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Molecular characterization of the incitant of cowpea bacterial blight and pustule, *Xanthomonas campestris* pv. *vignicola*

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Abstract

Strains of *Xanthomonas campestris* pv. *vignicola* (*Xcv*), isolated from cowpea leaves with blight or minute pustules and collected from various geographic areas, were selected on the basis of pathological and physiological features. All strains were analyzed for genotypic markers by two methods: ribotyping with *Eco*RI endonuclease, and RFLP analysis with a plasmid probe (*pth*B) containing a gene required for pathogenicity from *Xanthomonas campestris* pv. *manihotis*. Ribotyping revealed a unique pattern for all the strains that corresponded to the previously described ribotype rRNA7. Based on polymorphism detected by *pth*B among *Xcv* strains, nine haplotypes were defined. The observed genetic variation was independent of the geographic origin of the strains and of pathogenic variation. Some haplotypes were widely distributed, whereas others were localized. In some cases, we could differentiate strains isolated from blight symptoms and pustules according to haplotypic composition. However, in most cases, no significant differences were observed. Our results and the previous pathogenic and biochemical characterizations suggest that the strains isolated from leaves with blight symptoms or minute pustules belong to the same pathovar. We provide information on pathogen diversity that can be used to identify and characterize resistant germplasm.

Abbreviations: Xcv – Xanthomonas campestris pv. *vignicola*; *Xcm – Xanthomonas campestris* pv. *manihotis.*

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a staple crop of economic importance in the semi-arid to humid tropics of African countries and is a major source of protein for human nutrition. Cowpea production is limited by numerous pests and diseases (Bashir and Haptom, 1996), among which bacterial diseases are widespread and can cause losses as high as 92% (Kishun, 1989). *Xanthomonas campestris* pv. *vignicola* (*Xcv*) is the causal agent of bacterial blight of cowpea and induces cankers on stems and a broad range of symptoms on leaves (Patel, 1983). On the basis of genetic studies, Vauterin et al. (1995) recently proposed a reclassification of *Xanthomonas*, naming the cowpea bacterial blight organism *Xanthomonas axonopodis* pv. *vignicola*. However, because this change is not widely known and not yet accepted by the scientific community, we will use *Xanthomonas campestris* pv. *vignicola* to prevent confusion. Because of the variability of symptoms observed, Patel and Jindal (1982) proposed classifying the causal agent of bacterial pustule as a distinct pathovar, 'pv. *vignaeunguiculatae*'.

Vigna pubigera and *V. unguiculata* are described as natural hosts of both the blight and pustule causing bacterium. Differentiation between the blight and pustule bacteria are based on symptomatology on the host plant and some phenotypic features (Jindal and Patel, 1984). Pathogenic variation among strains of *Xcv* can be detected by using a range of cowpea varieties (Gitaitis, 1982; Gitaitis, 1983; Khatri-Chhetri et al., 1996a; Patel, 1983), but classification into races, or even into distinct pathovars, is not clear and needs improving. Recent attempts to distinguish the cowpea strains causing pustules from those causing blight by biochemical and physiological characterizations were unsuccessful (Khatri-Chhetri et al., 1995).

Molecular and serological techniques have been used to clarify the taxonomy of different xanthomonads pathogenic to the same host (Alvarez et al., 1994; Bragard et al., 1995; Leach et al., 1990; Verdier et al., 1994). Different molecular approaches, based on DNA polymorphism, have also been developed to study the population structure and assess the genetic variability within strains belonging to the same pathovar (Leach et al., 1992; Verdier et al., 1993). Ribotyping, based on the polymorphism of the conserved rRNA genes, is a highly discriminative method, that is currently used in phylogenetic studies of phytopathogenic bacteria (Berthier et al., 1993; Bragard et al., 1995; Nassar et al., 1994; Verdier et al., 1994). RFLP analysis with a selected plasmid probe (*pth*B) can distinguish between different strains of *Xanthomonas* at the pathovar level (Bragard et al., 1995; Verdier et al., 1994) and permit assessment of genetic variability of *Xanthomonas campestris* pv. *manihotis* (*Xcm*) field populations (Verdier et al., 1993). Probe *pth*B is a 5.6-kb *Eco*RI fragment that was cloned from an indigenous plasmid of *Xcm*. It harbors a pathogenicity gene related to the *avr/pth* gene family (Verdier et al., 1996). Avirulence (*avr*) genes mostly function as determinants of race specificity and are subjected to strong selection pressure (Leach & White, 1996). Because of their role in host-pathogen interactions, *avr* genes have been widely used as probes in RFLP studies of *X. oryzae* pv. *oryzae* (Adhikari et al., 1995; Nelson et al., 1994; Yashitola et al., 1997) and *Xanthomonas* pv. *mangifereindicae* (Gagnevin et al., 1997).

We aimed to characterize various strains of *Xcv* isolated from blight symptoms and pustules and to assess the genetic diversity of strains of various geographic origins. Our data from the genetic characterization does not support the proposed classification of *Xcv* into two different pathovars.

Materials and methods

Bacterial strains and virulence tests

The *Xcv* strains studied are listed in Table 1. Cowpea leaves showing leaf blight and pustule-like symptoms were collected in three African countries at different locations: two sites in Cameroon and in Niger, four in Nigeria. Within each site strains were collected from different cowpea cultivars. Further strains were obtained from international culture collections (Table 1). Reference strains of *Xanthomonas cassavae* (UPB 041), *Xcm* (NCPPB 1159) and *Xanthomonas campestris* pv. *phaseoli* (CIAT 214) were included in this study. All strains were stored as frozen glycerol stocks and were maintained on LPGA medium (5 g yeast extract, 5 g Bacto-peptone, 5 g glucose, 15 g agar, 1 liter distilled water, (pH 7.2). Liquid cultures for DNA extraction were grown on a medium containing 10 g peptone, 1 g casamino acids and 1 g yeast extract per liter. All strains were evaluated for pathogenicity on the susceptible cowpea genotype IT84D-449, according to the method described by Khatri-Chhetri et al. (1996b).

Ribotyping

Genomic DNA was extracted from a 5 ml culture grown overnight, following the method described by Ausubel et al. (1991). The DNA pellet was dissolved in 200 μl TE (10 mM Tris, 1 mM EDTA, pH 8.0). Approximately 5 μg DNA were digested with the enzyme *Eco*RI, as specified by the manufacturer (Eurogentec, Liege, Belgium). DNA fragments were separated by electrophoresis in 0.7% agarose-1 X TBE (40 mM Tris borate, 1 mM EDTA; pH 7.8) gels at 40 V for 4 h before alkali transfer to a nitrocellulose membrane. The probe used was a 16S + 23S rRNA probe from *Escherichia coli*, labelled with acetylaminofluorene (AAF) (Eurogentec, Liège, Belgium). Hybridization was performed as described by Berthier et al. (1993).

Restriction fragment length polymorphism (RFLP) analysis

Approximately 7 μg of genomic DNA were digested overnight at 37 °C with *Eco*RI (Eurogentec, Liège, Belgium), as described by the manufacturer. Digested DNA samples were run in an 0.7% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 7.6) at 30 V for 15 h prior to alkaline transfer on to

Table 1. Characteristics of *Xanthomonas campestris* pv. *vignicola* strains used in this study according to virulence and RFLP analysis

Origin	Locality	Strains[1]	Virulence[2]	Haplotype[3]
Field strains				
Cameroon	Maroua	1a, 1b2, 3b1, 3b2	L	p01, p01, p03, p03
		1b1, 4a1	M	p01, p01
		4b1	H	p02
	Garoua	2a1, 2b1	M	p01, p04
Nigeria	Baganda	7a1, 7b1, 8a2	M	NH, p01, p01
		6a1	H	p02
	Ibadan	13a2, 14b, 17b, 19a3, 19bk, 23a	L	p02, p02, p01, p01, p01, p01
		14a1, 15a, 16b, 19bg, 20b, 21a,	M	NH, p02, p01, p01, p01, p02
		21b1, 22a, 22a1, 22b3, 26a, 26ak	M	p02, p02, p02, p01, p01, p01
		15b, 17a, 24a2, 24b1, 25a, 26ag	H	p02, p02, p01, p01, p01, p01
	Kano	28a2	L	p02
		28a1, 29a, 32a1, 33b5, 35a3, 40a2	M	p02, p02, p02, nd, p06, p02
		28a1k, 33a1, 37b1, 37b2, 38a2	H	p02, p02, p02, p01, p02
		38a3, 39a4, 41a, 41a1, 42a4	H	p02, p02, p01, p01, p02
		36b4, 40b2	NP	NH
	Minjebi	34b5	NP	NH
		30b2	M	p01
		30a1	H	p01
Niger	Niamey	12a2	M	p02
	Cerra Kollo	47a, 53b1,	L	p07, p02
		44b1, 45a, 45b1,45b2, 51b3, 54b, 55a	M	p02, p02, p02, p02, p02, p01, p09
		44a, 46b2, 48a, 49a, 51a2, 53a, 56a	H	p02, p02, p02, p02, p08, p02, p02
Reference strains				
Venezuela		534	M	p01
		536a1g, 536b	H	p05, p05
Brazil		NCPPB 3187	L	p01
Nigeria		UPB 040	nd	20[4]
Sudan		NCPPB 2061	L	nd

Abbreviations: NCPPB — National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.; UPB — Unité de Phytopathologie Bacterial Collection, Louvain-La-Neuve, Belgium.
[1] Strains with the same number were isolated from the same plant showing, blight symptom (a), pustule-like symptom (b), small colony size (k), big colony size (g). Number following letter = colony type, most originate from one original strain, which differentiated after subculturing into different colony types.
[2] According to the scale described by Khatri-Chhetri et al. (1996a and 1996b), L — Low; M — Medium; H — High and NP — nonpathogenic; nd — not determined.
[3] *pth*B haplotype determined,as described in the text, for each strain respectively: nd — not determined; NH — no hybridization.
[4] As previously described by Verdier et al. (1994).

Hybond N^+, as recommended by the manufacturer (Amersham, France).

As probe, we used plasmid *pth*B which contained a 5.6-kb *Eco*RI fragment internal to a plasmid-borne pathogenicity gene from *Xcm* (Verdier et al., 1996). The probe was labelled with [^{32}P]dCTP (Amersham, France) by random priming, and hybridized to blots according to manufacturer' s instructions. Conditions of hybridization and washes were of high stringency. Filters were washed once with 2 × SSC, 0.1% SDS for 20 min; twice with 1 × SSC, 0.1% SDS for 15 min; and twice with 0.1 × SSC, 0.1% SDS for 10 min. The membranes were exposed to X-ray film at −80 °C with intensifying screens.

Data analysis

Each distinct RFLP banding pattern was regarded as a haplotype. Binary data were derived from the restriction fragment profiles of the strains by scoring the presence (1) or absence (0) of each band for each

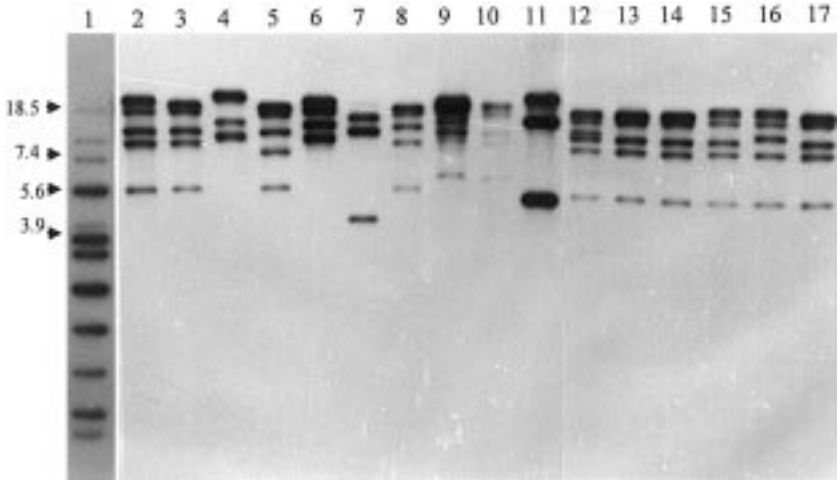

Figure 1. Southern blot analysis of *EcoR*I-digested genomic DNA from *X. campestris* pv. *vignicola* and reference strains probed with *pth*B and showing different haplotypes. Lane 1, RaoulI size marker (in kilobases) (Eurogentec, Liège, Belgium); lanes 2 and 15, p01 strains *Xcv* 19a3 and *Xcv* 54b, respectively; lanes 3, 13, 14 and 17, p02 strains *Xcv* 21b1, *Xcv* 51b3, *Xcv* 53b1 and *Xcv* 56a, respectively; lane 4, p03 *Xcv* 3b2; lane 5, p04 *Xcv* 2b1; lane 6, p05 *Xcv* 536b; lane 7, p06 *Xcv* 35a3; lane 8, p07 *Xcv* 47a; lane 9, Ht20 *Xcv* UPB040; lane 10, *X. cassavae* UPB041, Hzt16; lane 11, *X. campestris* pv. *manihotis* NCPPB 1159, Ht11; lane 12, p08 *Xcv* 51a2; lane 16, p09 *Xcv* 55a.

haplotype. The method described by Vera Cruz et al. (1996) was used. A pairwise comparison of strains was generated by NTSYS-pc (version 1.80, Exeter Biological Software, Setauket, NY) with Jaccard's coefficient of similarity. The data were used for single correspondence analysis (Version 6, SAS/IML Software: Usage and Reference; SAS/STAT User's Guide, SAS Institute, Inc., Cary, NC). The positions of the strains were determined on a three-dimensional graph that was drawn by the JMP program (version 3.1, SAS). The number of clusters was determined by consensus among three clustering statistics (SAS). Average genetic distances between and within clusters were obtained by the SAS/IML procedure.

Results

Ribotyping analysis

All the *Xcv* strains analysed were found to be identical by ribotyping and, thus, were grouped in the same ribotype (data not shown). The *Xcv* ribotype corresponds to ribotype 7, previously described by Verdier et al. (1994), and composed of five bands ranging in size from 1.5 to 9 -kb.

RFLP analysis with pth B *probe*

Among the 79 field strains examined for DNA polymorphism by RFLP analysis, using the plasmid probe *pth*B, nine haplotypes were defined (Figure 1). No hybridization was observed with five strains. The hybridization patterns generated were easy to interpret because of the small number of bands (3 to 6 bands scored per strain; Figure 1). A total of 14 band positions were scored. Two haplotypes (p01 and p02) were widely distributed. Haplotype p01 was observed among strains isolated in Africa and South America, and was found at more than one site in each African country (Table 1). In Africa, the most widely distributed *pth*B haplotype was p02; seven haplotypes were represented by only one or two strains (p03, p04, p05, p06, p07, p08, p09) and were site specific (Table 1). Strains from Cerra Kollo (Niger) were more diverse as five haplotypes were described within this site.

Forty-two strains isolated both from blight and pustule symptoms on the same plant were examined for DNA polymorphism (Table 1). These strains have the same number and differ by the letter following the number (a: isolated from blight symptom or b: isolated from pustule symptom) (Table 1). Strains from blight symptoms or pustules on the same plant could not be distinguished, except for strains 2a1 and 2b1, 4a1 and 4b1, and 17a and 17b) (Table 1). Particularly intriguing were two strains, 37b1 and 37b2, isolated from the same plant and the same type of symptom, that could be distinguished. These strains corresponded to different colony types. Reference strains from Brazil and Venezuela belonged to haplotypes p01 and p05. The *Xcv* strain UPB 040 belongs to a haplotype previ-

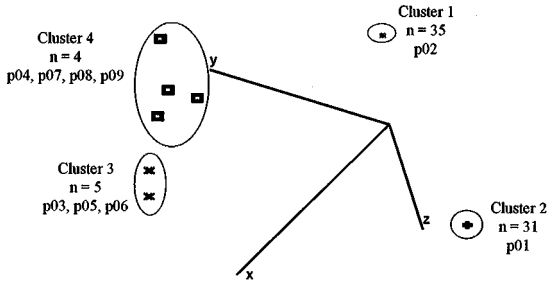

Figure 2. Three-dimensional graph from a single correspondence analysis (version 6, SAS/IML Software), showing the relationship of the *Xanthomonas campestris* pv. *vignicola* clusters after RFLP analysis with the plasmid probe *pth*B. The graph was drawn by the program JMP (version 3.1, SAS). Clusters were assigned by the average linkage method. The consensus among three clustering statistics, pseudo F^2, pseudo t^2, and cubic clustering criterion indicated four clusters. The first three dimensions (x, y, z) accounted for 54.5%, 44.9%, and 0.3% (total = 99.7%) of the variation for RFLP analysis. The haplotype and the number (n) of strains contained in each cluster are indicated. Symbols denote cluster members.

ously described by Verdier et al. (1994) but otherwise was not found in this study. Hybridization of strains *X. campestris* pv. *phaseoli*, pv. *manihotis* and pv. *cassavae* with the *pth*B probe gave different patterns (Figure 1, some data not shown).

Cluster analyses

Data obtained from the 74 field strains that showed a hybridization pattern and from reference strain NCPPB 3187 were included in the cluster analysis. The clustering criterion was the average linkage method and the number of clusters was determined by consensus among three principal clustering statistics (Vera Cruz et al., 1996). The 75 *Xcv* strains were grouped into four clusters (Figure 2). Two clusters were each represented by only one haplotype. Cluster 1 consisted of strains belonging to haplotype p02 isolated in Cameroon, Niger and Nigeria (Figure 2 and Table 1). Cluster 2 (strains showing the haplotype p01) also consisted mainly of strains of broad geographic origin (Table 1). Distances between and within clusters are indicated in Table 2. Clusters 3 and 4 contained a diverse group of strains and the within-cluster distance of clusters 3 and 4 was relatively high (0.57 and 0.48, respectively). Cluster 3 was represented by five strains that belonged to three haplotypes (p03, p05, p06) and were collected from diverse geographical regions (Cameroon, Venezuela and Nigeria, respectively). Cluster 4 consisted of four strains, one originating from Cameroon and the other three from Niger, and each belonging to a haplotype (p04, p07, p08, p09).

Table 2. Mean genetic distances between and within the four clusters generated by the analysis of 75 *Xanthomonas campestris* pv. *vignicola* strains analyzed with the *pth*B probe

Cluster	1	2	3	4
1	0.0			
2	0.16	0.0		
3	1.0	1.0	0.57	
4	0.40	0.41	1.0	0.48

Virulence of strains

Infiltration of bacteria into leaves of the susceptible cowpea genotype (IT84D 449) caused leaf and stem symptoms for all strains tested except strains 36b4 and 40b2 (Table 1). However, variation of virulence was detected among *Xcv* strains. Strains were classified as low, medium and highly virulent, according to Khatri-Chhetri et al. (1996b). Substantial variation was observed within most haplotype groupings. Most of the strains characterized as belonging to haplotypes p01 and p02 were highly virulent on cowpea, whereas strains belonging haplotypes p07 and p09 were weakly pathogenic. Some strains belonging to the same haplotype could be distinguished by the virulence analysis.

Discussion

Ribotyping has been suggested as a rapid way for comparing the genetic relationships among different bacteria (Grimont and Grimont, 1986). This technique differentiates among pathovars of *Xanthomonas* (Berthier et al., 1993), and also among closely related *Xanthomonas* strains having a similar phenotype or host (Bragard et al., 1995; Verdier et al., 1994). By using ribotyping, *X.c*. pv. *vignicola* can be clearly distinguished from other *Xanthomonas* pathovars. Nevertheless, strains originating from cowpea showing either blight symptoms or pustules could not be clearly distinguished. Thus, we conclude from the ribotyping results that all the *Xcv* strains we studied belonged to the same pathovar, supporting findings from studies on pathological and physiological studies by Khatri-Chhetri et al. (1995; 1996a; 1996b).

The probe containing *pth*B is of interest because the *pth*B gene is a member of the *avr*/*pth* gene family and plays a role in pathogenicity of *Xcm* (Verdier et al., 1996). RFLP analysis with this probe gave us additional insights into the pathogen variation. Most *Xcv* strains carry at least 3 to 6 DNA fragments that hybridize with the *Xcm pth*B gene. Hybridization of multiple fragments from two strains of *Xcv* was also observed, using another *avr/pth* gene family member as a probe (Swarup et al., 1992).

Clustering of strains from different regions of the same country or from different countries suggests patterns of pathogen migration that were probably a result of exchange of contaminated seeds. The pathogen can be transmitted through natural seed lots Gitaitis & Nilakhe (1982) or disseminated by insects (Gitaitis et al., 1986; Kaiser and Vakili, 1978).

An RFLP analysis with the *pth*B probe is particularly suitable for the rapid molecular characterization of *Xcv* because it allows discrimination among closely related but distinct bacterial strains. One site in Niger (Cerra Kollo) was particularly interesting as different haplotypes could be distinguished in the same area. This may have resulted from the diversity of cowpea genotypes planted there (K. Wydra, pers. comm.).

We compared the RFLP-*pth*B patterns characterized in this study with data obtained by the same analysis, using different pathovars of *Xanthomonas* (Berthier et al., 1993; Bragard et al., 1995; Verdier et al., 1994). We found that all the nine haplotypes identified were specific to *Xcv*. Unique profiles generated by the plasmid probe have been previously exploited for discerning different closely related strains forming a pathovar (Bragard et al., 1995; Verdier et al., 1993; Verdier et al., 1994).

Particularly intriguing is the fact that these sequences were carried by very different pathogens. DNA fragments that hybridized to the *pth*B *Xcm* probe were found in different strains of *Xanthomonas campestris*, including pv. *malvacearum*, pv. *translucens*, pv. *phaseoli* and pv. *cassavae* (Bragard et al., 1995; Verdier et al., 1994). Swarup et al. (1992) have also shown that multiple fragments homologous to *pth*A, a member of the *avr*/*pth* gene family, were present in different pathovars of *Xanthomonas*.

In *Xanthomonas*, members of the *avr*/*pth* gene family, located either chromosomally or on plasmids, are characterized by close similarity, and are strongly involved in plant-bacteria interactions (De Feyter and Gabriel, 1991; Leach & White, 1996). Their presence in phylogenetically distinct xanthomonads suggests that these genes may have transferred horizontally (Yang et al., 1994). Although demonstration of gene transfer has not yet been reported, presence of inverted repeat sequences in *avr*/*pth* genes suggest that they may transpose (De Feyter et al., 1993).

No hybridization to *pthB* was observed with five *Xcv* strains. Of these, three were found to be nonpathogenic and were not identified as belonging to pathovar *vignicola* (Khatri-Chhetri, pers. comm.). Two strains showed medium virulence and were identified by the Biolog test as *X. c.* pv. *carotae* and *X. c.* pv. *campestris* (Khatri-Chhetri, pers. comm.). Nevertheless, by ribotyping, the five strains were identified as belonging to pathovar *vignicola*. We confirmed the observations of different authors (Berthier et al., 1993; Bragard et al., 1995; Verdier et al., 1994) that, for bacterial strain identification, ribotyping is likely to be more useful than biochemical methods.

Although the presence of the *pth*B sequence is associated mostly with virulence in *Xcm*, it remains unclear whether the *pth*B sequence has a similar function in *Xcv*. Whether other nonpathogenic or low virulent *Xcv* strains also lack the target sequence is of research interest. Although probe *pth*B was cloned from an indigenous plasmid of *Xcm* we do not have evidence that the sequences found in *Xcv* and homologous to *pth*B are plasmid-borne.

Other approaches such as pathogenicity and biochemical tests support the distinctiveness of *Xcv* strains (Khatri-Chhetri et al., 1995; 1996a; 1996b). Nevertheless, phenotypic markers, including biotyping and serological systems, are less discriminative and more time consuming and difficult to apply to all strains. Ribotyping is a useful tool but involves the labor-intensive Southern blotting. This method could be simplified to become more widely adapted, for example, by using PCR amplification of the 16S rRNA gene, which permits the differentiation of pathovars of *Xanthomonas* (Nesme et al., 1995).

Other techniques, such as analysis of polymorphism after PCR with repetitive DNA elements (rep-PCR) have been applied successfully to generate specific DNA fingerprintings of *Xanthomonas* pathovars and strains (Louws et al., 1994; 1995; Vera Cruz et al., 1996). Such technique would be very useful for identifying bacterial strains of *Xcv*. RFLP with repetitive DNA probes has shown to be highly discriminative and has allowed the detection of genetic diversity among closely related strains of *X. oryzae* pv. *oryzae* (Ardales et al., 1996; Leach et al., 1992;

Vera Cruz et al., 1996) and *X.* pv. *mangiferaeindicae* (Gagnevin et al., 1997).

Substantial variation in virulence was observed within the strains of *Xcv* inoculated on a susceptible cowpea cultivar. The variation in virulence was observed within strains exhibiting the same haplotype, suggesting that differences in virulence occur independently of the genetic grouping as defined with the *pth*B probe. Such observations were described for *X. oryzae* pv. *oryzae* (Nelson et al., 1994). With *avrXa10* as a probe, various strains of *X. oryzae* pv. *oryzae* belonging to different physiological races were indistinguishable by DNA hybridization analysis (Nelson et al., 1994). In our study, virulence phenotype was not linked to haplotype. However no races of *Xcv* are known and no gene governing resistance in cowpea has been characterized. Recently, inoculation of various cowpea cultivars with some *Xcv* strains did not demonstrate a gene for gene interaction (K. Wydra, pers. comm.), and the strains selected for the virulence characterization did not represent the nine haplotypes distinguished by the present RFLP analysis. Further virulence analysis on cowpea genotypes is needed to determine whether the genetically distinct groups represent phenotypically distinct groups. As suggested by Adhikhari et al. (1995) and Nelson et al. (1994), we propose using one strain per haplotype and belonging to a different virulence class to inoculate cowpea germplasm.

We provided new information on the *Xanthomonas* strains collected from cowpea. We confirmed that all strains isolated from either blight symptoms or pustules belong to pathovar *vignicola*. The information we provided on the genetic and pathogenic diversity within *Xcv* strains is a prerequisite for selecting sources of disease resistance for crop breeding.

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