Development of a New Semiselective Medium for Isolating *Xanthomonas campestris* pv. *manihotis* from Plant Material and Soil

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ABSTRACT

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An effective control for bacterial blight of cassava (*Manihot esculenta*), caused by *Xanthomonas campestris* pv. *manihotis*, requires the use of non-contaminated cuttings and seeds. Using classical agar plating techniques for screening planting material for contamination has not been very successful because of the lack of a reliable semiselective agar medium. The pathogen grows slowly on general plating media and is easily overgrown by saprophytic bacteria during isolation from diseased plants. In an effort to develop a semiselective medium, the utilization of several carbon and nitrogen sources was studied. Results of these tests provided information used to design a basal medium allowing good growth of the target organism while suppressing growth of several common saprophytes. Additional selectivity was achieved by incorporating three antibiotics into the basal

medium. The new semiselective agar medium, designated cefazolin trehalose agar (CTA) medium, contained (per liter) 3.0 g of K_2HPO_4 , 1.0 g of NaH_2PO_4 , 0.3 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4Cl , 9.0 g of D(+)-trehalose, 1.0 D(+)-glucose, 1.0 g of yeast extract, 0.025 g of cefazolin, 0.0012 g of lincomycin, 0.0025 g of phosphomycin, 0.25 g of cycloheximide, and 14.0 g of agar. In comparison to a starch-based semiselective medium (SXM), plating efficiencies using pure cultures of 10 strains of *X. campestris* pv. *manihotis* were significantly higher on CTA, with an average of 85 and 50%, respectively. Likewise, isolation and recovery of *X. campestris* pv. *manihotis* from infected cassava leaves and contaminated soil were much higher on CTA than on SXM agar. When *X. campestris* pv. *manihotis* occurs in high concentrations in diseased tissue, the standard yeast trehalose glucose agar medium supplemented with 250 μ g of cycloheximide per ml appears to be satisfactory. The newly developed CTA medium should prove useful for control strategies to identify and remove infected planting material of cassava, as well as for basic ecological studies of the pathogen.

Cassava bacterial blight (CBB), caused by *Xanthomonas campestris* pv. *manihotis*, is a major constraint to cassava (*Manihot esculenta* Crantz) cultivation, and losses can be extremely severe in regions where highly susceptible cultivars are grown (22). On the basis of genotypic studies, Vauterin et al. (34) recently proposed a reclassification of *Xanthomonas*, renaming the CBB organism *X. axonopodis* pv. *manihotis*. However, since this change is not widely known and not yet accepted by the scientific community, we will use *X. campestris* pv. *manihotis* to avoid confusion. The pathogen was first reported in Brazil (4) and later observed in several additional countries of South America (20), Africa (14,33,35,38), and Asia (5). In Africa, losses have exceeded 50% (22).

X. campestris pv. *manihotis* is a Gram-negative, nonpigmented slender rod with a single polar flagellum (6). The pathogen penetrates via stomata or through wounds in epidermal tissues of leaves and young shoots, invades the vascular tissue, causes extensive breakdown of parenchymatous tissue (32), and results in water-soaking to brown-blighting symptoms (22). Since the pathogen invades its host systemically, transmission by vegetative propagation of cassava is often overlooked.

Sustainable control of the bacterial pathogen can be obtained by the use of integrated control measures (agronomic, biological, and chemical) in addition to varietal resistance (21). Breeding for resistance is a time-consuming process, and until cassava cultivars with durable resistance against CBB are available, the disease will need

to be controlled by hygienic measures. Since the pathogen is seed-borne (10,12,24) and survives as an epiphyte (9,12,24), sensitive detection methods are needed to screen seed lots and cuttings used for propagation.

In contrast to most other xanthomonads, colonies of *X. campestris* pv. *manihotis* are white on agar media and are, therefore, somewhat more difficult to distinguish from many naturally occurring saprophytes. In addition, the pathogen is often overgrown by saprophytic bacteria during isolation from infected plants; so a semiselective medium is needed. A starch-based medium (SXM) designed by Leksomboon et al. (19) for isolating *X. campestris* pv. *manihotis* proved not to be useful because most African strains failed to grow on SXM. Therefore, to facilitate control of the disease by eradicating infected cuttings and to carry out epidemiological studies, we designed a new semiselective agar medium.

MATERIALS AND METHODS

Bacterial strains. Twenty-nine original strains of *X. campestris* pv. *manihotis* were isolated in Africa, and eleven were received from collections (Table 1). The pathotype strain (GSPB no. 2337) was obtained from the International Collection of Microorganisms from Plants (ICMP no. 5741), New Zealand. New strains were isolated from leaves and stems of cassava plants from Benin, Nigeria, and Uganda and were maintained on yeast glucose chalk agar at 14°C (31). The freshly isolated strains were tested for pathogenicity on 6-week-old, susceptible plants of the local cultivar Agric from Benin as previously described (11).

One strain of *X. campestris* pv. *cassavae* and several epiphytic strains were included (Table 1). The bacteria were maintained on slants of yeast glucose chalk medium (31) by transferring every 6 months.

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For long-term preservation, strains were lyophilized in evaporated skim milk as a cryopreservative.

Carbon and nitrogen utilization. The capability to utilize different carbon compounds was determined by supplementing a defined agar medium (per liter: 3.0 g of K_2HPO_4 , 1.0 g of NaH_2PO_4 , 0.3 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of NH_4Cl , and 14.0 g of agar-agar no. 1 from Nordwald Co., Hamburg, Germany) with 1.0% of the following carbon sources: soluble potato starch (no. 101252; E. Merck AG, Darmstadt, Germany), α -D-lactose, D(+)-galactose, D(+)-cellobiose, D(+)-trehalose, melibiose, methylsuccinic acid, 2-oxoglutaric acid, bromosuccinic acid, citrate, L-proline, and glycerol (Sigma Chemical Co., St. Louis). Inorganic nitrogen sources were tested in the defined agar medium containing 1% glucose and no NH_4Cl . Peptone was tested as an organic nitrogen source and $(NH_4)_2SO_4$, NH_4Cl , and $NH_4H_2PO_4$ as inorganic nitrogen sources.

L-Glutamic acid and L-methionine were tested for their growth-promoting effects as proposed by Starr (30). In these experiments, 0.1% L-glutamic acid or 0.02% L-methionine was added to the defined agar medium containing 1% D(+)-trehalose. The pH of the basal medium was 7.1 prior to autoclaving and was not adjusted.

After determining the nutritional requirements of *X. campestris* pv. *manihotis*, growth of strains on the newly developed agar media was compared with growth on the SXM medium of Leksomboon et al. (19) (per liter: 0.8 g of K_2HPO_4 , 0.6 g of KH_2PO_4 , 0.7 g of yeast extract, 8.0 g of soluble starch, 1.0 g of glucose, and 14.0 g of agar) without addition of the antibiotics and biological stains. The number of colony forming units (CFU) was determined by dilution-plating methods, and plating efficiencies were expressed as the percentage of colonies grown on test media compared with the standard medium, which was nutrient glucose agar (NGA) (per liter: 8.0 g of nutrient broth, 10.0 g of glucose, 3.0 g of yeast extract, and 14.0 g of agar). Several semiselective media proposed for other xanthomonads were tested for the growth of *X. campestris* pv. *manihotis* including XCS (28) and modified D-5 media (18), Tween medium (23), and Tween-cellobiose medium (1,2) designed for *X. campestris* pv. *carotae*, *X. campestris* pv. *vesicatoria*, and *X. campestris* pv. *pelargonii*, respectively.

Resistance to antibiotics. The resistance of *X. campestris* pv. *manihotis* to antibiotics was determined by the technique of Bauer et al. (3). Commercially available discs containing different concentrations of antibiotics were used. Bacterial suspensions in 0.01 M $MgSO_4$ were prepared from 1- to 2-day-old NGA cultures, and the concentration was adjusted to approximately 1×10^8 CFU per ml. The bacterial suspension was streaked evenly onto the surface of the NGA medium with a cotton swab dipped into the inoculum. After 10 to 20 min, discs were placed on the agar with sterile forceps and gently pressed down to ensure contact. The plates were incubated at 28°C overnight, and the diameter of the inhibition zones were measured (including the 6-mm discs). A reading of 6 mm indicated no inhibition. The discs producing no inhibition zone did not necessarily indicate a suitable antibiotic for a *X. campestris* pv. *manihotis* medium, because the diffusion and solubility properties of the drugs might be limiting factors. For this reason, those antibiotics that showed no inhibition of *X. campestris* pv. *manihotis* by the disc technique of Bauer et al. (3), but that inhibited epiphytic bacteria (*Erwinia herbicola*, *Pseudomonas fluorescens*, and others) (discussed below), were further evaluated by incorporation of the antibiotics into basal yeast trehalose glucose agar (YTGA), as described below. Aliquots of 0.1 ml of each of three strains (2363, 2369, and 2592) were plated at concentrations of 10^3 and 10^2 CFU/ml onto each of three plates. Cefazolin (C5020; Sigma Chemical Co., St. Louis), lincomycin (L6004; Sigma), phosphomycin (P5396; Sigma), and thiamphenicol (T0261; Sigma) were included in these studies, because *X. campestris* pv. *manihotis* was reported to be resistant to the latter two antibiotics (13).

Combinations of several antibiotics were also tested in YTGA. In these experiments, cycloheximide was added at 250 µg/ml to inhibit fungal growth. Stock solutions of the antibiotics (100 mg of cefazolin in 10 ml of deionized water; 120 mg of lincomycin in 10 ml of 75% methanol; 25 mg of phosphomycin in 10 ml of deionized water, and 2,100 mg of cycloheximide in 8.4 ml of 75% ethanol) were filter-sterilized and stored in sterile screw-cap flasks at 5°C. All antibiotics were added aseptically to the basal medium at a temperature of 45°C after autoclaving.

TABLE 1. Origin of strains of *Xanthomonas campestris* pv. *manihotis* and other species used to evaluate semiselective media

GSPB ^a no.	Strain designation	Origin	Isolated	Isolated by
2363; 2373	J 12; J 17	Nigeria	1993	K. Wydra
	J 1 – J 11; J 13 – J 16, J 19	Nigeria	1993	K. Wydra
2364; 2593	I 1, I 2	Nigeria	1993	Wydra & Verdier
2296	CFBP 1860	Nigeria	1978	J. F. Daniel
2285	ORST 42	Nigeria	1978	J. F. Daniel
2360	O 4	Nigeria	1993	K. Wydra
2359	IITA 3c	Benin	1994	K. Wydra
	IITA 1, IITA 7, IITA 7a, IITA 7b, IITA 7c, Zoundja	Benin	1994	K. Wydra
2592	Xm 5	Benin	1993	K. Wydra
2275	ORST 36	Benin	1982	J. F. Daniel
2369	Ug 21	Uganda	1994	B. Boher
2286	ORST 41	Republic of Central Africa	1977	J. F. Daniel
2391	PC 3	Cameroon	1994	K. Wydra
2282	ORST 212	Cameroon	1992	V. Verdier
2272	ORST 187	Zaire	1987	B. Boher
2287	UPB 031 (<i>Xanthomonas campestris</i> pv. <i>cassavae</i>)	Rwanda	1977	H. Maraite
3001	CIAT 1203	Dominican Republic	1981	J. Lozano
2278	CIAT 1135	Taiwan	1975	J. Lozano
2289	HMB 068	Brazil	1941	W. H. Burkholder
2288	UnB 833	Brazil	1991	A. Takatsu
2337	ICMP 5741 ^y	Brazil	1965	C. F. Robbs
2356	CIAT 1214	Colombia	1982	J. Lozano
2348	CIAT 1222	Brazil	1984	J. Lozano
2350	NCPBP 2444	Colombia	1970	J. Lozano
2355	CIAT 1180	Venezuela	1977	J. Lozano
1713	<i>Pseudomonas fluorescens</i>	Hungary	1988	Z. Klement
450	<i>Erwinia herbicola</i> , NCPBP 601	United Kingdom	1958	R. A. Lelliott
	PB E1, PB E2, PB E2b, PB E4, PB E5a, PB E5b ^z	Benin	1993	K. Wydra

^a GSPB = Göttingen Collection (Sammlung) of Phytopathogenic Bacteria.

^y 2337 = Pathotype strain.

^z Typical epiphytic bacteria from cassava.

Efficiency and selectivity of the newly developed semiselective medium compared with SXM medium. Ten strains of *X. campestris* pv. *manihotis* (2337, 2348, 2350, 2355, 2356, 2359, 2360, 2364, 2369, and 2391) were used to evaluate growth on the newly designed, semiselective cefazolin trehalose agar (CTA) medium, in comparison with SXM and NGA. Bacterial cultures grown for 48 h at 30°C on NGA were suspended in sterile 0.85% NaCl (saline) and adjusted to a turbidity of OD₅₉₀ = 0.3 (corresponding to a viable cell count of approximately 1 × 10⁸ CFU/ml). Five serial 10-fold dilutions were made in sterile saline, and 0.1 ml from the 10⁻⁴ and 10⁻⁵ dilutions was plated onto each of three plates of the different media. Colony counts ranging from 10 to 1,000 were made after incubation for 48 h at 30°C and averaged.

Isolation of *X. campestris* pv. *manihotis* from naturally infected cassava leaves and recovery from inoculated leaf homogenates. Leaf samples from five cassava cultivars were collected during the dry season in February 1997 from Ina, North Benin. Samples were placed in paper bags and sent via courier mail to Göttingen and analyzed within a week. Small tissue pieces of cassava were homogenized with a sterile mortar and pestle in 10 ml of sterile saline. The homogenate was vortexed for approximately 30 s and aliquoted into 5 and 4.5 ml. The 5-ml homogenate was used for isolation and the latter for the recovery assay. After thoroughly mixing, the 5-ml homogenate was diluted serially to 10⁻³, and 0.1-ml aliquots of each 10-fold dilution were plated onto the media. Plates were incubated for 48 h at 30°C.

To determine recovery rates, the 4.5-ml homogenate prepared above was vortexed with 0.5 ml of a suspension of *X. campestris* pv. *manihotis*, strain 2359 (approximately 10⁶ CFU/ml), serially diluted, and plated onto the media (NGA, CTA, and SXM) as described above.

Isolation and recovery of *X. campestris* pv. *manihotis* from soil. Soil samples (dry, sandy clay) were collected from cassava fields in February 1997 from different localities in Cotonou, South Benin; Ina, North Benin; and Ibadan, West Nigeria. Soil samples were mailed to Göttingen the same way as cassava leaf samples.

One gram of field-collected soil was suspended in 5 ml of sterile saline, vortexed, diluted serially to 10⁻³, and 0.1-ml aliquots of each 10-fold dilution were plated onto the media in three repetitions and incubated as described above. To test recovery, 0.5 ml of a bacterial suspension (approximately 10⁶ CFU/ml) was added to 1 g of soil suspended in 4.5 ml and processed as described above.

RESULTS

Carbon and nitrogen utilization. Growth of *X. campestris* pv. *manihotis* on semiselective media proposed for other pathovars of *X. campestris* was unsatisfactory and, thus, none of these media were suitable for *X. campestris* pv. *manihotis*. All the carbon sources tested, except soluble starch, bromosuccinic acid, and citrate, supported good growth of the two strains tested (2275 and 2296). Because the growth of *X. campestris* pv. *manihotis* strains on starch medium was poor, an additional 225 strains of *X. campestris* pv. *manihotis* were tested for starch hydrolysis as previously described

TABLE 2. Plating efficiency (%) of three *Xanthomonas campestris* pv. *manihotis* strains on different nutrient media compared with the standard medium (NGA)²

Strains	NGA	SXM	YTGA	GTGA	GTA
2373	100	107	140	151	129
2592	100	77	147	160	145
2369	100	94	127	167	137
Colony diameter (mm)	2-3	2-3	2-3	1.5-3	1.5-2
After (h)	48 h	48 h	48 h	72 h	96 h

² NGA = Nutrient broth glucose agar; SXM = medium of Leksomboon et al. (19), tested without antibiotics and biological stains; YTGA = yeast trehalose glucose agar; GTGA = glutamate trehalose glucose agar; and GTA = glutamate trehalose agar.

(26,36). The results showed distinct differences in the ability of these strains to degrade starch (11,38). Therefore, starch was not chosen as a carbon source for *X. campestris* pv. *manihotis*. D(+)-Trehalose was found to be the best carbon source, because all strains of *X. campestris* pv. *manihotis* grew on it equally well. D(+)-Cellobiose was equivalent to D(+)-trehalose, but it had to be added aseptically after autoclaving. Both of the tested saprophytes, *P. fluorescens* (GSPB no. 1713) and *E. herbicola* (GSPB no. 450) grew on all carbon sources except bromosuccinic acid. Few differences were observed in growth of *X. campestris* pv. *manihotis* on the three inorganic nitrogen sources tested. Ammonium chloride (0.1%) appeared to be the best inorganic nitrogen source and was chosen.

The basal medium, designated glutamate trehalose agar (GTA) medium, contained the following ingredients per liter: 3.0 g of K₂HPO₄, 1.0 g of NaH₂PO₄, 0.3 g of MgSO₄·7H₂O, 1.0 g of NH₄Cl, 10.0 g of D(+)-trehalose, 1.0 g of L-glutamic acid, and 14.0 g of agar.

Evaluation and modification of basal GTA medium. Because *X. campestris* pv. *manihotis* colonies failed to grow on GTA beyond a 2-mm diameter after 96 h of incubation at 30°C, the initially formulated GTA medium was modified by (i) reducing trehalose to 0.9% and adding 0.1% glucose (glutamate trehalose glucose agar [GTGA] medium) and (ii) substituting 0.1% L-glutamic acid with 0.1% yeast extract (yeast trehalose glucose agar; YTGA). The pH was not adjusted and cycloheximide was added as above. The plate counts of three strains of *X. campestris* pv. *manihotis* on GTA, GTGA, YTGA, and SXM media were compared with NGA (Table 2). Plating efficiency on YTGA was higher than on SXM and NGA media (135 to 191% and 127 to 147%, respectively) and was equal to SXM and NGA in terms of colony diameter and growth rate. Plating efficiency was highest on GTGA, but bacterial growth was slower than on YTGA, which was, therefore, chosen for further studies.

Resistance to antibiotics. The two strains of *X. campestris* pv. *manihotis* and one strain of *X. campestris* pv. *cassavae* tested were resistant to cefazolin, lincomycin, and mupirocin and weakly resistant to Targocid (teicoplanin; Hoechst Marion Roussel, Frankfurt, Germany) (Table 3). The inhibition zones of *X. campestris* pv. *cassavae* were equal to or larger than those of *X. campestris* pv. *manihotis* strains. These four antibiotics were further tested with a larger group of *X. campestris* pv. *manihotis* strains using the single-disc method of Bauer et al. (3). These studies revealed that all the antibiotics tested, except cefazolin and lincomycin, inhibited some strains of *X. campestris* pv. *manihotis* (Table 3). Therefore, cefazolin and lincomycin were used in further studies as well as phosphomycin, which was reported not to affect *X. campestris* pv. *manihotis* (13). The maximum tolerable concentrations of cefazolin,

TABLE 3. The reaction of bacterial strains toward four selected antibiotics

Bacterial strains as received	Effect on <i>Xanthomonas campestris</i> pv. <i>manihotis</i> growth			
	Cefazolin	Lincomycin	Targocid	Mupirocin
J 1- J 15; J 17; J 19 ^x	0 ^y	0	0	0
Xm 5, Ug 21 ^x	0	0	0	0
IITA 1, 3c, 7, 7a, 7b, 7c ^x	0	0	0	0
Zoundja ^x	0	0	0	0
ORST 1 ^x	0	0	4	14
ORST 36 ^x	0	0	4	4
ORST 41, ORST 212 ^x	0	0	0	0
ORST 42 ^x	0	0	6	0
ORST 187 ^x	0	0	0	14
CFBP 1860 ^x	0	0	14	19
CIAT 1203 ^x	0	0	4	0
PB E1 ^z	26	0	0	0
PB E2	0	0	0	0
PB E2b	26	0	15	17
PB E4	14	0	0	0
PB E5a	24	0	0	0
PB E5b	30	0	16	18

^x *X. campestris* pv. *manihotis*.

^y Diameter of the inhibition zone in millimeters.

^z Typical accompanying bacteria.

lincomycin, and phosphomycin were 100, 1.2, and 2.5 µg/ml, respectively (Table 4).

Growth of *X. campestris* pv. *manihotis* on YTGA media supplemented with mixed antibiotics. After determining the optimal concentration for cefazolin, lincomycin, and phosphomycin, growth of *X. campestris* pv. *manihotis* was determined on three combinations of YTGA containing antibiotics and unmodified YTGA. Plating efficiencies ([average number of CFU per plate ÷ average number of CFU per plate on YTGA] × 100) were 80% with 25 µg of cefazolin per ml and 1.2 µg of lincomycin per ml; 70% with 50 µg of cefazolin per ml and 1.2 µg of lincomycin per ml; and 86% with 25 µg of cefazolin per ml, 1.2 µg of lincomycin per ml, and 2.5 µg of phosphomycin per ml. All the media contained 250 µg of cycloheximide per ml to inhibit fungal growth. These experiments resulted in the following composition of the final semiselective medium, designated CTA medium, per liter: 3.0 g of K₂HPO₄, 1.0 g of NaH₂PO₄, 0.3 g of MgSO₄·7H₂O, 1.0 g of NH₄Cl, 9.0 g of D(+)–trehalose, 1.0 g of D(+)–glucose, 1.0 g of yeast extract, 0.025 g of cefazolin, 0.0012 g of lincomycin, 0.0025 g of phosphomycin, 0.25 g of cycloheximide, and 14.0 g of agar.

Efficiency and selectivity of the newly developed semiselective medium compared with SXM medium. The media were compared for recovery of 10 strains of *X. campestris* pv. *manihotis* (Table 5). The CTA medium supported good growth of all 10 strains tested. Plating efficiencies for the 10 strains of *X. campestris* pv.

manihotis ranged from 69 to 102%, with an average of 85%. Colonies of *X. campestris* pv. *manihotis* were white to creamy white, convex, 1.5 to 4.0 mm in diameter after 2 days, visible after 48 h at 30°C, and had smooth margins. No morphological differences were observed between strains from different geographic origins. All strains of *X. campestris* pv. *manihotis* grew on SXM medium, and colonies appeared after 3 days of incubation at 30°C, measuring ≤1.5 mm in diameter. Colonies were round, convex, and because of color interference in the medium, bluish white. Plating efficiencies ranged from 5 to 80%, with an average of 50%. SXM medium allowed only slow growth of some of the strains tested (Fig. 1).

Isolation of *X. campestris* pv. *manihotis* from naturally infected cassava leaves and recovery of *X. campestris* pv. *manihotis* 2359 from leaf tissue homogenate on NGA, CTA, and SXM. Isolation of *X. campestris* pv. *manihotis* was positive on all three media for four of five samples (Table 6). Colonies of *X. campestris* pv. *manihotis* were countable after 48 h on CTA and NGA and after 72 h on SXM. Saprophytic bacteria associated with cassava leaves failed to grow on CTA, and *X. campestris* pv. *mani-*

TABLE 4. Growth of *Xanthomonas campestris* pv. *manihotis* strains on yeast trehalose glucose agar medium supplemented with different concentrations of cefazolin, lincomycin, phosphomycin, or thiamphenicol

Antibiotic	µg/ml	Strains ^y
Cefazolin	25	+
	50	+
	100	+
Lincomycin	1 ^z	+
	10 ^z	(+)
	100 ^z	–
Phosphomycin	2.5	+
	5.0	–
	10.0	–
Thiamphenicol	2.5	–
	5.0	–
	10.0	–

^y *X. campestris* pv. *manihotis* strains 2363, 2592, and 2369. + = Growth; (+) = slightly suppressed; and – = no growth.

^z Concentration of lincomycin is given in International Units (IU) and 1 IU ≡ 1.2 µg.

Fig. 1. Growth of *Xanthomonas campestris* pv. *manihotis* strain 2364 on cefazolin trehalose agar (CTA), medium of Leksomboon et al. (19) tested without antibiotics and biological stains (SXM), and nutrient broth glucose agar (NGA).

TABLE 5. Colony counts and plating efficiencies of 10 strains of *Xanthomonas campestris* pv. *manihotis* from different geographic origins on nutrient broth glucose agar (NGA), cefazolin trehalose agar (CTA), and medium of Leksomboon et al. (19) tested without antibiotics and biological stains (SXM) media

GSPB ^x no.	Source	No. of colonies per plate			Plating efficiency ^y (%)	
		NGA	CTA	SXM	CTA	SXM
2359	Benin	580 ± 95 ^z	400 ± 36	27 ± 29	69	5
2364	Nigeria	1,017 ± 120	737 ± 95	423 ± 116	73	42
2391	Cameroon	290 ± 30	267 ± 61	40 ± 10	92	14
2369	Uganda	797 ± 119	617 ± 40	150 ± 27	77	19
2360	Nigeria	930 ± 70	750 ± 87	380 ± 66	81	41
2355	Venezuela	674 ± 24	600 ± 17	516 ± 7	89	77
2350	Colombia	632 ± 18	601 ± 15	488 ± 8	95	77
2348	Colombia	550 ± 10	540 ± 13	429 ± 57	98	78
2356	Colombia	269 ± 5	245 ± 7	212 ± 10	91	79
2337	Brazil	601 ± 19	611 ± 2	478 ± 50	102	80

^x GSPB = Göttingen Collection (Sammlung) of Phytopathogenic Bacteria.

^y Plating efficiency = $\frac{\text{average no. of CFU on CTA or SXM plate}}{\text{average no. of CFU on NGA plate}} \times 100$.

^z Average number of colonies per plate ± standard error of the mean of three replications. Means of the total colony count for *X. campestris* pv. *manihotis* on NGA, CTA, and SXM media were 634, 537, and 314, respectively. Plating efficiencies for *X. campestris* pv. *manihotis* on CTA were significantly higher ($P = 0.05$) than that on SXM medium (19), when Tuckey's new multiple range test was applied.

hotis grew normally. In contrast, on NGA, either heavy growth of saprophytic bacteria overgrew colonies of *X. campestris* pv. *manihotis* or a few antagonistic bacteria suppressed its growth at dilutions of 10^0 and 10^{-2} (Fig. 2). A similar effect on nonselective media was also described for *X. campestris* pv. *campestris* (8). On SXM, saprophytes were inhibited and growth of *X. campestris* pv. *manihotis* cells was observed at dilution 10^0 , but no *X. campestris* pv. *manihotis* colonies were recovered at dilution 10^{-2} (Fig. 2). The total number of colonies of *X. campestris* pv. *manihotis* recovered from naturally infected leaf samples on CTA was much higher than that on SXM medium; colonies per plate averaged 436 and 250, respectively (Table 6). The recovery of *X. campestris* pv. *manihotis* on SXM medium required a higher concentration of the pathogen to be present in plant tissues as shown in Table 6.

Recovery of *X. campestris* pv. *manihotis* from infested soils on CTA, SXM, and NGA. Isolation of *X. campestris* pv. *manihotis* from three native soils collected from cassava-growing fields was unsuccessful. After adding bacterial suspensions to the soils,

Fig. 2. Isolation of *Xanthomonas campestris* pv. *manihotis* from naturally infected, dry season-collected cassava leaf samples on cefazolin trehalose agar (CTA), medium of Leksomboon et al. (19) tested without antibiotics and biological stains (SXM), and nutrient broth glucose agar (NGA). Eight saprophytic bacterial colonies (arrow S) inhibited the growth of *X. campestris* pv. *manihotis* colonies (arrow X) on NGA. Colonies of *X. campestris* pv. *manihotis* developed normally after 48 h at dilution 10^{-2} on CTA, but no colonies were visible on SXM.

recovery of *X. campestris* pv. *manihotis* on NGA was impeded at all dilutions (10^{-1} to 10^{-3}) by saprophytic soil bacteria. On CTA medium, colonies of *X. campestris* pv. *manihotis* were overgrown by saprophytes only at dilution 10^{-1} . The recovery of *X. campestris* pv. *manihotis* at dilution 10^{-3} was 100% and higher than at dilution 10^{-2} (Fig. 3), with 3 and 15 colonies of saprophytic soil bacteria, respectively. On SXM medium, many saprophytes grew at dilution 10^{-1} , but only a few grew at dilutions 10^{-2} and 10^{-3} . *X. campestris* pv. *manihotis* colonies were never detected on SXM within 48 h after plating.

DISCUSSION

Currently, CBB can most effectively be controlled by hygienic measures, since cassava cultivars with good resistance to CBB are not always available. Since the pathogen is seedborne (12,24) and survives as an epiphyte (12), sensitive detection methods are needed to screen seed lots and cuttings used for propagation. The most reliable method to detect bacteria in infected or infested seed and planting material is the use of semiselective agar media that suppress most of the accompanying epiphytic microflora and allow a

Fig. 3. Recovery of *Xanthomonas campestris* pv. *manihotis* strain 2359 from artificially infested soil on cefazolin trehalose agar (CTA), medium of Leksomboon et al. (19) tested without antibiotics and biological stains (SXM), and nutrient broth glucose agar (NGA) at dilution 10^{-3} .

TABLE 6. Isolation of *Xanthomonas campestris* pv. *manihotis* and contaminating bacteria from five naturally infected cassava leaves and recovery of *X. campestris* pv. *manihotis* 2359 from artificially inoculated leaf homogenates on cefazolin trehalose agar (CTA), medium of Leksomboon et al. (19) tested without antibiotics and biological stains (SXM), and nutrient broth glucose agar (NGA)

Cassava cultivar	Naturally infected tissue ^w						Artificially inoculated homogenate ^x		
	NGA		CTA		SXM		NGA	CTA	SXM
	<i>X. campestris</i> pv. <i>manihotis</i> ^y	Contaminating	<i>X. campestris</i> pv. <i>manihotis</i>	Contaminating	<i>X. campestris</i> pv. <i>manihotis</i>	Contaminating	<i>X. campestris</i> pv. <i>manihotis</i>	<i>X. campestris</i> pv. <i>manihotis</i>	<i>X. campestris</i> pv. <i>manihotis</i>
TMS 30572	38 ^z	11	27	0	0	0	1,284	1,230	556
084/00275	0	0	0	0	0	0	880	772	445
63397	158	2	102	0	72	0	1,295	1,150	508
087/00611	84	20	72	0	38	0	1,060	770	418
I82/00661	151	1	235	0	140	0	1,025	928	314
Total counts	431	34	436	0	250	0	5,544	4,850	2,241

^w CFU mean of two plates in dilution 10^{-2} .
^x Recovery of a strain of *X. campestris* pv. *manihotis* (2359) added to leaf homogenates.
^y Suspected colonies of *X. campestris* pv. *manihotis*.
^z Number of bacterial colonies per plate.

direct identification and recovery of the pathogen (27). Ideally, a selective medium should encourage growth of all viable cells of the target bacteria and concomitantly exclude or suppress growth of all other groups of bacteria (25). However, selective media are almost never entirely selective so that the term "semiselective" is probably more appropriate in most cases.

From the 12 carbon and 3 nitrogen compounds tested, D(+)-trehalose and ammonium chloride were selected as primary carbon and nitrogen sources, respectively, for the new semiselective medium. All 18 strains of *X. campestris* pv. *manihotis* tested readily utilized both substrates. To enhance the growth rate of *X. campestris* pv. *manihotis* and allow evaluation of plates after 48 h, glucose and yeast extract were added, both at 1.0 g/liter, to the final basal GTA medium. Of 20 antibiotics tested, 3 were included at optimal concentration. Cefazolin (16) inhibits Gram-negative bacteria, lincomycin (7) is active against Gram-positive bacteria, and phosphomycin (15,37) has broad antibacterial action. This combination of antibiotics inhibited most bacterial contaminants commonly found on cassava plants. However, it should be noted that the diameters of the zones of inhibition are different for each chemical, not only because the disc potencies vary, but because the diffusion and solubility properties of the antibiotics in NGA medium also vary (3). This may be the main reason for the discrepancy in the previously reported resistance of *X. campestris* pv. *manihotis* strains to 25 µg of thiamphenicol per ml (13), which was not confirmed in these studies when the antibiotics were added to the media in solution. The proposed semiselective CTA medium not only permits growth of *X. campestris* pv. *manihotis*, but also allows a good distinction of *X. campestris* pv. *manihotis* from saprophytes (Fig. 1) by colony characteristics.

The semiselective SXM medium designed by Leksomboon et al. (19) to isolate *X. campestris* pv. *manihotis* contains 0.8% soluble starch as a carbon source. However, analysis of 225 *X. campestris* pv. *manihotis* strains from the Göttingen collection revealed considerable variation in ability of this pathovar to grow on starch (11). Plating efficiencies of all 40 strains of *X. campestris* pv. *manihotis* tested in this study were significantly higher (Tuckey's new multiple range test) on CTA than on SXM agar, and colonies were countable after 48 h, in contrast to 72 h on SXM. Colony size was slightly reduced on SXM (Fig. 1). Other authors testing the growth of *X. campestris* pv. *manihotis* on the starch-containing media SM (8) and SX (29) also reported that plating efficiencies of *X. campestris* pv. *manihotis* strains were low (2 and 0%, respectively) compared with the TZC medium of Kelman (17). These findings were in line with our work testing 225 strains of *X. campestris* pv. *manihotis* (11,38). It can be assumed that the *X. campestris* pv. *manihotis* strains used by Leksomboon et al. (19) to evaluate SXM medium belonged to a group of strains that can degrade and metabolize starch as a primary energy source (Table 2). Our data show that starch is not a reliable carbon source for all strains of *X. campestris* pv. *manihotis*. We confirmed that two biological dyes, methyl violet 2B and methyl green, used in SXM medium (19) at 0.001 and 0.01 µg/ml, respectively, suppressed growth of most soil bacteria, but unexpectedly also growth of most *X. campestris* pv. *manihotis* colonies. Moreover, SXM agar was less transparent, and colonies of *X. campestris* pv. *manihotis* appeared bluish and were poorly differentiated morphologically from other starch-hydrolyzing bacteria.

Our isolation experiments from cassava leaves collected in the dry season showed that *X. campestris* pv. *manihotis* could be detected in low numbers on the new CTA medium; whereas on the standard NGA medium, no colonies of *X. campestris* pv. *manihotis* developed from undiluted samples. During the rainy growing season, when the CBB pathogen occurs in higher numbers, the use of the rather expensive semiselective CTA medium may not be necessary for routine testing. Instead, the pathogen should be easily isolated from diseased plants on yeast glucose agar medium (11), when supplemented with 200 ppm of cycloheximide. CTA medium

also proved to be very effective for isolating the pathogen from cassava field soils to which it had been added in low concentrations, whereas only 10% of the bacterial cells were reisolated on SXM medium (Fig. 3).

The newly developed semiselective CTA medium for *X. campestris* pv. *manihotis* should prove to be well suited for use in detecting infected plant material during vegetative propagation. Also, epidemiological studies will be considerably facilitated by the use of the semiselective medium.

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