SSM	255 ab	110 a	119 a	197 a	170 a
M-SSM	273 a	92 a	111 ab	186 a	166 a
YCA	232 b	0 c <sup>z</sup>	84 bc	156 ab	118 b

<sup>\*</sup>Powdered bean leaves (1/10 g) in 0.01 M phosphate buffer (pH 7.2) were serially diluted, and the last three dilutions were plated on the test media. Plates were incubated at 27 C for 5 days.

Effect of media age on recovery. Recovery of X. c. phaseoli was generally unaffected by age of the media up to 45 days. However, the time required for the absorption of water from the suspensions with all media decreased progressively with age.

## **DISCUSSION**

For epidemiological studies requiring the detection and isolation of X. c. phaseoli from diseased and infested bean tissues, and for estimations of populations of the pathogen, M-SSM appears to have several advantages over other semiselective media. For example, each of the 19 strains of the pathogen grew on M-SSM; colonies of the pathogen grew faster on it than on other media; and the medium required less time to prepare. In addition, M-SSM was highly effective for suppression of nontarget microbes. Colonies of X. c. phaseoli were easily distinguished from those of other bacteria. Fungal contaminants were inhibited by cycloheximide; nalidixic acid inhibited gram-negative, coccoid bacteria; and nitrofurantoin, a wide-spectrum antibacterial antibiotic (13), suppressed both gram-positive and certain nontarget gram-negative bacteria.

The colonies of X. c. phaseoli were distinctive and easy to identify. They developed rapidly, had a light yellow color, and were circular in shape with entire margins, convex, and surrounded by a zone of starch hydrolysis. Certain

nontarget species grew on M-SSM, but their colonies appeared later and were smaller than those of X. c. phaseoli; the colonies of nontarget species were not associated with starch hydrolysis or yellow pigmentation. The M-SSM medium stored in plastic bags for 45 days was equal to freshly prepared plates in recovering X. c. phaseoli from diseased bean leaf tissue.

The highest recovery of X. c. phaseoli in this study, 96%, was on MXP, which was very similar to that reported by Claflin et al (6). However, three of 19 strains did not grow on the medium (Table 2). A high concentration of gentamicin sulfate may account for this lack of growth. In dose-response studies, Trujillo (13) found that X. c. phaseoli tolerated 0.5  $\mu$ g·ml<sup>-1</sup> gentamicin sulfate, but growth was reduced. In contrast, strains used by Claflin et al (6) grew on media containing up to  $3 \mu \text{g·ml}^{-1}$ , based on colony counts. Differences between the present study and previous studies (6,13) may be attributed to differential sensitivity of strains of X. c. phaseoli to gentamicin sulfate. For example, two of three fuscous strains used here failed to grow on MXP, but the third developed small colonies (Table 2). Thus, no single medium appears best for all strains of the pathogen (10).

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<sup>&</sup>lt;sup>x</sup> Means of three replicates for each test medium. Within an experiment, means followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

<sup>&</sup>lt;sup>y</sup> Media MXP (6) and liquid SSM (13) were prepared as reported. Difco Bacto agar (15 g/L) was added to liquid SSM. Soluble starch, basic dyes, antibiotics, and agar were added to liquid SSM to give M-SSS. YCA = yeast extract-calcium carbonate agar.

No colonies of X. c. phaseoli were detected, because of high numbers of contaminants.