Techniques

A Semiselective Medium for the Isolation of *Xanthomonas campestris* pv. *juglandis* from Walnut Buds and Catkins

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ABSTRACT

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A medium was developed for the semiselective isolation of *Xanthomonas campestris* pv. *juglandis* from Persian (English) walnuts, *Juglans regia*. This brilliant cresyl blue-starch (BS) medium recovered 52–100% of the *X. campestris* pv. *juglandis* colony-forming units (cfu) compared to King's medium B (KB) when isolations were from naturally-infested walnut tissues, and eliminated 97% of the microorganisms found on KB. The starch hydrolysis zone surrounding *X. campestris* pv. *juglandis* colonies made them distinctive and easily distinguishable from the other bacteria that grew on BS. Six other *X. campestris* pathovars grew on BS medium: pv. *begoniae*, pv. *campestris*, pv. *incanae*, pv. *malvacearum*, pv. *phaseoli*, and pv. *vesicatoria*. The role of dormant and developing walnut buds and catkins as overwintering sites for *X. campestris* pv. *juglandis* was investigated by using the BS medium. Buds and catkins from two early blooming cultivars (Payne and Ashley) were infested, with the frequency of bud and catkin infestation ranging from 50–80% and 0–90%, respectively. The frequent infestation of walnut buds and catkins by *X. campestris* pv. *juglandis* indicated that this pathogen has a resident phase (sensu Leben) on walnuts.

Xanthomonas campestris pv. *juglandis* causes a leaf, twig, and nut blight of English (Persian) walnuts (*Juglans regia* L.) (15). The disease is of economic importance in California and Oregon and has been reported in other states (1,11,13,15). All of the cultivars grown in California are susceptible; however, cultivars with bloom periods that coincide with spring rains are the most severely blighted.

The majority of epidemiological data on walnut blight has been observational, fragmentary, and often contradictory (1–3,12,13). Consequently, how the pathogen survives, and the environmental factors that are conducive to inoculum buildup are not clearly understood. Gathering data on the epidemiology of this pathogen is dependent upon the development of techniques to allow the selective isolation and differentiation of *X. campestris* pv. *juglandis* from background walnut microflora. Techniques and media available for the quantitative study of *Xanthomonas* spp. under field conditions (7,16) were not applicable to the study of *X. campestris* pv. *juglandis* because these media lack the selectivity required for quantitative isolation of this pathogen.

This paper reports the development of brilliant cresyl blue-starch (BS) medium, a semiselective differential medium for the isolation

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of *X. campestris* pv. *juglandis* from walnut. This medium has been used to study the role of buds and catkins in the overwinter survival of *X. campestris* pv. *juglandis* on early blooming (15 March–1 April) walnut cultivars. A preliminary report has been published (14).

MATERIALS AND METHODS

Medium. A semiselective medium for the isolation of *Xanthomonas campestris* pv. *juglandis* was developed by incorporating selected carbon and nitrogen sources (17) into a basal medium composed of (g/L): KH_2PO_4, 1.5; Na_2HPO_4, 3.0; NH_4Cl, 1.0; $MgSO_4 \cdot 7H_2O$), 0.2; beef extract, 1.0, and Difco bacto agar, 15.0. Carbon and nitrogen sources were evaluated by comparing growth of five strains of *X. campestris* pv. *juglandis* with that of other bacteria that occur on walnut leaves and nuts. Media with various carbon and nitrogen sources were seeded with a cell suspension harvested from 72-hr-old cultures grown on King's B medium (KB). Serial dilutions of 1.0×10^8 colony-forming units (cfu)/ml suspensions were placed on test media at 0.1 ml per plate, spread with a sterile glass rod and incubated at 28 C. All bacterial strains used in these studies are listed in Table 1.

Bud and catkin sample collection. Dormant and developing buds and catkins were collected, between January and November, from orchards of the cultivars Payne and Ashley that had previously been blighted. Samples were collected by pruning 10- to 15-cm twig segments from the terminal growth of scaffold branches. Five to 15 trees were sampled per ochard by collecting eight to 12 twigs per tree. Twig samples collected during the growing season (March–October) were stripped of leaves to prevent infected foliage from contaminating buds and catkins in transit. Samples were transported to the laboratory in an ice chest and isolations usually were made within 24 hr after collection. Otherwise samples were stored at 4 C.

Apparently healthy buds and catkins were excised from twigs by using a sterile razor blade. Each bud and catkin sampled was suspended in 10 ml of sterile distilled water (SDW) and agitated intermittently on a vortex mixer for 30 min. A 0.1-ml aliquot from each suspension was transferred to each of the test media in petri dishes and spread with a sterile glass rod. The media were examined with a dissecting microscope and oblique lighting for colony growth after 48–72 hr of incubation at 28 C.

Recovery of *X. campestris* pv. *juglandis* from naturally-infested materials. The efficiency of BS medium in recovering *X. campestris* pv. *juglandis* from naturally-infested walnut buds and catkins was compared to that of KB, SX agar (16), and D-5 medium (7). Ten buds and 10 catkins of cultivar Payne were sampled and processed as described above. Three plates of each medium were seeded with 0.1 ml of the suspension that was prepared from each bud and catkin. Colony counts were made after 72 hr at 28 C and were expressed as the percentage of colony-forming units recovered on KB.

Comparative recovery of *X. campestris* pathovars. Twenty-eight bacterial strains representing 12 *X. campestris* pathovars (Table 2) were seeded onto BS medium to determine if it could serve as a general isolation medium for *X. campestris* pathovars. All strains were grown on yeast-dextrose-$CaCO_3$-peptone (YDCP) medium prior to being tested on BS. Strains were initially mass streaked on BS to qualitatively assess growth. Those strains that grew when mass streaked were studied quantitatively by comparing the number of colonies recovered on BS to the number recovered on KB. Strains grown for 72 hr at 28 C on YDCP were harvested and suspended in SDW. Serial dilutions from these stock suspensions were plated (0.1 ml per plate) on BS and KB. Plates were incubated at 28 C and comparison counts were made after 48–72 hr.

RESULTS

Medium. A semiselective medium for the isolation and differentiation of *X. campestris* pv. *juglandis* from walnut buds and catkins was developed and tested. This brilliant cresyl blue-starch (BS) medium contained (g/L): potato starch, 10.0; $K_2HPO_4 \cdot 3H_2O$, 3.0; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 2.0; L-methionine, 0.25; nicotinic acid, 0.25; L-glutamate, 0.25 (Sigma Chemical Company, St. Louis, MO 69178); brilliant cresyl blue, 0.01 (J. T. Baker, Phillipsburg, NJ 08865, 55% total dye content): methylene green, 0.01 (Allied Chemical and Dye Corporation, New York, NY), and Difco bacto agar, 15.0. The pH of the medium was adjusted to 6.8–7.0, before autoclaving, with 8.0 ml/L of 1N NaOH. The medium could be stored in bottles and remelted when needed.

Xanthomonas campestris pv. *juglandis* appeared on the medium as small, round, pale blue, opalescent colonies with intact margins, surrounded by an opaque zone due to starch hydrolysis (Fig. 1). Plates could be flooded with a half-strength solution of Burke's Iodine to precipitate the unhydrolyzed starch and enhance the appearance of the hydrolysis zone (18). This treatment, however, killed the viable bacteria and generally was not necessary.

Adonitol, D-aspartate and potato starch showed promise as selective carbon sources for isolation of the pathogen. Starch was selected as the carbon source for the final medium because growth of *X. campestris* pv. *juglandis* on starch media produced a distinctive zone of hydrolysis around the colonies.

Comparative recovery of *X. campestris* pv. *juglandis* from walnuts. The percentage of *X. campestris* pv. *juglandis* colony-forming units recovered on BS medium compared to KB varied from 51.7 to 100%. Ninety-seven percent of the other bacteria that grew on KB did not develop on BS. The percentage of colony-forming units of the pathogen on SX agar and D5 medium compared to KB averaged 5.7 and 0%, respectively. Kado's D-5 medium for general isolation of *X. campestris* pathovars did not recover *X. campestris* pv. *juglandis* either from walnut buds and catkins or when inoculated with laboratory-grown cultures. Schaad and White's SX agar, which was developed for isolating *X. campestris* from soil, contains methyl violet B which reduced the growth rate of *X. campestris* pv. *juglandis* so that only pinpoint colonies (that did not produce starch hydrolysis zones) developed after 5 days of incubation.

To determine the accuracy of identifying *X. campestris* pv. *juglandis* on BS medium, 90 representative isolates were selected from field isolations on the basis of colony morphology and starch

TABLE 1. *Xanthomonas campestris* pathovar strains used to evaluate a newly developed selective isolation medium

Pathovar	Strain no.	Source
begoniae	854	Author, Berkeley, CA
campestris	855,857,XC1, XC2, X26, X28	Author, Berkeley, CA
holcicola	X26, X28	D. Sands, Bozeman, MT
incanae	867, 870	Author, Berkeley, CA
juglandis	1025,1060	Author, Berkeley, CA
	4544, 35	D. Dye, New Zealand
	3D5	C. Kado, Davis, CA
malvacearum	875, 876	Author, Berkeley, CA
oryzae	XO 101, XO 115, XO 120	A. Kelman, Madison, WI
phaseoli	880	Author, Berkeley, CA
pruni	APB, SCB	M. Davis, New Brunswick, NJ
hordei	X21, X24, X25	D. Sands, Bozeman, MT
vesicatoria	884, 891	Author, Berkeley, CA
vitians	895	Author, Berkeley, CA

hydrolysis. All of these isolates produced yellow mucoid colonies, which are characteristic of xanthomonads when streaked on YDCP. Fifty-three of these isolates were then compared serologically to *X. campestris* pv. *juglandis* ATCC #11329 by using a drop agglutination technique (19). Forty-nine of the 53 (92.4%) were agglutinated by *X. campestris* pv. *juglandis* antiserum. A random selection of 25 isolates that showed serological similarity to ATCC #11329 were tested for pathogenicity by inoculating nutlets of the cultivar Payne with an aqueous cell suspension ($\sim 1 \times 10^8$ cfu/ml) containing corundum which was applied with a stiff camel's hair brush (3 cm wide). All isolates caused typical blight symptoms and the pathogen was consistently reisolated from the infected nutlets on BS. Ten control nuts inoculated as above with SDW did not develop blight lesions. The four isolates that were not agglutinated by *X. campestris* pv. *juglandis* antiserum were lost and could not be tested for pathogenicity.

Recovery of other *X. campestris* pathovars on BS. Eight of 12 *X. campestris* pathovars tested grew on BS medium (Table 2). Of these, *X. campestris* pv. *juglandis*, *X. campestris* pv. *malvacearum*, and *X. campestris* pv. *vesicatoria* were, on the average, recovered with greater efficiency on BS than on KB. The recovery of strains capable of growth as single colonies on BS ranged from 22 to 737.5% compared to KB. No differences in plate counts were observed between 48 and 72 hr of incubation. However, starch hydrolysis zones were most easily discerned after 72 hr at 28 C. *X. campestris* pv. *holcicola*, *X. campestris* pv. *oryzae*, *X. campestris* pv. *hordei*, and *X. campestris* pv. *vitians* did not grow on BS medium even when mass streaked. With the exception of *X. campestris* pv. *vitians*, all these strains grew poorly on KB and YDCP, even after repeated subculturing on these media. *Xanthomonas campestris* pv. *oryzae* did not grow in single colonies even on rich media and did not utilize starch as a primary carbon source.

Recovery of *X. campestris* pv. *juglandis* from walnut buds and catkins. In the orchards studied, buds infested with *X. campestris* pv. *juglandis* ranged from 5 to 80% with bacterial populations up to 4.0×10^5 cfu per bud (Table 3). Of the catkins sampled, the percent infested ranged from 0 to 90 with single catkin populations up 1.75×10^4 cfu. Orchards for this study were selected from areas of the state where annual heavy spring rains initiated regular blight epidemics. Only orchards of cultivars Payne or Ashley were used since these bloomed the earliest and were the most severely blighted. In seven of the eight orchards sampled, blight control procedures consisted of two to three applications of copper applied during pistillate bloom (March–April). In these orchards *X. campestris* pv. *juglandis* caused 20–40% yield losses and greater than 45% of the buds and catkins were infested. In one orchard, where eight to 10 applications of copper were used, 5% of the buds and none of the catkins were infested and yield losses due to blight were about 3%.

TABLE 2. Comparative recovery of pure cultures of 12 *Xanthomonas campestris* pathovars

X. campestris pathovar	Isolate no.	Colonies per plate[a] BS	KB	Percent recovery[a] (BS/KB × 100)
begoniae	854	86	120	71.6
campestris	855	73	331	22.0
	857	0[b]	···	0
	XC1	334	163	200.0
	XC2	72	275	26.1
holcicola	X26	0	···	0
	X28	0	···	0
incanae	867	233	424	54.9
	870	0	···	0
juglandis	1025	333	175	127.0
	1060	107	84	190.2
	4574	172	244	70.5
	35	201	218	92.2
	3D5	71	136	52.2
malvacearum	875	362	281	128.8
	876	382	207	184.5
oryzae	XO 101	0	···	0
	XO 115	0	···	0
	XO 120	0	···	0
phaseoli	880	135	65	207.6
	881	127	233	54.5
hordei	X21	0	···	0
	X24	0	···	0
	X25	0	···	0
vesicatoria	884	295	40	737.5
	891	525	512	102.5
vitians	895	0	ND	0

[a] Plate counts all adjusted to the 10^{-7} dilution. BS = brilliant cresyl blue-starch agar; KB = King's B agar.

[b] Counts of zero represent isolates that did not grow when mass-streaked on BS agar and were, therefore, not tested in quantitative isolation studies.

DISCUSSION

The BS medium proved to be effective for studying the epidemiology of *X. campestris* pv. *juglandis* because of its high selectivity and differentiating capacity. The medium was used to quantitatively determine the nature and extent of walnut bud and catkin infestation by *X. campestris* pv. *juglandis*.

Potato starch (Difco) was used in the medium as it dissolved more readily than soluble starch (Mallinckrodt) and the resulting

TABLE 3. Isolations of *Xanthomonas juglandis* from dormant buds and catkins of early blooming walnut cultivars[a]

Cultivar	Infested buds (%)	Infested catkins (%)
Ashley	80	90
Ashley	80	90
Ashley	70	50
Ashley	5	0
Payne	60	70
Payne	75	55
Payne	45	58
Payne	70	55

[a] 20 to 40 buds and catkins were collected at each location sampled and plated on BS medium. The numbers represent the percent of buds and catkins on each sample that harbored *X. campestris* pv. *juglandis*.

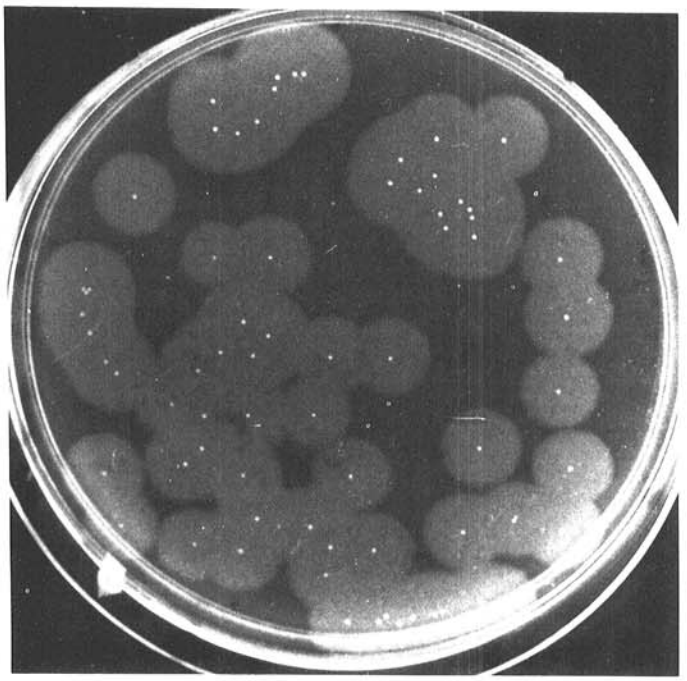

Fig. 1. *Xanthomonas campestris* pv. *juglandis* colonies on brilliant cresyl blue-starch medium (BS) after 72 hr of incubation at 28 C. Clear starch hydrolysis zones surrounding the colonies allowed rapid differentiation of the pathogen from other bacteria that grew on BS medium.

medium was more transparent. This transparent quality enhanced the appearance of the hydrolysis zones around *X. campestris* pv. *juglandis* colonies and facilitated identification of the pathogen. L-glutamate, L-methionine, and nicotinic acid provided growth factors which increased the growth rate of the pathogen (4).

Brilliant cresyl blue was an important selective agent in this medium, as it inhibited many Gram-positive and most Gram-negative bacteria with the exception of those found in the Pseudomonadaceae (6). Methylene green suppressed the growth of other Gram-positive bacteria encountered when isolating *X. campestris* pv. *juglandis* from walnut tissues. Because of the selective nature of brilliant cresyl blue and the organic acid constituents, oxidase-negative, fluorescent *Pseudomonas syringae* group bacteria were the most frequently encountered contaminant on BS medium. These bacteria did not readily hydrolyze the starch in the medium and thus were easily distinguished from *X. campestris* pv. *juglanids.*

The efficient recovery of *X. campestris* pv. *malvacearum*, *X. campestris* pv. *phaseoli*, *X. campestris* pv. *pruni*, and *X. campestris* pv. *vesicatoria* on BS medium indicated that this medium might also be applicable to studying the ecology of these pathogens. The high variability observed in the recovery of *X. campestris* pathovars on BS medium was not surprising considering their nutritional heterogeneity and the relative nutritional austerity of this medium (5).

A serious problem encountered in the development and evaluation of any selective medium concerns the nature and history of the bacterial strains that are tested. Because of the potential development of auxotrophs by repeated mass transfer, many of the strains that exist in culture collections may not have the same properties as wild-type strains. In our studies freshly isolated strains tended to grow better and were more readily recovered than were those that had been stored. In some cases, stored strains did not grow well even on nutritionally rich media. Thus, the use of laboratory-grown strains for evaluating selective media may give little indication of the sensitivity the medium may exhibit when isolations are made from naturally-infected plant parts.

With the BS medium, variability in recovery efficiency among strains of a given *X. campestris* pathovar appeared to be inversely related to the length of time isolates had been in storage. *X. campestris*, isolate 857, which had been in storage for 12 yr, did not grow on BS medium while XC1, freshly recovered from cabbage in the Salinas Valley prior to testing, was recovered with high efficiency. Similarly, *X. campestris* pv. *juglandis* isolates 35 and 3D5, originally isolated in 1956 and 1968, respectively, were recovered with much lower efficiency that isolates 1025 and 1060, which were fresh field isolates.

The frequent recovery of abundant *X. campestris* pv. *juglandis* inoculum from buds and catkins indicates that this pathogen has a resident phase (sensu Leben [9,10]) on walnuts (8). These data help explain Ark's observation (1) that early season leaf infections always occurred following rains even when obvious sources of inoculum, such as twig cankers and blighted catkins, were lacking. These data further suggest that controls should be directed at reducing inoculum at these overwintering sites and that the use of materials to eradicate *X. campestris* pv. *juglandis* from dormant buds and catkins needs to be investigated.

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