# Pathological and Molecular Characterization of Xanthomonas campestris Strains Causing Diseases of Cassava (Manihot esculenta)

VALÉRIE VERDIER,<sup>1\*</sup> BERNARD BOHER,<sup>1</sup> HENRI MARAITE,<sup>2</sup> AND JEAN-PAUL GEIGER<sup>1</sup>

Laboratoire de Phytopathologie, ORSTOM, 34032 Montpellier, France,<sup>1</sup> and Unité de Phytopathologie, Faculté des Sciences Agronomiques, Université Catholique de Louvain, B 1348 Louvain la Neuve, Belgium<sup>2</sup>

Received 6 June 1994/Accepted 26 September 1994

Fifty-one strains representing Xanthomonas campestris pv. manihotis and cassavae and different pathovars occurring on plants of the family Euphorbiaceae were characterized by ribotyping with a 16S+23S rRNA probe of Escherichia coli and by restriction fragment length polymorphism analysis with a plasmid probe from X. campestris pv. manihotis. Pathogenicity tests were performed on cassava (Manihot esculenta). Histological comparative studies were conducted on strains of two pathovars of X. campestris (vascular and mesophyllic) that attack cassava. Our results indicated that X. campestris pv. manihotis and cassavae have different modes of action in the host and supplemented the taxonomic data on restriction fragment length polymorphism that clearly separate the two pathovars. The plasmid probe could detect multiple restriction fragment length polymorphisms among strains of the pathovar studied. Ribotyping provides a useful tool for rapid identification of X. campestris pathovars on cassava.

Two pathovars of Xanthomonas campestris are pathogenic to cassava (Manihot esculenta) and cause different diseases (23). X. campestris pv. manihotis is the causal agent of cassava bacterial blight and typically induces angular leaf spot symptoms and a systemic infection which leads to wilting and dieback. X. campestris pv. cassavae, associated with cassava bacterial necrosis, induces angular leaf spots very similar to those produced by X. campestris pv. manihotis; however, systemic invasion of the vessels has not been observed (23, 24). X. campestris pv. manihotis was first reported in South America and now has a worldwide distribution, while X. campestris pv. cassavae is, at present, restricted to the East African Highlands (23).

Because of the similarities of the leaf symptoms in these two pathovars, Robbs et al. (30) proposed considering X. campestris pv. cassavae to be a yellow variant of X. campestris pv. manihotis. At first, the only character distinguishing the pathovars was pigmentation (24). Attempts to distinguish these two pathovars have included biochemical and pathogenic characteristics (24, 25), serology (7, 11), membrane profiles analysis (5a), electrophoretic patterns and DNA-DNA hybridization (33), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein patterns (34), and fatty acid methyl esters analysis (38); these attempts showed that the two pathovars could be differentiated. Van den Mooter et al. (33) demonstrated that X. campestris pv. cassavae was phenotypically more heterogeneous than X. campestris pv. manihotis.

On the basis of different observations, Maraite (23) suggested that cassava is not the primary host of X. campestris pv. cassavae. Some overlap in the host range among isolates from members of the family Euphorbiaceae have been reported (31, 33), which could confirm this hypothesis.

The characterization of the X. campestris pathovars, which is

currently based on host and symptom specificity, remains difficult, particularly when the host plant of a xanthomonad is not known and also with the nonpathogenic xanthomonads which occur epiphytically on healthy or diseased plants (10, 35). An illustration is given by the xanthomonadin-producing strains of *X. campestris* which have been isolated from cassava leaves in Colombia. On the basis of different analyses, these Colombian isolates were found to be related to *X. campestris* pv. cassavae (7), *X. campestris* pv. manihotis (5a, 15), or *X. campestris* pv. poinsettiicola (33). Deviant *X. campestris* pv. cassavae has been isolated from cassava leaf spots in Niger (13), recently clustered in *X. campestris* pv. cassavae (34, 38).

Molecular approaches based on DNA polymorphisms have been developed for the taxonomic study of plant-pathogenic bacteria. Rapid methods, based on specific PCR amplification, have been used recently for the detection and identification of Xanthomonas species (19, 21). Restriction fragment length polymorphism (RFLP) analysis is a highly discriminative method currently used to describe the pathogen population structure (20). Different probes could be used to detect and differentiate restriction polymorphisms in the pathogen genome. rRNA genes have highly conserved sequences, and their potential usefulness in the identification and phylogenetic studies of bacteria has been demonstrated (2, 9). Specific sequences from genomic or plasmid DNA, such as repetitive elements and insertion sequences, provided useful probes for the assessment of genetic diversity and also allowed a better understanding of the pathogen population structure (1, 4, 8, 18, 28, 29). Both ribotyping and RFLP analysis with the use of different DNA probes facilitated the study of the population structure of X. campestris pv. manihotis, and the results obtained suggested a clonal population structure for this pathogen in Africa (37).

The present study was undertaken to determine if X. campestris pv. manihotis and cassavae are closely related and to clarify the relationships between these pathovars and the various strains found on cassava and other members of the Euphorbiaceae. We also examined strains of X. campestris pv.

PM 287

ORSTOM Fonds Documentaire N° § 41.049 Lp 1 Cote • B

4478

2 3 FEV. 1995

<sup>\*</sup> Corresponding author. Mailing address: Laboratoire de Phytopathologie, ORSTOM, 911 Av. Agropolis, 34032 Montpellier, France. Phone: (33.1) 67 61 75 87. Fax: (33) 67 54 78 00. Electronic mail address: verdier@orstom.orstom.fr.

وروار فروار والالالار

cassavae from various African countries, comparing the data with our previous data on an African population of *X. campestris* pv. manihotis (37). In this paper we propose a rapid and reproducible method to identify strains found on cassava.

# MATERIALS AND METHODS

Bacterial strains. The strains studied are listed in Table 1. All strains were stored as frozen glycerol stocks and were grown on YPGA medium (5 g of yeast extract per liter, 5 g of Bacto Peptone per liter, 5 g of glucose per liter, and 15 g of agar per liter [pH 7.2]). For the DNA extraction, the bacteria were grown in liquid medium containing peptone (10 g liter<sup>-1</sup>), Casamino Acids (1 g liter<sup>-1</sup>), and yeast extract (1 g liter<sup>-1</sup>) (pH 7.2). Five ribotypes were previously characterized among a worldwide collection of X. campestris pv. manihotis isolates (37). X. campestris pv. manihotis strains representative of each ribotype were included in this study. Two yellowpigmented bacteria recently isolated from cassava leaves in Colombia were included in the study (strains CIAT1187 and CIAT1192). These isolates had not been characterized. The strain UPB008, isolated in 1973 from leaf symptoms in Zaire. had not been further characterized since isolation.

**Phytopathogenicity tests.** The pathogenicity of all strains was tested on 6-week-old, greenhouse-grown cassava plants (susceptible cultivar Fetonegbodgi from Togo) at 28°C and 80% relative humidity.

Stem inoculations were done by previously described methods (26). The evolution of the symptoms over 5 weeks was issessed, and the observed reactions were rated 4 weeks after noculation according to the following scale: 0, no reaction; 1, small dark area around the inoculation point extending less than 2 mm in diameter; 2, dark necrotic area extending more than 5 mm from the inoculation point; 3, formation of exudates on stem; 4, wilting and defoliation.

Leaves were inoculated by placing  $20-\mu l$  droplets of a bacterial suspension calibrated at  $10^8$  CFU/ml ( $A_{260}$ , 0.1) in a small hole (diameter, 2 mm) previously punched out with a cork borer. Leaves were treated with sterile distilled water as control. Angular leaf spots around the hole were observed after 7 days of incubation. The average surface of five lesions on each of the leaves inoculated was estimated, and differences among means were examined by the Kruskal-Wallis test for mean separation.

Light microscopy. Pieces of inoculated tissues were fixed in 0.1% (vol/vol) glutaraldehyde–4% *para*-formaldehyde in 0.05 M cacodylate buffer (pH 7.3) for 4 h at room temperature. After washing and dehydration, they were embedded in LR white resin (London Resin Co.). Polymerized blocks were sectioned into 1.5- $\mu$ m-thick sections, stained in 1% toluidine blue in 1% aqueous sodium borate, and observed with a Leitz (Diaplan) microscope.

**Ribotyping.** Isolation of total DNA, restriction digestion vith the enzyme *Eco*RI (Boehringer GmbH, Mannheim, Germany), and DNA blotting were performed as previously described (2, 37). A nonradioactive acetylaminofluorene-labeled 16S–23S rRNA probe from *Escherichia coli* (9) was used in our study. Hybridization and immunoenzymatic detection of hybridizing fragments were performed as previously described (2) and as specified by the manufacturer (Eurogentec, Liege, Belgium). Acetylaminofluorene-labeled pBR322 hybridized with the DNA fragments of the standard Raoul I set. The presence or absence of each hybridizing fragment was observed on the nitrocellulose membrane and coded as 1 or 0, espectively; the size was estimated from the reference marker (Raoul I). Band density was not taken into account. All the experiments were done at least twice for each strain.

**RFLP** analysis. Plasmid F3 consists of a 5.4-kb *Eco*RI fragment cloned from an indigenous plasmid of the *X. campestris* pv. manihotis CFBP1851 in the Bluescript M13 vector by standard methods (22). This fragment is an internal region of the 13-kb *Hind*III (pBSF2) fragment previously studied (36, 37). It has been shown that this DNA fragment (pBSF2) contains pathogenicity genes of *X. campestris* pv. manihotis (36). The 5.4-kb *Eco*RI fragment was isolated from agarose gels with a Geneclean Kit (Bio 101. La Jolla, Calif.) and labeled by random priming with the Multiprime kit (Amersham, Les Ulis, France). Prehybridization and hybridization (2 h) were performed at 65°C under conditions described by the manufacturer (Amersham).

**Hierarchical cluster analysis.** The similarity between individual strains was estimated from the number of matching bands in the ribotype patterns by using the Sorensen-Dice coefficient (12),  $S = 100 \times [2 N_{ab}/(N_a + N_b)]$ , where  $N_{ab}$  is the number of matching bands between a pair of strains, and  $N_a$  and  $N_b$ , respectively, are the numbers of bands present in strain a and in strain b. The coefficient was calculated by the procedure SIMIL-Dice with the logiciel R (A. Vaudor, Laval, Canada). Cluster analysis was performed by a UPGMA method with the same logiciel.

### RESULTS

Pathogenic characteristics. Leaves treated with sterile water did not show any visible reaction (Fig. 1A). The same experiment was done with leaves inoculated with the two Colombian yellowish isolates (CIAT1187 and CIAT1192) as well as X. campestris pv. cassavae LMG672 (Table 2). Strains belonging to X. campestris pv. vignicola, poinsettiicola, euphorbiae, and ricini, as well as two Colombian isolates (strains UPB137 and CIAT1165) and the deviant X. campestris pv. cassavae strains, induced a typical necrotic area around the inoculation point (Fig. 1B). The sizes of the water-soaked areas induced by X. campestris pv. cassavae varied greatly and differences were detected (Fig. 1C; Table 2). All the X. campestris pv. manihotis strains except strain UPB079 induced symptoms, and most of these strains caused significantly larger water-soaked areas than did X. campestris pv. cassavae strains (Fig. 1D; Table 2). Great variation in the size of water-soaked lesions were observed for X. campestris pv. cassavae and X. campestris pv. manihotis strains, which were clustered in six significantly different groups by the Kruskal-Wallis test (Table 2). After inoculation into the stem, strains of X. campestris pv. euphorbiae, ricini, and poinsettiicola, deviant X. campestris pv. cassavae strains, and two Colombian isolates (UPB137 and CIAT1165) induced a local dark-brown reaction around the inoculation point without any evolution (Table 2). Reactions induced by X. campestris pv. cassavae strains vary greatly: no or poor reactions were observed with some isolates, whereas a cortical dark area extended very slowly after inoculation of a few isolates; wilting of the plant was never induced (Table 2). Most of the X. campestris pv. manihotis strains caused a systemic infection in the stem, leading to the formation of exudates. Differences in the speed of symptom formation were evident; however, we clearly differentiated five X. campestris pv. manihotis strains which failed to induce wilting of the plant (Table 2).

Light microscopy. Light microscopy of transverse sections in leaf samples at the wound level gave information about the nature of the different reactions observed (Fig. 1). In control leaves, a fine brown ring of necrotic cells developed around the

# 4480 VERDIER ET AL.

TABLE 1. Strain collection

X. campestris pathovar	Strain <sup>4</sup>	Host	Country and year of isolation
Cassavae	NCPPB101* (LMG673, UPB054)	Manihot esculenta	Malawi, 1951
	LMG672 (UPB038)	Manihot esculenta	Rwanda, 1977
	UPB030	Manihot esculenta	Rwanda, 1977
	UPB032	Manihot esculenta	Rwanda, 1977
	UPB033	Manihot esculenta	Rwanda, 1977
	UPB035	Manihot esculenta	Rwanda, 1977
	UPB037 (LMG5264)	Manihot esculenta	Rwanda, 1977
	UPB039	Manihot esculenta	Rwanda, 1977
	UPB041 (LMG5265)	Manihot esculenta	Rwanda, 1977
	UPB043	Manihot esculenta	Rwanda, 1977
	UPB044 (LMG5267)	Manihot esculenta	Rwanda, 1977
	UPB045 (LMG5268)	Manihot esculenta	Rwanda 1978
	UPB046	Manihot esculenta	Rwanda 1978
	LIPB047	Manihot esculenta	Rwanda, 1978
	LIPR040	Manihot esculenta	Dwondo 1078
	UPR051 (I MC5260)	Manihot esculenta	Rwanda 1976
	IIPP052 (IMC5270)	Muninoi escuenta	Rwanua, 1976 Dwords, 1078
	$\frac{\text{UDD050}(\text{LMC5270})}{\text{UDD050}(\text{LMC52764})}$	Muninoi escuenta	Kwanua, 1976
	UPB039 (LMC704) $UPD146 (LMC5071)$	Maninot escuenta	Tanzania, 1978
	UPB146 (LMG52/1)	Manihot esculenta	Kenya, 1979
Manihotis	ORSTX27	Manihot esculenta	Togo, 1989
	UPB079 (LMG778)	Manihot esculenta	Brazil, 1978
	CIAT1129	Manihot esculenta	Venezuela, 1974
	NCPPB1834* (UPB055)	Manihot esculenta	Brazil, 1965
	UPB009 (LMG768, NCPPB3058)	Manihot esculenta	Zaire, 1973
	LMG777 (UPB078)	Manihot esculenta	Brazil, 1978
	LMG779 (UPB080)	Manihot esculenta	Brazil 1978
	ORST2 (CIAT1061)	Manihot esculenta	Venezuela 1971
	ORST5	Manihot esculenta	Brazil 1074
	ORST7	Manihot esculanta	Brazil 1073
	LIPB070 (NCPPB1160)	Manihot asculanta	Brazil, 1975
	CEBP1851 (CIAT1111)	Manihot esculenta	Colombia 1974
	ATCC22280 (NCDDD1150)	Muninoi esculenta	Deseil 1041
	NCDDD249	Maninoi escuenta	Drazii, 1941
	I M C 774 (I ID D 0 c 0)	Maninot escuenta	Brazil, 1954
	CIAT1125	Maninot escuenta	Taiwan, 1978
	CIATTI35	Manihot esculenta	Taiwan, 1975
	NCPPB2444	Manihot esculenta	<ul> <li>Colombia, 1970</li> </ul>
	CIAT117	Manihot esculenta	Brazil, 1974
	CIAT1122	Manihot esculenta	Venezuela, 1974
Deviant cassavae	UPB899 (LMG8048)	Manihot esculenta	Niger, 1987
	UPB900 `	Manihot esculenta	Niger, 1987
Poinsettijoola	LIDD072* (NCDDD501)		T
romsetticola	LMC5402	Eupnorbiae puicherrima	111dia, 1950
	LMG5403	Euphorbiae pulcherrima	New Zealand, 1972
Euphorbiae	LMG863* (NCPPB1828)	Funhorbiae alcaluphoides	Sudan 1965
<b>-</b>	LMG7402 (NCPPB2067)	Fuphorbiae alcalyphotaes	Sudan, 1966
		Euphoronae aneusphonaes	Sulan, 1900
Ricini	UPB075* (NCPPB1063)	Ricinus communis	Ethiopia, 1961
	UPB076 (NCPPB1324, LMG862)	Ricinus communis	Hong Kong, 1962
	· · · · · · · · · · · · · · · · · · ·		
Vignicola	UPB040 -	Vigna sinensis	Nigeria, 1977
Unknown	LIPB008	Manihat esculenta	7 aire 1073
	LIPB137 (CIAT1164 I MC 5244)	Manihat asculanta	Colombia $1076$
	CIAT1165 (I MC5242 IDD124)	Manihot coulentu	Coloration 1970
· .	CIAT 1107 (LARCE)245, UPD150)	Manihot esculentu	Colombia 1970
		Maninot escuenta	Colombia, 1992
- 4	CIAI 1192	Maninot esculenta	Colombia, 1992

<sup>a</sup> Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; CIAT, Xanthomonas collection, Centro Internacional de Agricultura Tropical, Cali, Colombia; UPB, Collection of H. Maraite, Louvain La Neuve, Belgium; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; ORST, Xanthomonas collection, Laboratoire de Phytopathologie, ORSTOM, Montpellier, France; \*, a pathovar reference strain.

edges of the hole exposed to droplets of distilled water. Behind it, a faint translucent halo composed of hypertrophic cells showing hyperplastic activity was visible (wound repair zone) preceding the unaltered mesophyll parenchyma (Fig. 1A). Leaves inoculated with the Colombian isolates UPB137 and CIAT1165, as well as with strains belonging to all other pathovars, showed the necrotic ring, which enlarged during incubation. The clear wound repair zone was visible, and no



FIG. 1. Symptomatological (left) and histological (right) comparison of the development of three isolates on cassava leaf. (A) Water control; (B) strain UPB137; (C) X. campestris pv. cassavae UPB053; (D) X. campestris pv. manihotis ORSTX27. The left-hand panels illustrate lesion aspects. The central spot is the inoculation hole (diameter, 2 mm). The first black ring (larger in panel B) corresponds to the necrotic zone. It precedes a narrow translucent border (zone of hyperplasia and hypertrophy) visible in panels A, B, and C. In panels C and D, the mesophyll is occupied by bacteria and extracellular matrix. The right-hand panels illustrate transverse sections corresponding to the pictures above. A breviations: NZ, necrotic zone; ZHH, zone of hyperplasia and hypertrophy; BIZ, bacterium-invaded zone; HZ, healthy tissues; ue, upper  $\epsilon_{-}$  dermis; le, lower epidermis; m, mesophyll; vb, vascular bundle. Bar, 0.1 mm.

bacteria were present in the mesophyll beyond this zone (Fig. 1B). Leaves inoculated with all strains of *X. campestris* pv. cassavae except one (LMG672) showed the necrotic ring and the translucent halo. The latter was thinner than in the control leaf. Intercellular spaces in the mesophyll were occupied by bacteria and exopolysaccharides. Spongy parenchyma cells began to collapse, and palisade cells remained turgid (Fig. 1C). In leaves inoculated with *X. campestris* pv. manihotis ORSTX27, the clear wound repair zone was absent (Fig. 1D). Immediately beyond the necrotic ring, the water-soaked mesophyll was heavily colonized by bacteria producing extracellular matrix. With the exception of the upper epidermal cells, all the cells had collapsed (Fig. 1D).

**Ribotyping.** Ribotypes obtained with some strains are shown in Fig. 2. Strains of *X. campestris* pv. cassavae were homogeneous, with 19 of the 20 strains tested belonging to the same ribotype (Table 2). Strain UPB008, isolated in Zaire, showed the typical ribotype pattern of *X. campestris* pv. cassavae strains. The ribotype pattern observed for *X. campestris* pv. Casavae UPB037 was similar to that of the *X. campestris* pv. vignicola strain (Fig. 2). Faint hybridizing bands which could not be systematically visualized were not taken into account.

A total of 326 strains of X. campestris pv. manihotis had been previously ribotyped; a total of five ribotypes were observed (37) and are shown in Fig. 2. Ribotypes of X. campestris pv. manihotis were clearly different from the general ribotype pattern of the X. campestris pv. cassavae strains. Three fragments (9, 2, and 1.5 kb) were common to both X. campestris pv. cassavae strains and X. campestris pv. manihotis strains. The 1.5-kb fragment was common to all strains tested except for the two yellowish Colombian isolates, CIAT1187 and CIAT1192.

The two strains of X. campestris pv. ricini had the same ribotype. The Colombian strain, UPB137, showed a distinct pattern, with four bands in common with the ribotype of X. campestris pv. ricini strains (Fig. 2). Each strain of X. campestris pv. poinsettiicola gave distinct patterns (Fig. 2). Strains of X. campestris pv. euphorbiae showed a ribotype similar to that of the X. campestris pv. poinsettiicola strain (Fig. 2; Table 2). The deviant strains of X. campestris pv. cassavae had the same

#### VERDIER ET AL. 4482

3

# TABLE 2. Hybridization and pathogenicity results

	Country of	RFLP group <sup>a</sup> with:		Pathogenicity			
Strain		rRNA	F3 Eco	Lesion surface (mm <sup>2</sup> ) <sup>b</sup>	Class <sup>c</sup>	Stem reaction <sup>d</sup>	
X. campestris manihotis							
ORSTX27	Togo	1	1	$57.7 \pm 4.5$	f	4	
UPB079 (LMG778)	Brazil	1	2	LC		1	
CIAT1129	Venezuela	1	3	$23.8 \pm 7.6$	с	4	
NCPPB1834 (UPB055)	Brazil	1	4	$50.8 \pm 5.8$	f	3	
UPB009 (LMG768, NCPPB3058)	Zaire	1	5	$44.8 \pm 11.1$	e	2	
LMG777 (UPB 078)	Brazil	2	6	$17.5 \pm 7.4$	b	3	
LMG779 (UPB 080)	Brazil	2	4	$42.7 \pm 13.6$	3	4	
ORST2 (CIAT1061)	Venezuela	2	8	$23 \pm 5.9$	с	2	
ORST5	Brazil	2	8	$52.8 \pm 13.2$	f	4	
ORST7	Brazil	2	9	$35.8 \pm 14.1$	d	3	
UPB70 (NCPPB1160)	Brazil	3	10	$17.6 \pm 4.8$	Ь	3	
CFBP1851 (CIAT1111)	Colombia	3	11	$6.6 \pm 2$	а	3	
ATCC23380 (NCPPB1159)	Brazil	4	12	$11.9 \pm 6.9$	ь	2	÷,
NCPPB348	Brazil	4	12	$24.3 \pm 7.1$	С	4	÷.
LMG774 (UPB060)	Taiwan	4	13	$62.6 \pm 25.1$	f	4	1
CIAT1135	Taiwan	4	13	$13.7 \pm 3.4$	ь	2	
NCPPB2444	Colombia	5	14	$50.1 \pm 10.9$	f	4	Ń
CIAT1117	Brazil	5	14	$50.1 \pm 10.9$	f	4	
CIAT1222	Venezuela	5	15	$46.7 \pm 4$	e	4	11.
X. campestris pv. cassavae							
LMG 672 (UPB 038)	Rwanda	6	16	LC		0	1,3
UPB029 (LMG 671, NCPPB3061)	Rwanda	6	16	$4.3 \pm 2.7$	а	. 0	
UPB030	Rwanda	б	16	$31.2 \pm 5.1$	d	2	
UPB035	Rwanda	6	16	$51 \pm 8.9$	f	1	9 - C
UPB039	Rwanda	6	16	$25.2 \pm 5$	с	· 2	1.
UPB041 (LMG5265)	Rwanda	б	16	$22 \pm 4.6$	С	2	λ.,
UPB046	Rwanda	6	16	$39.6 \pm 4.7$	d ·	2	. <sup>1</sup>
UPB047	Rwanda	6	16	$47.1 \pm 13$	e	2	j.
NCPPB 101 (LMG673, UPB 054)	Malawi	6	17	$5.7 \pm 5.3$	а	0	2. 1. juli
UPB032 -	Rwanda	6	17	$25.3 \pm 4.2$	с	2	÷.,
UPB033	Rwanda	6	17	$18.4 \pm 2.4$	b	0	
UPB043	Rwanda	6	17	$33.3 \pm 1.9$	d .	1 .	
UPB044	Rwanda	б	17	$15.2 \pm 7.4$	b	1	÷.,
UPB045 (LMG5268)	Rwanda	6	17	$28.6 \pm 3.4$	C	. 1	÷.
UPB049	Rwanda	б	17	$32.7 \pm 9.2$	d	2	
UPB051 (LMG5269)	Rwanda	б	17	$16.4 \pm 5.9$	b.	1	11
UPB053 (LMG5270)	Rwanda	6	17	$16.5 \pm 6.3$	ь	2	. <b>*</b> ,
UPB008	Zaire	6	17	$2.4 \pm 1.2$	а	0	2
UPB059 (LMG764)	Tanzanie	6	18	$22.8 \pm 4.7$	с	0	÷.,
UPB146 (LMG5271)	Kenya	6	18	$16.3 \pm 6.4$	Ь	1	
UPB037 (LMG5264)	Rwanda	7	19	$19.1 \pm 6.2$	ь	1	
X. campestris pv. vignicola				<b>f</b>			
UPB040	Nigeria	7	20	NR⁄		1 ;	ЯĽ,
X. campestris pv. poinsettiicola							
UPB073 (NCPPB581)	India	10	22	NR		1	. di
LMG5403	New Zealand	11	$\mathrm{NH}^{g}$	NR		1	
X. campestris euphorbiae							1. 1. 41
LMG863 (NCPPB1828)	Sudan	10	25	NR		1	ja.
LMG7402 (NCPPB2067)	Sudan	10	25	NR		1	
X. campestris pv. ricini							
UPB075 (LMG861, NCPPB1063)	Ethiopia	12	23	NR		1 4 4.4	1.1
UPB076 (NCPPB1324, LMG862)	Hong Kong	12	24	NR		1	Ĉ.
Undetermined pathovars							
UPB899 (LMG8048)	Niger	8	21	NR		1	
UPB900	Niger	8	_21	NR		1	• •
UPB137 (CIAT1164, LMG5244)	Colombia	9	NH	NR		1	S.
CIAT1165 (LMG5243, UPB136)	Colombia	9	NH	NR		1	
Undetermined strains	<b></b>			* ~			
CIAT 1187	Colombia	13	NH	LC		1	
CIAT 1192	Colombia	13	NH	LC		1	

<sup>a</sup> RFLP groups with rRNA probe and F3Eco plasmid probe. Endonuclease EcoRI was used. <sup>b</sup> Lesion surface calculated from means of five replicate leaves. The means  $\pm$  standard deviations are shown. <sup>c</sup> Values were clustered in six classes (a to f) in increasing surface order. Each class is significantly different from the others at P < 0.05 by the Kruskal-Wallis test for variability. Values among each class are not significantly different at P < 0.01 by the same test. <sup>d</sup> Stem reaction measured according to the scale described in the text. Numbers are not comparable across rows. <sup>e</sup> LC, some reaction as observed with the control leaf. <sup>f</sup> NR, necrotic reaction.

<sup>8</sup> NH, no hybridization.

Vol. 60, 1994

kb 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



FIG. 2. EcoRI ribotypes of X. campestris pathovars. Lanes: 1, 10, and 17, DNA marker Raoul (Eurogentec); 2, pv. vignicola (strain UPB 040); 3 and 4, pv. cassavae (strains UPB037 and UPB035, respectively) (lane 4 represents the typical ribotype pattern); 5 to 9, the five different ribotypes observed for strains of pv. manihotis (strains UPB070, ATCC23380, LMG777, CIAT1117, and NCPPB1834, respectively); 11, deviant pv. cassavae (strain UPB899); 12, pv. ricini (strain UPB075); 13. unknown pathovar (strain UPB137), 14, pv. euphorbiae (strain MG7402); 15 and 16, pv. poinsetticola (strains UPB073 and LMG 403, respectively). The ribotypes are identified in Table 2.

pattern, clearly distinct from the other ribotypes obtained (Fig. 2; Table 2).

A cluster analysis was performed on the basis of the ribotype patterns of 50 strains tested, and a dendrogram was produced (Fig. 3). Three main clusters appeared in the dendrogram. A cluster containing X. campestris pv. manihotis strains was clearly distinct from the X. campestris pv. cassavae group. According the cluster analysis, the strains of X. campestris pv. cassavae copeared to be most closely related to the strains of X. campestris pv. poinsettiicola and euphorbiae. Members of this cluster were 80% or more related to each other. A cluster enclosing X. campestris pv. ricini, the deviant X. campestris pv. cassavae strains, and two yellowish Colombian isolates was clearly distinct from the two other groups.

RFLP patterns with the plasmid probe. The hybridization patterns obtained with the DNA plasmid probe (F3Eco) used in this study are simple to interpret. Fewer bands were produced than with the probe pBSF2 (37). The DNA probe allowed different subgroups to be distinguished within X. npestris pv. cassavae and X. campestris pv. manihotis, respecavely (Fig. 4). Three different hybridization patterns were observed among strains of X. campestris pv. cassavae belonging to the same ribotype (Fig. 4; Table 2). The strains from Tanzania and Kenya had a similar hybridization pattern, clearly distinct from that of the strains from Rwanda and Malawi (Fig. 4). X. campestris pv. cassavae UPB037, which was clearly differentiated by ribotyping (Fig. 2), also showed a distinct hybridization pattern with the plasmid probe (Fig. 4). A high degree of polymorphism was detected among strains of X. campestris pv. manihotis, with 15 hybridization profiles o<sup>1</sup> served among the 19 strains tested (Table 2). Hybridization " th X. campestris pv. vignicola, ricini, and poinsettiicola (strain UPB073) and deviant X. campestris pv. cassavae gave different patterns, respectively (Fig. 4; Table 2). No hybridization was observed with the four Colombian yellowish isolates or with one strain of X. campestris pv. poinsettiicola (LMG 5403).

### DISCUSSION

Ribotyping has been suggested as a rapid way of comparing the genetic relationships among different bacteria (2, 9, 37). The similarities of leaf symptoms induced by *X. campestris* pv. c. savae and manihotis have raised the question whether the first pathovar could be a yellow variant of the second one or an unrelated taxon. By using ribotypes, X. campestris pv. cassavae can be clearly distinguished from strains of X. campestris pv. manihotis. Furthermore, on the basis of ribotype patterns, a quantitative measure of the genetic relationships between the strains that occur on cassava and those that occur on other members of the Euphorbiaceae (Euphorbiae pulcherrima, E. alcalyphoides, and Ricinus communis) was expressed.

Our results confirmed previous reports which have revealed that X. campestris pv. manihotis and cassavae can be differentiated from each other (25, 33, 34). Moreover, hybridization with the DNA plasmid probe revealed genomic polymorphism among strains of X. campestris pv. cassavae. The strains studied have been isolated in various East African countries and are representative of the geographical diversity within the pathovar. A significant finding is the fact that X. campestris pv. cassavae was found in Zaire (Yangambi). Until now, it has been detected only in East African countries (24).

Our results showed that X. campestris pv. cassavae is more heterogeneous than African strains of X. campestris pv. manihotis, which had previously shown a clonal population structure (37). Nevertheless, a high degree of polymorphism was detected among South American strains of X. campestris pv. manihotis, which confirmed previous reports (26, 37). Genetic diversity of strains within pathovars of X. campestris had also been described for various pathogens (2, 17, 32).

One strain of X. campestris pv. cassavae (LMG672) failed to induce any symptoms on cassava. This strain was described as a phenotypically aberrant isolate but was considered an authentic member of this pathovar (33). Our results confirmed that this strain belongs to this pathovar.

There is still some confusion in the literature concerning the pathovar naming of the yellowish strains (UPB137 and CIAT 1165) isolated in Colombia. Elango et al. (7) observed that these strains were serologically similar to X campestris pv. cassavae. On the basis of the study of the isozymic patterns (15) and on the peptide profile analysis (5a), the same isolates were found to be phylogenetically more closely related to X. campestris pv. manihotis than to the African strains of X. campestris pv. cassavae. On the other hand, Van den Mooter et al. (33) have shown that these isolates were genetically and electrophoretically very closely related to X. campestris pv. poinsettiicola. Our results show that these Colombian isolates can be clearly differentiated from other pathovars that occur on cassava and other members of the Euphorbiaceae. Moreover, the lack of hybridization with the plasmid probe confirmed that these strains are not related to X. campestris pv. manihotis.

On the basis of ribotyping results, the other two Colombian strains (CIAT1187 and CIAT1192) could be clearly separated from the other *Xanthomonas* strains. The 1.5-kb fragment which is absent in these strains seemed to be characteristic of the *Xanthomonas* pattern (2). Moreover, these strains do not induce any reaction on cassava stems or leaves. These non-xanthomonad isolates may exist as epiphytes or saprophytes on cassava.

Deviant strains of X. campestris pv. cassavae have been characterized in Niger (13). From the SDS-PAGE protein pattern and fatty acid methyl esters composition, Vauterin et al. (34) and Yang et al. (38) clustered these strains in the X. campestris pv. cassavae group. We clearly demonstrated that the deviant strains of X. campestris pv. cassavae were distinguishable from strains of X. campestris pv. cassavae and were related to X. campestris pv. ricini. These strains produced a brown diffusible pigment, as did strains of X. campestris pv. ricini (13). The possibility remains that these nonpathogenic





isolates on cassava are pathogenic *Xanthomonas* strains on other members of the Euphorbiaceae. Pathogenicity of the strains studied on a wide host range should be determined for better identification and for taxonomic studies.

X. campestris pv. poinsettiicola appears to be very heterogeneous in protein profiles (34) and fatty acid methyl esters profiles (38). RFLP analysis confirmed these previous results and also showed that strains of X. campestris pv. euphorbiae are closely related to X. campestris pv. poinsetticola. Sabet et al. (31) have reported an overlap in the host range of X. campestris pv. poinsettiicola (infecting E. pulcherrima and M. esculenta) and X. campestris pv. euphorbiae (infecting E. alcalyphoides, E. pulcherrima, and R. communis). The limited geographical distribution of X. campestris pv. cassavae, its heterogeneity, and, particularly, its absence in South America, the center of origin of cassava, suggested that cassava is not the primary host of X. campestris pv. cassavae (23). This hypothesis accords with the overlap observed in the host range of X. *campestris* strains isolated from members of the Euphorbiaceae. In Kenya, an overlap was also reported in the distribution of *X. campestris* pv. cassavae and manihotis (27).

The pathogenicity assay on leaves was used as a method to determine the level of aggressiveness of bacterial strains. This inoculation method had been used on detached leaves of *Citrus aurantifolia* (16). On *Manihot esculenta*, the method is useful for distinguishing, at the pathovar level, the capacity to colonize the host plant, and it also allows the detection of strain diversity or heterogeneity in *X. campestris* pv. manihotis and cassavae. Pathogenicity tests were conducted at 28°C; however, lesion development caused by *X. campestris* pv. cassavae could be limited at such temperature. Indeed, the optimum temperature for disease development is about 25°C for *X. campestris* pv. cassavae and 30°C for *X. campestris* pv. manihotis (24).

X. campestris pv. manihotis and cassavae offer a good opportunity for initial comparative studies of tissue colonization. The histological studies provided information on the Vol. 60, 1994





FIG. 4. Southern hybridization of *Eco*RI-digested genomic DNA of strains of *X. campestris* pathovars probed with <sup>32</sup>P-labeled clone F3*Eco*. Lanes: 1 and 2, pv. manihotis (strains ATCC23380 and NCPPB 2444, respectively); 3 to 10, pv. cassavae (strains UPB008, UPB146, UPB059, UPB045, UPB044, UPB041, LMG672, and UPB037, respectively); 11 and 12, deviant pv. cassavae (strains UPB900 and UPB899, respectively); 15 and 14, pv. ricini (strains UPB075 and UPB076, respectively); 15, pv. vignicola (strain UPB 040); 16, pv. poinsetticola (strain UPB073). The patterns are identified in Table 2.

processes associated with colonization of the same host by two different pathogens. Both *X. campestris* pv. manihotis and cassavae were characterized by colonization of the mesophyll intercellular spaces. In contrast to *X. campestris* pv. manihotis, systemic invasion of the xylem vessels by *X. campestris* pv. cassavae was not observed. The reason for these differences in pathogenic behavior is not clear. Extracellular enzymes produced by strains of *X. campestris* play a major role in pathogenicity (5), and Dow et al. (6) reported distinct differences between vascular and mesophyllic crucifer pathogens in the pattern of extracellular proteases produced.

Infection of X. campestris pv. cassavae in the stem led to the occurrence of a brown necrotic reaction (24) similar to the vascular hypersensitive response described by Kamoun et al. (14) with mesophyllic pathogens of crucifers. Coinoculation of X. campestris pv. cassavae and manihotis resulted in an inhibition of the vascular development of X. campestris pv. manihotis (3). This suggests that X. campestris pv. cassavae could induce a specific defensive response by the plant. On the other hand, the vascular pathogen (X. campestris pv. manihotis) may have the ability to overcome the defensive reaction. Kamoun et al. 14) have reported the role of hrpX genes in a similar type of reaction. Further cytochemical and molecular investigations may lead to a clearer understanding of these different cassavapathogen interactions.

## ACKNOWLEDGMENTS

We thank Claude Bragard and Paul Calatayud for their help in conducting the cluster analysis and C. Lozano (CIAT, Cali, Colombia) for providing isolates from Colombia.

### REFERENCES

- Berthier, Y., D. Thierry, M. Lemattre, and J. L. Guesdon. 1994. Isolation of an insertion sequence (IS1051) from Xanthomonas campestris pv. diffenbachiae with potential use for strain identification and characterization. Appl. Environ. Microbiol. 60:377– 384.
- Berthier, Y., V. Verdier, J. L. Guesdon, D. Chevrier, J. B. Denis, G. Decoux, and M. Lemattre. 1993. Characterization of *Xanthomonas* campestris pathovars by rRNA gene restriction patterns. Appl. Environ. Microbiol. 59:851–859.
- 3. Boher, B. Unpublished data.
- Cook, D., E. Barlow, and L. Sequeira. 1991. DNA probes as tools for the study of host-pathogen evolution: the example of *Pseudomonas solanacearum*, p. 103–108. *In* Kluwer Academic Publishers (ed.), Advances in molecular genetics of plant-microbe Interac-

tions. Kluwer Academic Publishers, The Netherlands.

- 5. Daniels, M. J., C. E. Barber, J. M. Dow, S. A. Han, S. A. Liddle, M. A. Newman, J. E. Parker, S. D. Soby, and T. G. J. Wilson. 1993. Plant and bacterial genes involved in interactions between Xanthomonas and crucifers, p. 423–433. In Kluwer Academic Publishers (ed.), Advances in molecular genetics of plant-microbe interactions. Kluwer Academic Publishers, The Netherlands.
- 5a.Dos Santos, R. M. D. B., and J. C. Dianese. 1985. Comparative membrane characterization of *Xanthomonas campestris* pv. cassavae and *X. campestris* pv. manihotis. Phytopathology 75:581-587.
- Dow, J. M., M. A. Fan, M. A. Newman, and M. J. Daniels. 1993. Differential expression of conserved protease genes in cruciferattacking pathovars of *Xanthomonas campestris*. Appl. Environ. Microbiol. 59:3996–4003.
- Elango, F. N., J. C. Lozano, and J. F. Peterson. 1981. Relationships between Xanthomonas campestris pv. manihotis, X.c. pv. cassavae and Colombian yellowish isolates, p. 96–106. In J. C. Lozano (ed.), Proceedings of the 5th International Conference on Plant-Pathogenic Bacteria. Centro Internacional de Agricultura Tropical, Cali, Colombia.
- Gabriel, D. W., J. E. Hunter, M. T. Kingsley, J. W. Miller, and G. R. Lazo. 1988. Clonal population structure of *Xanthomonas campestris* and genetic diversity among citrus canker strains. Mol. Plant-Microbe Interact. 1:59–65.
- Grimont, F., D. Chevrier, P. A. D. Grimont, M. Lefevre, and J. L. Guesdon. 1989. Acetylaminofluorene-labelled ribosomal RNA for use in molecular epidemiology and taxonomy. Res. Microbiol. 140: 447–454.
- Hildebrand, D. C., N. J. Palleroni, and M. N. Schroth. 1990. Deoxyribonucleic acid relatdness of 24 xanthomonad strains representing 23 Xanthomonas campestris pathovars and Xanthomonas fragariae. J. Appl. Bacteriol. 68:263–269.
- Ikotun, T. 1981. Some characteristics that distinguish Xanthomonas cassavae from Xanthomonas manihotis. Fitopatol. Bras. 6:1– 14.
- Jackson, D. A., K. M. Somers, and H. H. Harvey. 1989. Similarity coefficients: measures of co-occurrence and association or simply measures of occurrence. Am. Nat. 133:436–453.
- Janse, J. D., and M. Defrancq. 1988. Characterization of bacterial strains isolated from *Manihot esculenta* and of strains of *Xan*thomonas campestris pv. oryzae and X. campestris pv. ricini from Niger. Phytopathol. Mediterr. 27:182–185.
- Kamoun, S., H. V. Kamdar, E. Tola, and C. I. Kado. 1992. Incompatible interactions between crucifers and *Xanthomonas* campestris involve a vascular hypersensitive response: role of the hrpX locus. Mol. Plant-Microbe Interact. 5:22-33.
- Kimura, O., and J. C. Dianese. 1983. Proteic isoenzymic characterization of the pathovars of *Xanthomonas campestris* which attack cassava. Pesqui. Agropecu. Bras. 18:1215–1228.
- Lawson, R. H., M. M. Dienelt, and E. L. Civerolo. 1989. Histopathology of Xanthomonas campestris pv. citri from Florida and Mexico in wound-inoculated detached leaves of Citrus aurantifolia: light and scanning electron microscopy. Phytopathology 79: 329-335.
- Leach, J. E., M. L. Rhoads, C. M. Vera Cruz, F. F. White, T. W. Mew, and H. Leung. 1992. Assessment of genetic diversity and population structure of *Xanthomonas oryzae* pv. oryzae with a repetitive DNA element. Appl. Environ. Microbiol. 58:2188–2195.
- Leach, J. E., F. F. White, M. L. Rhoads, and H. Leung. 1990. A repetitive DNA sequence differentiates *Xanthomonas campestris* pv. oryzae from other pathovars of *X. campestris*. Mol. Plant-Microbe Interact. 3:238-246.
- Leite, R. P., G. V. Minsavage, U. Bonas, and R. E. Stall. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria. Appl. Environ. Microbiol. 60:1068–1077.
- Leung, H., R. J. Nelson, and J. E. Leach. 1993. Population structure of plant pathogenic fungi and bacteria, p. 157-205. *In* J. H. Andrews and I. C. Tommerup (ed.), Advances in plant pathology, vol. 10, Academic Press, Inc., San Diego, Calif.
- Maes, M. 1993. Fast classification of plant-associated bacteria in the *Xanthomonas* genus. FEMS Microbiol. Lett. 113:161-166.

4486 VERDIER ET AL.

- 22. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maraite, H. 1993. Xanthomonas campestris pathovars on cassava: cause of bacterial blight and bacterial necrosis, p. 18-24. In J. G. Swings and E. L. Civerolo (ed.), Xanthomonas. Chapman & Hall, Ltd., London.
- 24. Maraite, H., and D. Perreaux. 1978. Comparative symptom development in cassava after infection by Xanthomonas manihotis or X. cassavae under controlled conditions, p. 18–26. In E. R. Terry, G. J. Persley, and S. C. A. Cook (ed.), Cassava bacterial blight in Africa: past, present and future. Reports of an interdisciplinary workshop, IITA, Ibadan, Nigeria. COPR Publishing Co., London.
- 25. Maraite, H., and J. Weyns. 1979. Distinctive physiological, biochemical and pathogenic characteristics of *Xanthomonas maniho*tis and *Xanthomonas cassavae*, p. 103–117. In H. Maraite and J. A. Meyer (ed.), Diseases of tropical food crops. Université Catholique de Louvain, Louvain La Neuve, Belgium.
- 26. Maraite, M., J. Weyns, O. Yinkwan, P. Lipembra, and D. Perreaux. 1981. Physiological and pathogenic variations in *Xanthomonas campestris* pv. manihotis, p. 358–368. *In J. C. Lozano (ed.)*, Proceedings of the 5th International Conference on Plant Pathogenic Bacteria, Centro Internacional de Agricultura Tropical, Cali, Colombia.
- Onyango, D. M., and A. H. Ramos. 1978. La bactériose du manioc au Kenya, p. 26–29. *In* E. R. Terry, G. J. Persley, and S. C. A. Cook
   (ed.), Cassava bacterial blight in Africa: past, present and future. Reports of an interdisciplinary workshop, IITA, Ibadan, Nigeria. COPR Publishing Co., London.
- Pruvost, O., J. S. Hartung, E. L. Civerolo, C. Dubois, and X. Perrier. 1992. Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. citri, the causal agent of citrus bacterial canker disease. Phytopathology 82:485-490.
- 29. Qhobela, M., and L. E. Claffin. 1992. Eastern and southern African strains of *Xanthomonas campestris* pv. vasculorum are distinguishable by restriction fragment length of DNA and polyacryl-

amide gel electrophoresis of membrane proteins. Plant Pathol. **41**:113–121.

- Robbs, C. F., R. D. Ribeiro, O. Kimura, and F. Lakiba. 1972. Variacoes em Xanthomonas manihotis (Arthaud Berthet) Starr. Rev. Soc. Bras. Fitopatol. 5:67-75.
- Sabet, K. A., F. Ishag, and O. Khalil. 1969. Studies on the bacterial diseases of Sudan crops. VII. New records. Ann. Appl. Biol. 63: 357–369.
- 32. Stall, R. E., C. Beaulieu, D. Egel, N. C. Hodge, R. P. Leite, G. V. Minsavage, H. Bouzar, J. B. Jones, A. M. Alvarez, and A. A. Benedict. 1994. Two genetically diverse groups of strains are included in *Xanthomonas campestris* pv. vesicatoria. Int. J. Syst. Bacteriol. 44:47-53.
- 33. Van den Mooter, M., H. Maraite, L. Meiresonne, J. Swings, M. Gillis, K. Kersters, and J. De Ley. 1987. Comparison between Xanthomonas campestris pv. manihotis ISPP list 1980 and Xanthomonas campestris pv. cassavae ISPP list 1980 by means of phenotypic, protein electrophoretic, DNA hybridization and phytopathological techniques. J. Gen. Microbiol. 133:57–71.
- Vauterin, L., J. Swings, and K. Kersters. 1991. Grouping of Xanthomonas campestris pathovars by SDS-PAGE of proteins. J. Gen. Microbiol. 137:1677-1687.
- 35. Vauterin, L., J. Swings, K. Kersters, M. Gillis, T. Mew, M. N. Schroth, N. J. Palleroni, D. C. Hidebrand, D. E. Stead, E. L. Civerolo, A. C. Hayward, H. Maraite, R. E. Stall, A. K. Vidaver, and J. F. Bradbury. 1990. Towards an improved taxonomy of *Xanthomonas*. Int. J. Syst. Bacteriol. 40:312–316.
- 36. Verdier, V., C. Boucher, P. Barberis, and B. Boher. Unpublished data.
- Verdier, V., P. Dongo, and B. Boher. 1993. Assessment of genetic diversity among strains of *Xanthomonas campestris* pv. manihotis. J. Gen. Microbiol. 139:2591–2601.
- Yang, P., L. Vauterin, M. Vancanneyt, J. Swings, and K. Kersters. 1993. Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. Syst. Appl. Microbiol. 16:47– 71.