APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4478–4486
0099-2240/94/$04.00+0

Vol. 60, No. 12

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

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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.

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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^{-1}), Casamino Acids (1 g liter^{-1}), and yeast extract (1 g liter^{-1}) (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 yellow-pigmented 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 assessed, and the observed reactions were rated 4 weeks after inoculation 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-μl 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-μm-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 with 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, respectively; 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 *Hin*dIII (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), $S = 100 \times [2N_{ab}/(N_a + N_b)]$, 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

TABLE 1. Strain collection

X. campestris pathovar	Strain[a]	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
	UPB047	*Manihot esculenta*	Rwanda, 1978
	UPB049	*Manihot esculenta*	Rwanda, 1978
	UPB051 (LMG5269)	*Manihot esculenta*	Rwanda, 1978
	UPB053 (LMG5270)	*Manihot esculenta*	Rwanda, 1978
	UPB059 (LMG764)	*Manihot esculenta*	Tanzania, 1978
	UPB146 (LMG5271)	*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, 1974
	ORST7	*Manihot esculenta*	Brazil, 1973
	UPB070 (NCPPB1160)	*Manihot esculenta*	Brazil, 1941
	CFBP1851 (CIAT1111)	*Manihot esculenta*	Colombia, 1974
	ATCC23380 (NCPPB1159)	*Manihot esculenta*	Brazil, 1941
	NCPPB348	*Manihot esculenta*	Brazil, 1954
	LMG774 (UPB060)	*Manihot esculenta*	Taiwan, 1978
	CIAT1135	*Manihot esculenta*	Taiwan, 1975
	NCPPB2444	*Manihot esculenta*	Colombia, 1970
	CIAT117	*Manihot esculenta*	Brazil, 1974
	CIAT1122	*Manihot esculenta*	Venezuela, 1974
Deviant cassavae	UPB899 (LMG8048)	*Manihot esculenta*	Niger, 1987
	UPB900	*Manihot esculenta*	Niger, 1987
Poinsettiicola	UPB073* (NCPPB581)	*Euphorbiae pulcherrima*	India, 1950
	LMG5403	*Euphorbiae pulcherrima*	New Zealand, 1972
Euphorbiae	LMG863* (NCPPB1828)	*Euphorbiae alcalyphoides*	Sudan, 1965
	LMG7402 (NCPPB2067)	*Euphorbiae alcalyphoides*	Sudan, 1966
Ricini	UPB075* (NCPPB1063)	*Ricinus communis*	Ethiopia, 1961
	UPB076 (NCPPB1324, LMG862)	*Ricinus communis*	Hong Kong, 1962
Vignicola	UPB040	*Vigna sinensis*	Nigeria, 1977
Unknown	UPB008	*Manihot esculenta*	Zaire, 1973
	UPB137 (CIAT1164, LMG5244)	*Manihot esculenta*	Colombia, 1976
	CIAT1165 (LMG5243, UPB136)	*Manihot esculenta*	Colombia, 1976
	CIAT 1187	*Manihot esculenta*	Colombia, 1992
	CIAT 1192	*Manihot esculenta*	Colombia, 1992

[a] 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

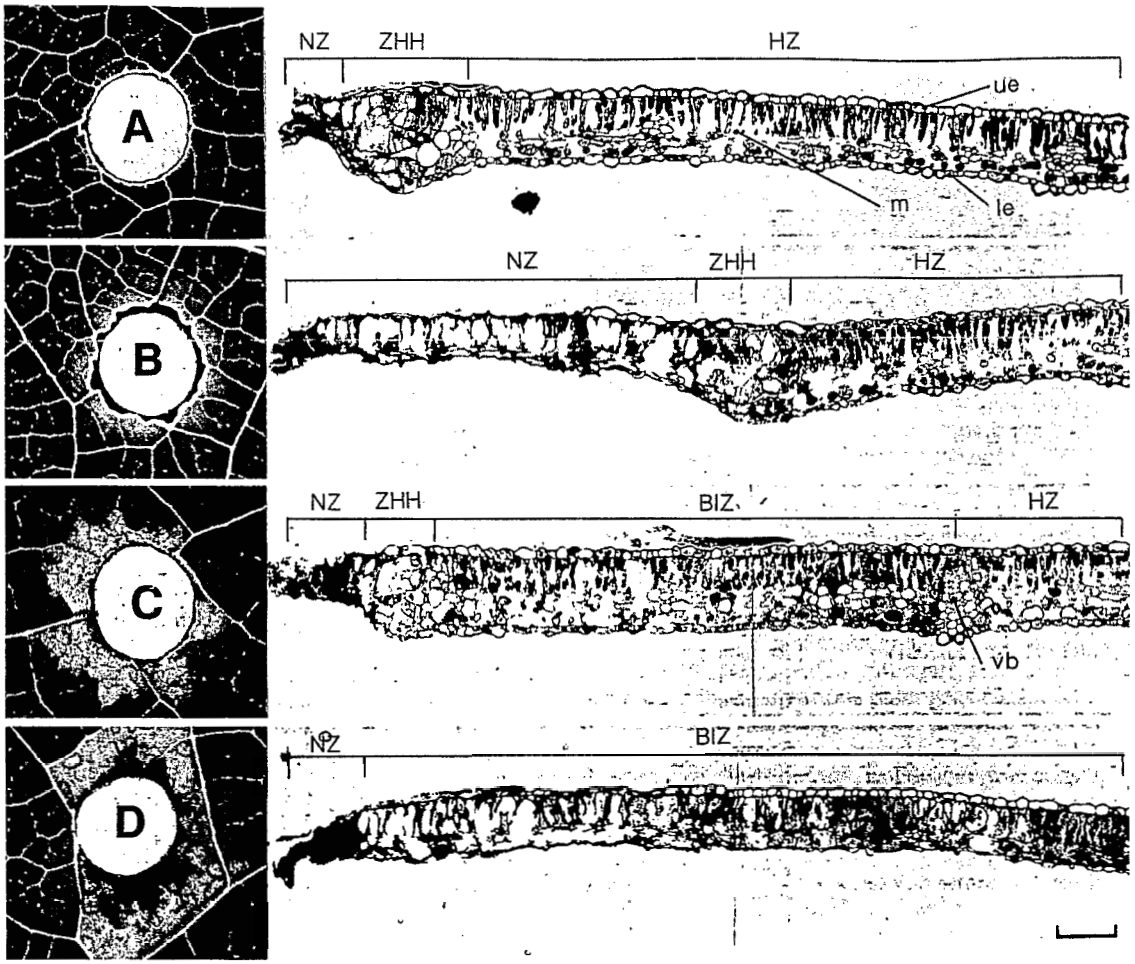

FIG. 1. Symptomatological (left) and histological (right) comparison of the development of three isolates on cassava leaf. (A) Water control; (B) strain UPB137; (C) *X. campestris* pv. cassavae UPB053; (D) *X. campestris* pv. manihotis ORSTX27. The left-hand panels illustrate lesion aspects. The central 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. Abbreviations: NZ, necrotic zone; ZHH, zone of hyperplasia and hypertrophy; BIZ, bacterium-invaded zone; HZ, healthy tissues; ue, upper epidermis; 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. cassavae 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

TABLE 2. Hybridization and pathogenicity results

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

[a] RFLP groups with rRNA probe and F3*Eco* plasmid probe. Endonuclease *Eco*RI was used.
[b] Lesion surface calculated from means of five replicate leaves. The means ± standard deviations are shown.
[c] 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.
[d] Stem reaction measured according to the scale described in the text. Numbers are not comparable across rows.
[e] LC, some reaction as observed with the control leaf.
[f] NR, necrotic reaction.
[g] NH, no hybridization.

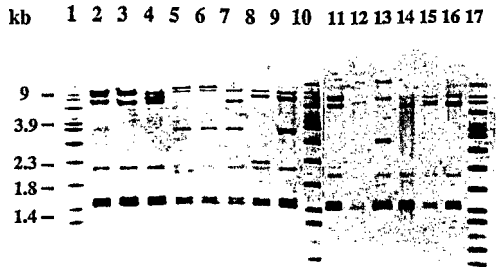

FIG. 2. *Eco*RI ribotypes of *X. campestris* pathovars. Lanes: 1, 10, and 17, DNA marker Raoul (Eurogentec); 2, pv. vignicola (strain UPB 040); 3 and 4, pv. cassavae (strains UPB037 and UPB035, respectively) (lane 4 represents the typical ribotype pattern); 5 to 9, the five different ribotypes observed 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. poinsettiicola (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 to the cluster analysis, the strains of *X. campestris* pv. cassavae appeared 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 (F3*Eco*) 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. campestris* pv. cassavae and *X. campestris* pv. manihotis, respectively (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 observed among the 19 strains tested (Table 2). Hybridization with *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. cassavae 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 nonxanthomonad 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

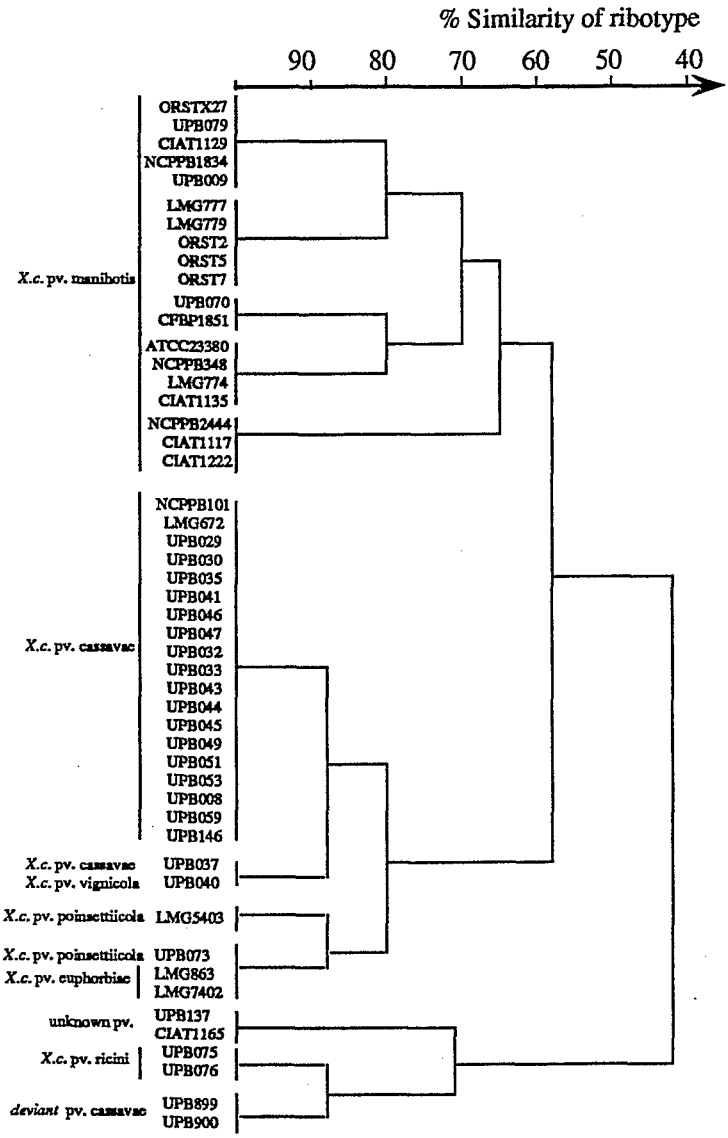

FIG. 3. Clustering of strains of *X. campestris* according to similarity of *Eco*RI ribotypes. All the strains listed in Table 1 were included, except two yellowish Colombian isolates (strains CIAT1187 and CIAT1192).

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

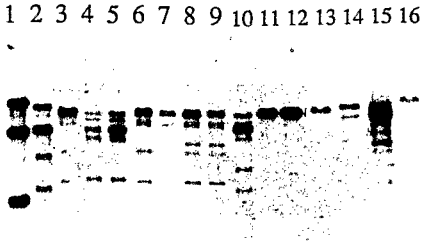

FIG. 4. Southern hybridization of *Eco*RI-digested genomic DNA of strains of *X. campestris* pathovars probed with ^{32}P-labeled clone F3*Eco*. Lanes: 1 and 2, pv. manihotis (strains ATCC23380 and NCPPB 2444, respectively); 3 to 10, pv. cassavae (strains UPB008, UPB146, UPB059, UPB045, UPB044, UPB041, LMG672, and UPB037, respectively); 11 and 12, deviant pv. cassavae (strains UPB900 and UPB899, respectively); 13 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 cassava-pathogen 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.

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