Techniques

An Agar Medium for the Isolation and Identification of *Xanthomonas campestris* pv. *vesicatoria* from Seed

K. Sijam, C. J. Chang, and R. D. Gitaitis

First and second authors: Department of Plant Pathology, University of Georgia, Georgia Experiment Station, Griffin 30223; third author: Department of Plant Pathology, University of Georgia, Coastal Plain Experiment Station, Tifton 31793. Present address of first author: Department of Plant Protection, Universiti Pertanian Malaysia, 43400 Serdang, Selangor D.E., Malaysia.
This study was supported by state and Hatch funds.
Accepted for publication 1 March 1991 (submitted for electronic processing).

ABSTRACT

Sijam, K., Chang, C. J., and Gitaitis, R. D. 1991. An agar medium for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria* from seed. Phytopathology 81:831-834.

An agar medium was developed for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria,* the causal agent of bacterial spot of pepper and tomato. The new medium, designated as CKTM, contained soy peptone, Bacto tryptone, dextrose, L-glutamine, L-histidine, ammonium phosphate, potassium phosphate, magnesium sulfate, calcium chloride, Pourite, and agar. Selectivity was afforded with cycloheximide, bacitracin, neomycin, cephalexin, 5-fluorouracil, tobramycin, and Tween 80. Strains of *X. c. vesicatoria* were easily distinguished from strains of other pathovars of *X. campestris* by the formation of a clear ring around their colonies. The ring appeared 1–2 days after colony transfer or 3–4 days after serial dilutions were plated. Minute tan to white crystals of various intensity formed in the clear ring. *X. c. vesicatoria* was detected in 15 of 22 seed lots. Recovery of *X. c. vesicatoria* from tomato seed ranged from 17.7 to 100% on CKTM compared with 6.3 to 44.4% on Tween B medium. In addition, recovery of *X. c. vesicatoria* on CKTM medium was qualitatively superior to that of Tween B medium, with a greater reduction of contaminating microflora.

Additional keywords: Capsicum annuum, Lycopersicon esculentum, seed assay.

Growth of nontarget bacteria can be a problem in the detection and identification of target bacteria from diseased plant material, and from seed, soil, and weed samples. A semiselective medium can aid in the growth and identification of the target bacterium. Several selective and semiselective media have been developed for phytopathogenic bacteria that enable such differentiation (3,4,11–14), and several of them are specific for pathovars of *Xanthomonas campestris* (2,3,11–14). In addition, some of these same media have been used in seed assays for the determination of contamination with pathogenic bacteria.

X. c. pv. *vesicatoria,* the causal organism of bacterial spot of

pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.), was suggested to be seedborne. In early studies, seed from "suspected" contaminated seed lots were grown out and occurrence of symptoms recorded (5,7). Bashan et al (1) recovered the bacterium from pepper seed by using the leaf enrichment medium. Semiselective Tween media have been used to isolate the bacterium from soil (Tween B), plant materials (Tween A), and tomato seeds (Tween B) (10,11). A modification of Tween B medium by reducing cephalexin, tobramycin, and boric acid by 46, 50, and 67%, respectively, apparently increased its efficacy by supporting the growth of eight strains of *X. c. vesicatoria* (8). All of these media, however, still allow growth of several other pathovars of *X. campestris* as well as numerous nonpathogenic xanthomonads and saprophytes that produce culture characteristics similar to *X. c. vesicatoria,* i.e., the formation of zones of white crystals that consist of calcium salts of fatty acids (C. J. Chang, *unpublished*).

The present work was conducted to determine the effectiveness of a newly developed semiselective medium in differentiating strains of *X. c. vesicatoria* from other pathovars of *X. campestris* on colony morphology and to evaluate this medium for isolation of this pathovar from seed.

MATERIALS AND METHODS

Medium formulation. An isolation medium, designated as CKTM, was adapted from combination of Tween B (10,11) and a semiselective medium (CS20ABN) developed for *X. c. campestris* (2,3).

CKTM was prepared by adding and autoclaving the following components in 900 ml of distilled water: 1 drop of Pourite (Analytical Products, Inc., Belmont, CA), 2 g of soy peptone (Scotts Laboratories, Fiskeville, RI), 2 g of Bacto tryptone, 1 g of dextrose, 6 g of L-glutamine, 1 g of L-histidine, 0.8 g of $(NH_4)_2$ HPO_4, 1 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of anhydrous $CaCl_2$, and 12 g of Bacto agar. Ten milliliters of Tween 80 (tissue culture grade) was autoclaved separately and added immediately after the medium was removed from the autoclave. The following antibiotics were added to the cooled medium (45 C) in the following amounts: 0.5 ml of cycloheximide (stock: 1 g in 5 ml of methanol), 2 ml of bacitracin (stock: 1 g in 20 ml of distilled water), 0.5 ml of neomycin sulfate (stock: 0.4 g in 20 ml of distilled water), and 100 ml of sterile distilled water containing 65, 12, and 0.4 mg of cephalexin, 5-fluorouracil, and tobramycin, respectively.

All ingredients for CKTM were dissolved one at a time to avoid clumping and formation of precipitates when Tween 80 was added. Leaving a stir bar in the flask of the medium while it is being autoclaved and using Pourite are recommended to avoid the production of bubbles. After Tween 80 was added, the medium immediately appeared cloudy but turned clear when cool.

Bacterial strains. Pathovars of *X. campestris* and pectolytic strains of xanthomonads used to evaluate the selectivity of the medium are listed in Table 1. All strains were maintained in 2 ml of sterile tap water in screw cap vials at 4 C. When needed, the bacteria were streaked onto yeast dextrose calcium carbonate (YDC) agar plates, and pure cultures of the bacteria were either spot inoculated onto CKTM or Tween B medium or suspensions of approximately 10^8 colony-forming units (cfu)/ml were made, serially (1:9) diluted, and 0.1-ml aliquots of the dilutions were spread on both media. After 48–72 h of incubation at 28 C, colony morphologies were compared.

Seed assay. Twenty-two tomato seed lots supplied by Campbell Institute for Research and Technology, Davis, CA, were assayed for *X. c. vesicatoria.* Twelve grams of seed (approximately 5,000 seeds) from each seed lot were shaken in 100 ml of peptone buffer, pH 7.0, containing (per liter) 5.3 g of KH_2PO_4, 8.6 g of Na_2HPO_4, and 1 g of Bacto peptone (10) for 2 h at room temperature with a constant speed of 115 rpm on Gyrotory Shaker Model 62 (New Brunswick Scientific, New Brunswick, NJ). The suspension was filtered through a double layer of cheesecloth into a centrifuge tube and spun at 12,000 *g* for 30 min at 10 C. The resulting supernatant was discarded, and the pellet was resuspended in 2 ml of phosphate buffer, pH 7.2. The suspension was serially (1:9) diluted, and 0.1-ml aliquots of the dilutions were spread over three plates each of CKTM and Tween medium B. Plates were incubated at 28 C for 3–5 days and the number of colonies of *X. c. vesicatoria* and other bacteria was recorded and related to gram weight of seed.

Young and mature fruits of pepper (cv. Yolo) were collected from either diseased plants or artificially inoculated pepper plants grown in the greenhouse. Pepper plants were artificially inoculated with *X. c. vesicatoria* as described elsewhere (4). Flowers of plants at the first flower stage of growth were sprayed with a suspension of sterile distilled water containing approximately 10^6 cfu/ml of *X. c. vesicatoria* with a chromatographic atomizer. Plants were inoculated twice. Fruits were harvested at various stages of maturity, and the seeds removed aseptically. Seeds were dried overnight on sterile paper towels under a Laminair flow hood (Environmental Air Control, Inc., Hagerstown, MD). To determine the number of contaminated seeds in the sample of harvested seeds, approximately 100–300 seeds (from five fruits) were placed on CKTM medium. Plates were incubated at 28 C for 7–14 days. The number of contaminated seeds was recorded.

Effect of Tween 80 concentration on colony differentiation. Concentrations of Tween 80 at 2.5, 5.0, 10, 15, 20, or 25 ml/L were tested for their effect on the ring or zone surrounding the colony of *X. c. vesicatoria.*

Pathogenicity test. Suspect colonies of *X. c. vesicatoria* obtained from the seed assay were purified by streaking onto YDC medium. All isolates (one isolate for each seed lot) and representative strains of *X. c. vesicatoria* were suspended in phosphate buffer (pH 7.2)

TABLE 1. Characteristics of pathovars of *Xanthomonas campestris* and pectolytic xanthomonads on CKTM medium and Tween B medium

Bacterial strains	Number	Clear ring on CKTM[a]	Lipolytic zone on Tween B[b]
X. c. pathovars			
vesicatoria	18[c]	+[d]	+
campestris	3	−	+
phaseoli	2	−	+
raphani	3	−	+
malvacearum	1	−	+
translucens	6	+	+
incanae	1	−	+
Pectolytic xanthomonads	9	−	+

[a] Formation of a clear ring around the colony.
[b] Formation of minute crystals around the colony.
[c] Of the 18 strains, three were from Florida, three from North Carolina, and 12 from Georgia.
[d] + and − represent formation and no formation, respectively, of either the clear ring or the lipolytic zone.

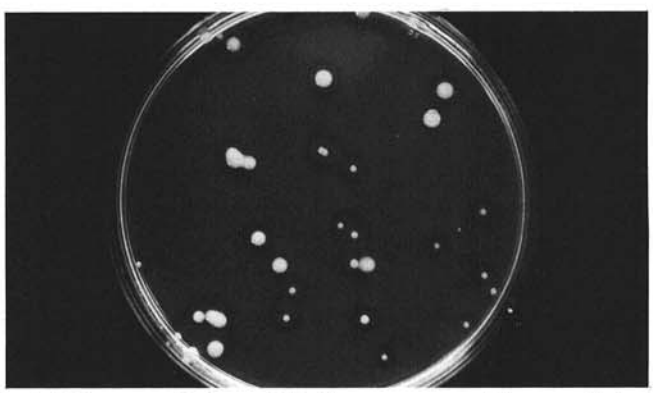

Fig. 1. Colony morphology of *Xanthomonas campestris* pv. *vesicatoria* on CKTM medium. Note clear ring formation around individual colonies.

TABLE 2. Recovery of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) from commercial tomato seed lots on CKTM and Tween B medium

Seed lot	Number of colony-forming units[a]				Xcv (%)	
	Xcv		Total bacteria[b]			
	CKTM	Tween B	CKTM	Tween B	CKTM	Tween B
307	70	52	180	120	38.9	43.3
308	130	54	280	420	46.4	12.9
309	110	80	360	260	30.6	30.8
310	96	82	320	460	30.0	17.8
311	90	72	260	420	34.6	17.1
312	200	140	320	380	62.5	36.8
314	460	200	2,600	3,200	17.7	6.3
315	120	60	520	520	23.1	11.5
317	220	114	540	800	40.7	14.3
318	180	120	480	280	37.5	42.8
319	0	0	780	980	0.0	0.0
320	220	160	500	360	45.2	44.4
321	0	0	0	160	0.0	0.0
322	0	0	0	140	0.0	0.0
323	0	0	0	50	0.0	0.0
324	0	0	0	112	0.0	0.0
325	0	0	40	100	0.0	0.0
326	20	0	20	40	100.0	0.0
327	30	20	56	58	53.6	34.5
328	0	0	0	40	0.0	0.0
162	120	80	240	320	50.0	25.0
720	80	60	320	300	25.0	20.0

[a]Twelve grams of seed per lot was washed in buffer and washings were plated on three plates of each medium.
[b]Total bacteria included *X. c. vesicatoria* and saprophytes.

and tested for pathogenicity on tomato (cv. Floradade) and pepper (cv. Yolo) plants. Suspensions containing approximately 10^8 cfu/ml of bacteria were used for inoculation. Suspensions of each isolate were atomized with a chromatographic sprayer onto leaf surfaces of 4- to 6-wk-old plants. Three tomato and three pepper plants were used for each isolate. Control plants were sprayed with phosphate buffer. After inoculation, plants were placed in a moist chamber for 48 h, and then were moved to greenhouse benches for incubation for 2–4 wk to observe symptom development. The pathogenicity test was repeated twice. Reisolation of the *X. c. vesicatoria* from inoculated leaves was done by placing the leaf disks on CKTM as well as on YDC medium. The inoculated leaves were surface-sterilized before removing lesions with a hole puncher.

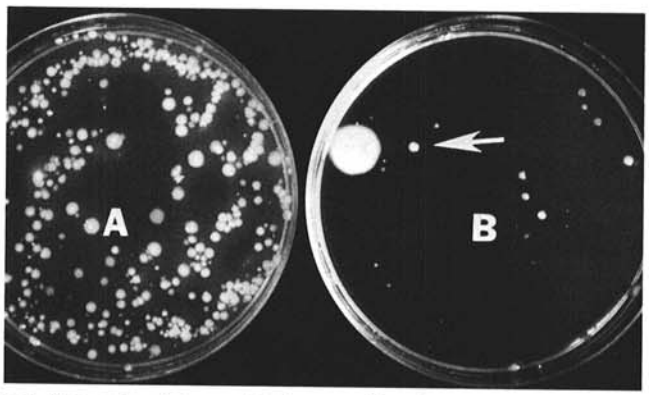

Fig. 2. Comparison between **A,** Tween medium B; and **B,** CKTM medium for the selective isolation of *Xanthomonas campestris* pv. *vesicatoria* from a commercial tomato seed lot. Note a clear ring (arrow) surrounding colony of *X. c. vesicatoria* on CKTM medium.

RESULTS

Colony differentiation. All isolates of *X. c. vesicatoria* on CKTM appeared as circular, raised, yellow colonies surrounded by a clear ring (Fig. 1). This clear ring or zone formed as early as 1–2 days after spot inoculation onto the medium, or 3–4 days after dilutions were plated. Either tan to white minute crystals formed in the clear ring (pepper strain) or dense white haloes developed around the colonies inside the clear ring (tomato strain) (C. J. Chang, *unpublished*). The size of clear rings varied among strains of *X. c. vesicatoria.* The diameter of clear ring increased with an increase in incubation time. Other pathovars of *X. campestris* and opportunistic xanthomonads, except *X. c. translucens,* could be distinguished from *X. c. vesicatoria* by the absence of a clear ring around the colony (Table 1). Instead, these pathovars and opportunistic xanthomonads only produced a dense white halo around the colony without the initial formation of a clear ring. On Tween B medium, pathovars of *X. campestris* including *X. c. vesicatoria* and the opportunistic xanthomonads developed a zone of white crystals around the colony and were indistinguishable from each other.

Recovery of *X. c. vesicatoria* from seed. *X. c. vesicatoria* was detected in 15 of 22 tomato seed lots (Table 2). On CKTM medium, recovery of *X. c. vesicatoria* increased and in a majority of samples the number of contaminating saprophytes decreased (Table 2 and Fig. 2). Colonies of *X. c. vesicatoria* were larger and easier to distinguish from contaminating saprophytes (Fig. 2). The number of *X. c. vesicatoria* isolates detected ranged from 17.7–100% of total bacteria recovered on CKTM compared with 6.3–44.4% on Tween B medium.

Out of 17 fruit samples tested, seven were positive for the presence of *X. c. vesicatoria* in seeds. The highest detectable level (15%) was obtained from one fruit sample.

Effects of Tween concentration on colony differentiation. The clear ring or zone around colonies of *X. c. vesicatoria* was greatly affected by concentration of Tween 80. No clear ring formation was observed when the concentration of Tween was below 10 ml/L. Concentrations of Tween 80 above 10 ml/L inhibited the formation of the dense white halo around colonies of tomato strains of *X. c. vesicatoria* (data not shown). However, at higher concentrations (15, 20, and 25 mg/L), the formation of the clear ring was not affected.

Pathogenicity test. All presumptive isolates of *X. c vesicatoria* on CKTM medium produced typical bacterial spot symptoms 2–3 wk after inoculation of both pepper and tomato plants. Isolates recovered from diseased leaves produced a yellow colony surrounded by a clear ring typical of *X. c. vesicatoria* on CKTM.

DISCUSSION

Many plant pathogenic bacteria can be identified by the use of artificial culture media. Distinguishing characteristics can be enhanced by the incorporation of selected chemicals or antibiotics. Tween B medium has been used in the identification of *X. c. vesicatoria.* KBr enhances the yellow dibromo-methoxyphenylpolyene pigment, and Tween 80 in conjunction with $CaCl_2$ demonstrates lipolysis (11). However, most pathovars of *X. campestris* and contaminating bacteria associated with seed have lipolytic activity highly similar to *X. c. vesicatoria.* Even though some contaminants can be distinguished easily from other xanthomonads by the color of their colonies, others are not so easily distinguished. CS20ABN (2,3), the semiselective medium developed for *X. c. campestris,* was able to support growth of *X. c. vesicatoria,* but could not be used to differentiate it from other pathovars of *X. campestris* (C. J. Chang, *unpublished*). Modifications of CS20ABN were made based on the lipolytic activity of *X. c. vesicatoria* and its inability to hydrolize starch. These modifications resulted in the formulation of CKTM. The clear ring around colonies of *X. c. vesicatoria,* 1–2 days after colony transfer, is a reliable diagnostic feature of *X. c. vesicatoria* and a rapid technique for identifying the bacterium in mixed cultures. The only other pathovar of *X. campestris* tested that forms a clear ring around its colony is *X. c. translucens.* This may not constitute a problem in seed assay because neither tomato nor pepper is considered as a host plant of *X. c. translucens* as reported by Leyns et al (9).

Selectivity of the medium was afforded by the use of cycloheximide, bacitracin, neomycin sulfate, cephalexin, 5-fluorouracil, and tobramycin. Cycloheximide specifically inhibits fungi, and its concentration in the medium can be increased without adversely affecting the recovery of *X. c. vesicatoria.* Cephalexin specifically inhibits *Erwinia herbicola;* 5-fluorouracil effectively eliminates fluorescent pseudomonads; and tobramycin is very effective against other pseudomonads that inhabit plant tissues.

Although CKTM did not eliminate all contaminants found on seed, the benefit of using the medium in seed and plant assays is significant. None of the pectolytic xanthomonads tested displayed a clear ring colony characteristic. This is most useful since the pectolytic xanthomonads are weak pathogens but can be found associated with both seed and plant material (6).

LITERATURE CITED

1. Bashan, Y., Okon, Y., and Henis, Y. 1982. Long-term survival of *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper seeds. Phytopathology 72:1143-1144.
2. Chang, C. J., Donaldson, R., Crowley, M., and Pinnow, D. 1989. Comparison of the isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds in four semiselective media. (Abstr.) Phytopathology 79:1140.
3. Chang, C. J., Donaldson, R., Crowley, M., and Pinnow, D. 1990. Isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds using a highly selective medium. Pages 533-539 in: Proc. Int. Conf. Plant Path. Bact., 7th. Z. Klement, ed. Budapest, Hungary.
4. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78:121-126.
5. Gardner, M. W., and Kendrick, J. B. 1923. Bacterial spot of tomato and pepper. Phytopathology 13:307-315.
6. Gitaitis, R. D., Sasser, M. J., Beaver, R. W., McInnes, T. B., and Stall, R. E. 1987. Pectolytic xanthomonads in mixed infections with *Pseudomonas syringae* pv. *syringae, P. syringae* pv. *tomato,* and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper transplants. Phytopathology 77:611-615.
7. Higgins, B. B. 1922. The bacterial spot of pepper. Phytopathology 12:501-516.
8. Kim, H. M., and Schaad, N. W. 1989. Modified Tween medium for detecting *Xanthomonas campestris* pv. *vesicatoria* in tomato seeds. (Abstr.) Phytopathology 79:1140.
9. Leyns, F., De Cleene, M., Swings, J-G., and De Ley, J. 1984. The host range of the Genus *Xanthomonas.* Bot. Rev. 50:308-356.
10. McGuire, R. G., and Jones, J. B. 1989. Detection of *Xanthomonas campestris* pv. *vesicatoria* in tomato. Pages 59-62 in: Detection of Bacteria in Seed and Other Planting Material. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. American Phytopathological Society, St. Paul, MN.
11. McGuire, R. G., Jones, J. B., and Sasser, M. 1986. Tween media for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant materials. Plant Dis. 70:887-891.
12. Norman, D., and Alvarez, A. 1989. A rapid method for presumptive identification of *Xanthomonas campestris* pv. *dieffenbachiae* and other xanthomonads. Plant Dis. 73:654-658.
13. Randhawa, P. S., and Schaad, N. W. 1984. Selective isolation of *Xanthomonas campestris* pv. *campestris* from crucifer seeds. Phytopathology 74:268-272.
14. Schaad, N. W., and Forster, R. L. 1985. A semiselective agar medium for isolating *Xanthomonas campestris* pv. *translucens* from wheat seeds. Phytopathology 75:260-263.