AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB)

Gilda Sanchez, Silvia Restrepo, Myriam-Cristina Duque, Martin Fregene, Merideth Bonierbale, and Valérie Verdier

Abstract: Cassava bacterial blight (CBB) is caused by *Xanthomonas axonopodis* pv. *manihotis (Xam)*. Resistance is found in *Manihot esculenta* and, in addition, has been introgressed from a wild relative, *M. glaziovii*. The resistance is thought to be polygenic and additively inherited. Ninety-three varieties of *M. esculenta* (Crantz) were assessed by AFLPs for genetic diversity and for resistance to CBB. AFLP analysis was performed using two primer combinations and a 79.2% level of polymorphism was found. The phenogram obtained showed between 74% and 96% genetic similarity among all cassava accessions analysed. The analysis permitted the unique identification of each individual. Two *Xam* strains were used for resistance screening. Variation in the reaction of cassava varieties to *Xam* strains was observed for all plant accessions. The correlation of resistance to both strains, had a coefficient of 0.53, suggesting the independence of resistance to each strain. Multiple correspondence analysis showed a random distribution of the resistance/susceptibility response with respect to overall genetic diversity as measured by AFLP analysis. A total heterozygosity index was calculated to determine the diversity within clusters as well as among them. Our results demonstrate that resistance to CBB is broadly distributed in cassava germplasm and that AFLP analysis is an effective and efficient means of providing quantitative estimates of genetic similarities among cassava accessions.

Key words: amplified fragment length polymorphism, genetic base, resistance screening, *Xanthomonas axonopodis* pv. *manihotis*.

Résumé : La bactériose du manioc est causée par *Xanthomonas axonopodis* pv. *manihotis (Xam)*. La résistance observée chez *Manihot esculenta* a été introduite à partir de l'espèce sauvage *M. glaziovii*. Elle est supposée polygénique et héritée de manière additive. Quatre-vingt-treize variétés de *M. esculenta* (Crantz) ont été évaluées à la fois par la technique AFLP et pour leur résistance/sensibilité à la bactériose. L'analyse AFLP a été faite avec deux combinaisons d'amorces primers et montre un taux de polymorphisme de 79,2%. Le phénogramme obtenu montre entre 74% et 96% de similarité entre les variétés analysées. L'analyse permet l'identification de chaque variété montrant ainsi qu'il n'y a pas de duplicat au sein de l'échantillon utilisé. Deux souches de *Xam* ont été utilisées pour l'évaluation de la résistance. On observe une variabilité dans la réaction des variétés aux deux souches de *Xam*. La corrélation de la résistance à *Xam* indique un coefficient de 0,53, suggérant que la résistance à chaque souche de *Xam* est indépendante. Une analyse de correspondance multiple met en évidence une distribution au hasard de la réaction résistance/sensibilité par rapport à la diversité génétique évaluée par AFLP. Un index total d'hétérozygotie est calculé afin de déterminer la diversité aussi bien entre les groupes qu'au sein des groupes obtenus. Nos résultats montrent que la résistance est largement distribuée au sein des variétés de manioc et que l'AFLP est une méthode efficace et performante qui permet une évaluation quantitative de la similarité entre les variétés de manioc.

Mots clés : polymorphisme de la longueur des fragments amplifiés, base génétique, évaluation de la résistance, *Xanthomonas axonopodis* pv. *manihotis*.

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Introduction

Cassava (*Manihot esculenta* Crantz) is a starchy root crop that feeds about 500 million people throughout the tropics. It is produced mainly by small farmers for food and small-scale industrial use. The genetic resources available for cassava improvement include improved breeding lines, landraces, and wild relatives of the crop. Information about the structure of these gene pools is important in the development of sound strategies for germplasm conservation and use (Bonierbale et al. 1994). Cassava bacterial blight (CBB), a disease that is economically significant in Latin America and Africa, is caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), a systemic and epiphytic pathogen (Lozano and Sequeira 1974). Yield losses range between 12% and 100%. The disease also affects the quality of planting material (Lozano 1975; Boher and Verdier 1994).

The deployment of resistant varieties is the major method of CBB control. Resistance has been found in *M. esculenta* and in its wild relative *M. glaziovii* (Hahn 1978). It is thought to be polygenic and additively inherited (Lozada 1990), but the genes involved in the resistance response have not yet been identified (Verdier et al. 1997). Resistance is expressed as a reduced rate of disease development in stems (Umemura and Kawano 1983), with the number of infected xylem vessels lower in resistant than in susceptible cultivars (Kpémoua et al. 1996). Defense mechanisms have been characterised and include phenol production and xylem vessel occlusion by lignin-like compounds (Kpémoua et al. 1996).

Genetic studies show that South American *Xam* populations are more genetically diverse than African populations (Verdier et al. 1993). Recently, geographical differentiation of the pathogen was shown in Colombia (Restrepo and Verdier 1997). Although variation in the aggressiveness of *Xam* strains has been reported (Maraite and Meyer 1975; Restrepo and Verdier 1997), no clear interactions between cassava cultivars and strains have been yet established. The most limiting factor in such studies is the lack of a suitable way of choosing the cassava varieties that can be used for establishing an appropriate set of host differentials. Currently, more than 6000 accessions are available in the world cassava collection held at CIAT. The use of molecular marker technology has provided the potential to speed up plant improvement for a variety of objectives including disease resistance. Specifically, understanding the genetic relationships between resistant and susceptible accessions is the first step in choosing the most appropriate segregating populations for mapping possible new sources of resistance.

Amplified fragment length polymorphism (AFLP) is a reliable, PCR-based marker system for obtaining quantitative estimates of genetic relationships (Vos et al. 1995). This technique is advantageous because variability can be assessed at a large number of independent loci, and data are obtained quickly and reproducibly (Majer et al. 1996; O'Neill et al. 1997). Since polymorphism is detected as the presence or absence of amplified restriction fragments, AFLPs are usually considered dominant markers (Mackill et al. 1996; Powell et al. 1996). The resulting DNA fingerprint provides a large number of genetic markers and the multiplex ratio, defined as the number of information points analysed per experiment, is much higher than for other types of markers (Powell et al. 1996). AFLP is reported to be more informative than RAPD, isoenzymes, nuclear RFLP, or cDNA RFLP, in establishing relationships within other crops (Hill et al. 1996; Mackill et al. 1996; Sharma et al. 1996; Hongtrakul et al. 1997). Phenetic trees based on AFLP data are consistent with known taxonomic relationships and similar to those developed with RFLP data (Hill et al. 1996; Sharma et al. 1996). Recently, AFLPs have been used to evaluate cassava diversity, to estimate genetic relationships in the genus *Manihot*, and to identify putative species-specific markers useful for germplasm classification (Roa et al. 1997).

This study aims to (*i*) describe the distribution of genetic variability within a large group of accessions representing the genetic diversity available in cassava germplasm; (*ii*) assess their resistance or susceptibility of these accessions to bacterial blight in order to determine if resistance is general or specific; and (*iii*) help in identifying accessions that could be selected for improvement programs and in studies of host–pathogen interaction.

Material and methods

Plant material

Ninety-three accessions of *Manihot esculenta* (Crantz), from the international core collection and the cassava improvement program at CIAT (Centro Internacional de Agricultura Tropical) were analysed (Table 1). The accessions included were either landraces (coded by their origin) or improved clones classified as ELITE in relation to agronomic traits, such as resistance to biotic or abiotic stresses and yield. The ELITE accessions are coded as follows: CG and CM are CIAT cassava improvement program-controlled crosses; SG and SM are CIAT cassava improvement open-pollinated crosses. The landraces are coded by geographic origin (Table 1).

Resistance screening

CBB resistance screening was conducted on the 93 cassava accessions. Two *Xanthomonas axonopodis* pv. *manihotis* strains, CIAT 1117 and Orst X27, previously characterised as virulent were used (Verdier et al. 1994; Kpémoua et al. 1996). For long-term storage, *Xam* strains were conserved in glycerol at –80°C. Strains were streaked on LPGA media (5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} glucose, and 15 g l^{-1} agar) 12 h before inoculation. The cassava plants for testing were grown from mature stem cuttings in sterile soil. Ten plants per accession and per each bacterial strain were tested in the greenhouse at 28:19°C day:night temperatures, under a 12 h daylight photoperiod, and 80% relative humidity, in Cali, Colombia. Stems were inoculated as described by Verdier et al. (1994). Disease progress was recorded at 7, 14, and 30 d after inoculation, with the following disease severity rating: score 0 = no disease symptoms; 1 = necrosis around the inoculation point; 2 = gum exudation on stem; 3 = wilting of one or two leaves and exudation; 4 = wilting of more than two leaves; 5 = complete wilting and dieback. For the present analysis, plants with a disease reaction of ≤ 3 were grouped as resistant, while those with a disease reaction of > 3 were classified as susceptible.

DNA extraction and purification

DNA was extracted using some modifications of the Gilbertson et al. (1991) method. Samples of 8-mo-old leaf tissue were collected and ground in liquid nitrogen, then extracted in extraction buffer (100 mM Tris–HCl at pH 8.0; 50 mM EDTA at pH 8.0;

Table 1. Source and origin of the cassava accessions used in this study.

	Cluster		Disease reaction[b]			Cluster		Disease reaction	
Accession[a]	UPGMA	MCA	X–27	CIAT1117	Accession	UPGMA	MCA	X–27	CIAT1117
CG1141–1	A	3	S	S	MBRA872	C	6	S	S
CG402–11	G	4	S	S	MBRA881	C	8	R	R
CG501–1	D	2	S	S	MBRA886	C	7	R	R
CG501–15	D	1	R	R	MBRA900	B	8	R	R
CG501–16	D	2	S	S	MBRA902	A	6	S	S
CG501–18	D	1	R	S	MBRA908	C	8	S	S
CG501–2	D	1	R	R	MBRA929	B	8	S	S
CM1335–4	D	2	S	R	MBRA99	B	8	S	S
CM1585–13	D	1	S	S	MCOL1438	B	8	S	S
CM2177–2	A	4	R	S	MCOL1505	B	7	S	S
CM2623–1	E	9	R	S	MCOL1522	F	n.d.	S	S
CM2952–1	F	9	R	R	MCOL1684	B	8	S	S
CM2952–2	F	9	R	S	MCOL1939	B	7	R	R
CM2952–3	F	9	R	R	MCOL2041	B	7	S	S
CM3171–8	F	9	S	S	MCOL2061	B	9	S	S
CM3306–19	F	2	R	R	MCOL2066	H	2	S	R
CM3306–25	F	2	R	S	MCOL22	H	4	S	R
CM3306–4	A	3	S	S	MCOL2215	I	n.d.	S	S
CM3320–8	F	9	R	R	MCOL2261	J	n.d.	S	S
CM4013–1	F	9	S	S	MCOL647	B	n.d.	S	R
CM4063–6	E	9	R	S	MCUB5	B	7	S	S
CM4733–4	F	0	S	S	MCUB74	B	8	R	R
CM4772–4	F	9	S	S	MECU82	B	6	R	R
CM507–37	A	5	S	S	MIND4	B	7	S	R
CM523–7	A	5	R	R	MIND48	F	9	S	R
CM5286–3	D	9	S	S	MMEX59	B	8	R	S
CM6438–14	D	2	S	R	MNGA19[c]	B	6	R	R
CM6855–3	A	7	S	S	MNGA2	B	6	S	S
CM6921–3	A	5	S	S	MPAR110	B	8	S	S
CM7389–9	G	3	S	S	MPTR19	B	6	S	S
HMC1	F	9	R	S	MTAI1	F	0	S	S
MARG11	A	7	R	R	MVEN25	A	9	S	S
MBOL3	F	n.d.	S	S	MVEN45	A	7	R	R
MBRA106	F	0	S	R	SG104–264	B	6	S	S
MBRA108	F	8	R	R	SG105–11	A	6	R	R
MBRA110	B	8	R	S	SG107–35	A	7	R	R
MBRA12	F	9	S	S	SG427–87	F	7	S	S
MBRA159	F	0	R	S	SG536–1	F	9	S	S
MBRA200	F	0	S	S	SG638–6	J	n.d.	R	S
MBRA383	F	9	S	S	SM1031–2	A	5	S	R
MBRA429	A	5	S	S	SM1053–12	G	4	S	S
MBRA545	B	8	R	R	SM328–1	A	7	R	R
MBRA579	B	6	S	R	SM494–2	B	9	R	R
MBRA605	C	5	S	S	SM524–1	G	2	S	S
MBRA685	B	6	R	R	SM627–5	C	5	S	S
MBRA784	C	6	S	S	SM667–1	D	2	R	S
					SM853–7	H	3	S	S

Note: n.d. = not determined, varieties taken out from the second MCA.

[a]Accession codes for improved materials are CG or CM (controlled cross), SG or SM (open-pollinated cross). Landraces are codified as follows: MBra, Brazil; MArg, Argentina; MBol, Bolivia; MCol, Colombia; MCub, Cuba; MEcu, Ecuador; MInd, Indonesia; MMex, Mexico; MPar, Paraguay; MPtr, Puerto Rico; MTai, Thailand; and MVen, Venezuela.

[b]Plants with a disease reaction of ≤3 were grouped as resistant (R), and those with a score >3 were classified as susceptible (S).

[c]MNGA2 and MNGA19 are elite varieties from IITA (International Institute of Tropical Agriculture, Nigeria).

500 mM NaCl; 100 mM β-mercaptoethanol and 1% PVP40 with 20% SDS, shaken and incubated at 65°C for 20 min. Next, proteins were removed from the samples by addition of 5 mL cold potassium acetate (5 M) followed by precipitation with cold isopropanol (80%), then stored overnight at –20°C. Samples were centrifuged at 14 000 rpm and the supernatant was removed. The pellet was

washed with 500 µL of 70% ethanol, dried for 30 min, and then resuspended on cold TE buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA at pH 8.0). DNA was treated with RNAse (Sigma) for 20 min at 37°C, and visualised on an agarose gel (0.8%). The DNA was quantified, using a Hoefer DyNA Quant 200 fluorometer. It was also verified for the absence of nucleases, which can interfere with AFLP screening, as follows: a 1 µg sample of DNA was tested with magnesium chloride at a final concentration of 10 mM, incubated at 37°C for 2 h, then visualised on 0.8% agarose gel.

Amplified fragment length polymorphism method

AFLPs were performed as described by Vos et al. (1995), using 500 ng of genomic DNA. Each DNA sample was digested with *Eco*RI and *Mse*I (New England BioLabs). Ligation was performed using specific adapters (*Eco*RI Adapter 1-5′ CTC GTA GAC TGC GTA CC 3′; Adapter 2-5′ AAT TGG TAC GCA GTC 3′; and *Mse*I Adapter 1-5′ GAC GAT GAG TCC TGA G 3′; Adapter 2-5′ TAC TCA GGA CTC A-T 3′). The pre-amplification step was carried out using primers +1/+1 (*Eco*RI primer + A- PE1-5′ GAC TGC GTA CCA AAT CA 3′, and *Mse*I primer + G- PM1-5′ GAT GAG TCC TGA GTA AG 3′). Primer labelling was with the *Eco*RI primer +3 using 32^{γ} d[ATP]. This reaction was incubated for 30 min at 37°C. The second amplification reaction was made using 5 µL of the dilution (1:20) from the first PCR as DNA template, the labelled *Eco*RI primer, and the *Mse*I primer +3 in a PCR final volume of 20 µL. Two sets of primers, out of nine previously tested on 9 of the 93 accessions (A. Bernal and M. Fregene 1997, unpublished results), were selected. PE1A * PM1A (PE1A-5′ GAC TGC GTA CCA AAT CAAC 3′ * PM1A-5′ GAT GAG TCC TGA GTA AGTA 3′) and PE1B * PM1D (PE1B-5′ GAC TGC GTA CCA AAT CACG 3′* PM1D-5′ GAT GAG TCC TGA GTA GGT 3′) were used, based on the level of polymorphism and banding pattern found. A control DNA sample was used each time the reaction was performed. An aliquot of 3 µL of reaction product was mixed with 2 µL of formamide dye, denatured for 3 min at 100°C, and chilled on ice. The reaction products were size-fractionated on 6% polyacrylamide denaturing gels on a SequiGen (BioRad) sequencing apparatus. Electrophoresis was carried out for 2 h in 1 × TBE at 40 V cm^{-1} and 50°C. The gels were covered with Saran Wrap, dried under vacuum for 1 h at 80°C, and exposed 24 h on x-ray films (Kodak X-omat LS) for autoradiographs.

Data analyses

AFLPs were generated using two pairs of primers, PE1A * PM1A and PE1B * PM1D. Each clearly variable marker was treated as a separate character and scored as either present (1) or absent (0) across the 93 accessions evaluated. Genetic similarities between pairs of accessions were estimated using the Nei and Li formula (1979), $Gs = 2C(ij) / N(ij)$, where Gs is the measure of genetic similarity between the ith and jth accession, $C(ij)$ is the number of bands shared by i and j, and $N(ij)$ the total sum of scored bands. This definition of similarity excludes from the calculation those bands that are absent in both individuals, because mutual absence cannot necessarily be attributed to a common cause. Weighting the bands that coincide by a factor of 2 permits better differentiation of individuals with low levels of similarity (Tohme et al. 1996). All the similarity analyses were performed in NTSYS-pc (Version 1.80, Rohlf 1994). Phenograms were constructed by employing the DICE option for the similarity coefficient, the UPGMA (unweighted pair group method of averages) clustering method of Sneath and Sokal (1973), and the TREE option to display results graphically. A correlation index was calculated between the similarity matrices resulting from the two different primer combinations to analyse the complementarity or redundancy of information.

Multiple correspondence analysis (MCA) was conducted to evaluate the contribution of specific active variables (AFLP products) to the clustering observed among genotypes. The cophenetic correlation coefficient indicates the extent to which the clustering of genotypes accurately represents the estimates of genetic similarity of the accessions. The MCA analysis allows further insights on the relationships between AFLP genotypes and the identification of bands with large effects on clustering. For this purpose the active variables were those corresponding to the presence or absence of bands; resistance or susceptibility were used as supplementary variables. The analysis was done by employing the CORRESP procedure of SAS (version 6–11; 1989).

In order to define the distribution of diversity found within the germplasm group the coefficient of genetic diversity was calculated. This coefficient describes the genetic differentiation relative to the total population and is given by $Gst = Dst / Ht$, where Dst is the average gene diversity between subpopulations, (including the comparisons of subpopulations with themselves) and Ht is the genetic diversity in the total population, defined by Nei (1973) as $Ht = Hs + Dst$ ($Hs = 1 - Js$, and Js is the average gene diversity between subpopulations). Thus, the genetic diversity in the total population can be analysed as the genetic diversities within and between subpopulations (Nei 1973).

Using the resistance data categorised as resistant (R) or susceptible (S) as described previously, a correlation index was calculated to determine the independence or correlation of resistance to the different *Xam* strains.

Results

Resistance screening

Variation in the reaction of cassava varieties to *Xam* strains was observed for all plant materials: 13% of all varieties were resistant only to X-27, 11% only to CIAT1117, 25% were resistant to both strains, and 51% were susceptible to both strains (Table 1). The correlation between resistance to strains X-27 and CIAT1117 had a value of 0.53, suggesting independence of resistance to the two strains and the likely presence of more than one gene for resistance.

AFLP analysis

The primer pairs PE1A * PM1A and PE1B * PM1D were chosen from the larger set of primer pairs, based on their relatively higher polymorphism (50 and 26 polymorphic bands, respectively) and resolution of bands in a subset of this germplasm. All 93 accessions were tested, using both primer combinations. A total of 96 bands, 63 from the first combination and 33 from the second was scored (Fig. 1). Of these, only 13 and 7 bands, respectively, were monomorphic, showing a 79% level of polymorphism with this primer combination. The matrices of genetic similarity estimated by the Nei-Li coefficient, based on AFLP patterns from the two separate primer combinations, showed low correlation (r = 0.12). This suggests that each combination provided different and therefore complementary information. The analysis permitted differentiation of all individuals, indicating that the collection samples used did not contain genetic duplicates. Repetitions, using the same set of primers and several accessions, consistently yielded the same number and pattern of polymorphic bands (data not shown).

UPGMA cluster analysis

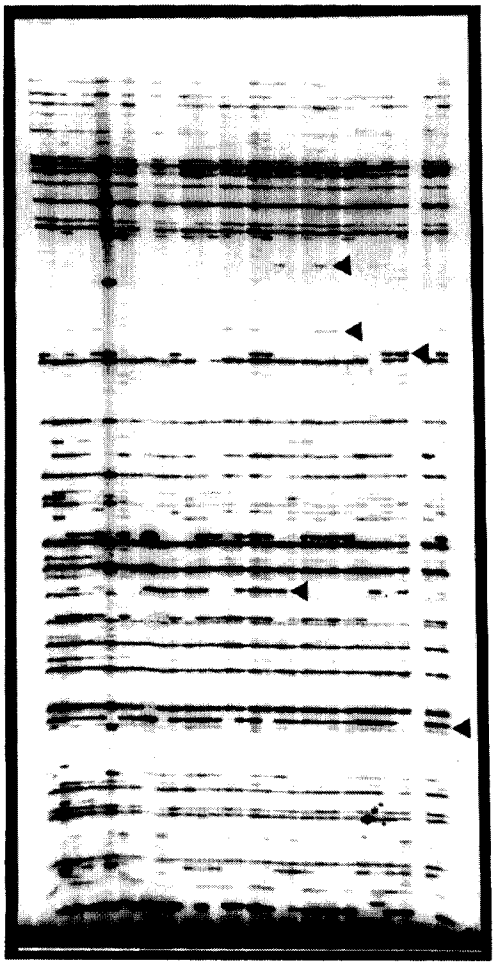
The phenogram obtained shows between 74% and 96% genetic similarity (Fig. 2) among all cassava accessions. The highest similarity was found between two open-pollinated clones (Cluster A), which share the same pistillate parent. At 80% genetic similarity, 10 clusters were defined. Cluster A is composed of landraces from Argentina, Venezuela, and Brazil, and some improved clones; members of this cluster do not show a defined pattern of resistance or susceptibility to either *Xam* strains considered (Table 1). The second and largest cluster (B) is composed of landraces and improved varieties from all three cassava-growing continents (Africa, Asia, and Latin America). The African varieties are improved clones with Brazilian landraces in their pedigrees. CBB resistance response is highly variable in this group (Fig. 2, Table 1). Cluster C is composed of predominantly Brazilian varieties, showing different resistance responses, with genetic similarities ranging from 79% to 94%. Clusters D and E consist of improved varieties developed at CIAT with variable response to CBB. Cluster F, the second largest, is composed of improved varieties, Indonesian, Colombian, Thai, and Brazilian landraces. Cluster G is made up of improved varieties all susceptible to both *Xam* strains. Cluster I has only one member, a Colombian landrace that is susceptible to both strains. Clusters H and J contain Colombian landraces and two improved clones with variable responses to *Xam*.

As expected, most closely related clones, such as the CG501 series, which are a full-sib family, clustered together having genetic similarities between 87% and 91% (Fig. 2). Besides reflecting similar pedigree, this confirms the reliability and additive nature of this molecular data. However, other full-sib families clustered in different groups. For example, CM3306 had one of its three members in cluster A and two members in cluster F, with 80% genetic similarity. The level of heterozygosity in the parental lines could explain the different degrees of genetic similarity found in these two families; cassava is strongly outcrossing and maintains high levels of heterozygosity. Clusters H, I, and J are made up of Colombian landraces having slightly higher levels of genetic diversity (genetic similarity ranges from 74% to 89%).

Multiple correspondence analysis

The multiple correspondence analysis showed all the accessions as a compact group in which the first three axes explain 19% of the variation (data not shown). Specific bands that distinguished accessions and permitted their simpler classification were not detected. However, in this analysis, one group of accessions differs from the compact, primary one. By excluding the accessions grouped in this latter cluster: (MCOL2261, MCOL647, MBol3, MCOL1522, MCOL2215, and SG638-1), and performing a separate multiple correspondence analysis with the remaining varieties, it was possible to resolve differences among varieties of the primary group. With a cophenetic correlation coefficient value of 0.93, ten well-defined clusters were found (Fig. 3), with varieties grouped on the basis of their distance, reflecting both CBB response (Table 1) and geographic origin. It is noteworthy that some clusters are composed of accessions susceptible to both *Xam* strains (e.g., cluster 3), and others, like cluster 7, are composed of accessions that were either resistant or susceptible to both strains, with only one exception. In some cases accessions coming from the same full-sib family were either located in the same cluster or in a cluster close by (e.g., accessions from CG501 are located in either cluster 1 or 2; Fig. 3). When accessions were plotted on the basis of their resistance or susceptibility to either *Xam* strain or to both, a random distribution was found (data not shown), instead of resistant varieties grouping together, thus confirming our findings with UPGMA. A total heterozygosity index was calculated to find diversity within clusters, as well as among them (Table 2); the genetic differentiation coefficient found was 0.249 suggesting that the diversity within groups is higher than among them.

Fig. 1. Example of AFLP autoradiograph obtained using primer combination PE1A * PM1A. Arrows indicate some polymorphic bands among cassava accessions.



Discussion

Our results demonstrate that AFLP analysis effectively and efficiently provided quantitative estimates of genetic similarities related to the distribution of variability among cassava accessions. This finding is supported by Roa et al. (1997), who reported similar results using samples from different *Manihot* species. In the scoring of autoradiographs, all

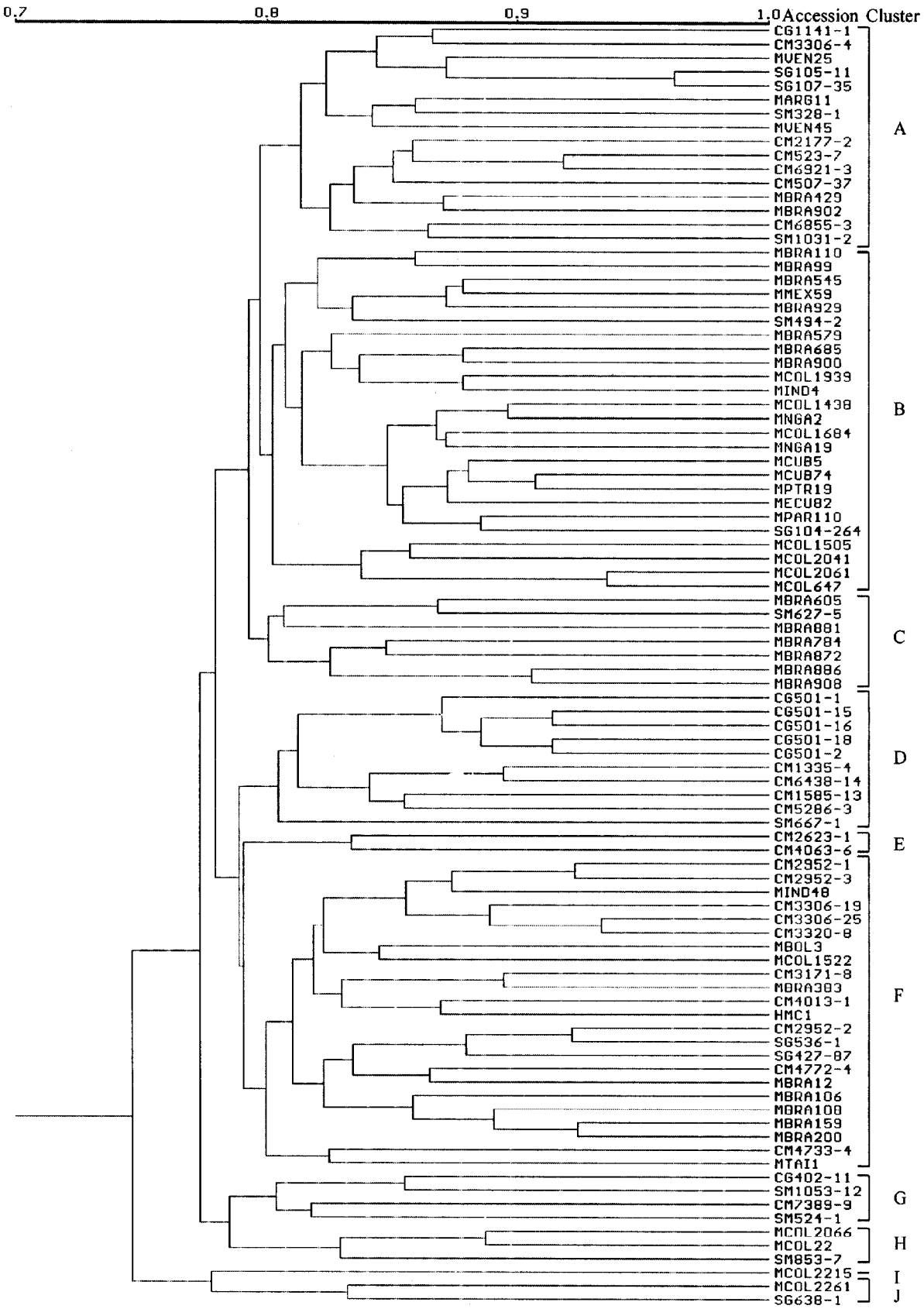
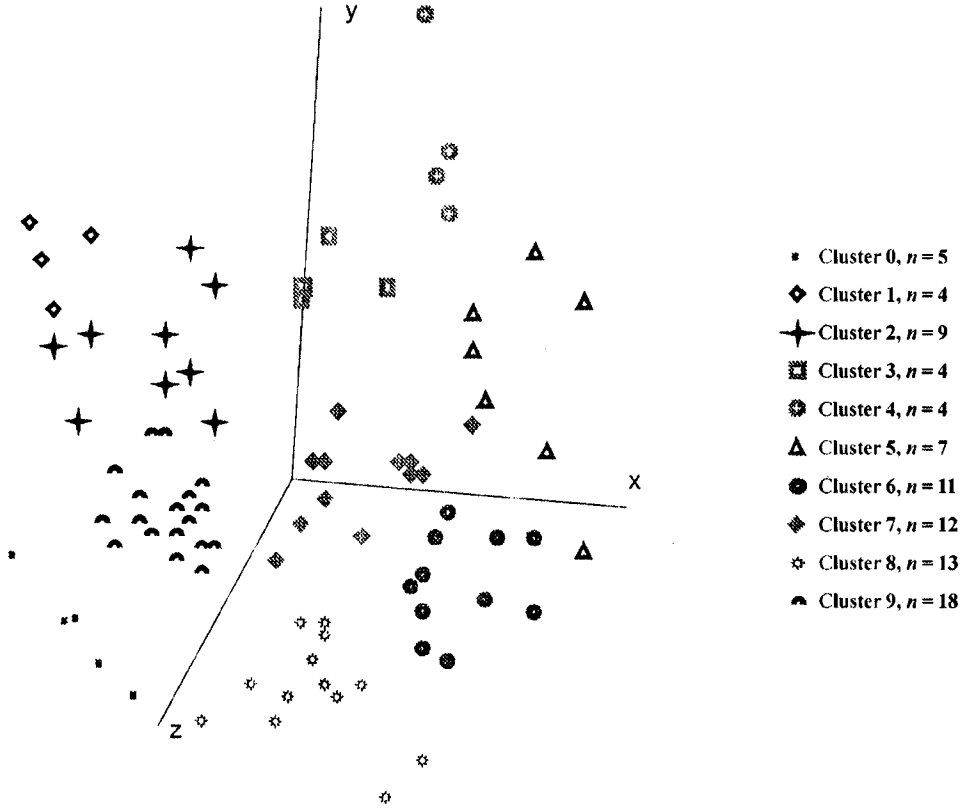


Fig. 2. Phenogram obtained from AFLP data, showing the distribution in 10 clusters (A to J) of 93 cassava accessions. See Table 1 to identify resistant or susceptible accessions.

Fig. 3. Clusters obtained from multiple correspondence analysis. See Table 2 to identify the accession names by cluster.



fragments of the same size in different genotypes were considered to represent the same character. This assumption is difficult to confirm without extensive genetic analysis or hybridisation studies. However, the correspondence of the AFLP data with analyses based on other types of markers indicates that the scoring of multiple fragments as one locus is uncommon (Hill et al. 1996).

AFLP analysis has several advantages over other marker techniques for the analysis of genetic diversity. A major advantage is the short time required to assay large numbers of DNA loci. Compared with RAPD, AFLP analysis detects about 12 times the number of polymorphic loci per assay in soybean varieties (Vogel et al. 1994 cited in Hill et al. 1996; Maugham et al. 1996). AFLP also provides a high number of markers that can be screened per experiment and the results are highly reproducible (Cervera et al. 1996; Majer et al. 1996).

AFLP analysis yielded 20 to 50 bands per individual and per combination (for primer sets PE1A * PM1A and PE1B * PM1D), more than previously reported for RAPD and RFLP data on similar accessions of *M. esculenta* (Bonierbale et al. 1994). We also found a high rate of polymorphism (near 78%) which agrees with previous reports of cassava AFLP studies (M. Fregene, personal communication). AFLP is also more informative than other methods used to date to characterize cassava germplasm such as isoenzymes, RFLPs, and RAPDs (Beeching et al. 1993; Lefevre and Charrier 1993; Marmey et al. 1994).

One objective in characterising resistance to CBB in source populations is to assess the likely effects of selection for this character on the overall genetic diversity. Genetic advance for traits of primary and secondary importance depends on the identification of recombinant individuals in variable populations that carry favourable alleles from their parents. Because the genetic diversity of a breeding population is reduced by selection, the theoretical rate of gain declines. If a trait has a narrow genetic base, that is, it is present only in closely related individuals, selection for the trait may result in greater reduction of overall diversity. The coefficient of genetic differentiation (Table 2) *Gst* = 0.249, clearly shows that the difference between clusters explains 25% of total heterogeneity for this material. A higher diversity was therefore found within clusters than between them. In this case, the value of *Gst* agrees with the multiple correspondence analysis, in which the distance between samples of the same cluster is bigger than between clusters, thus suggesting that the group of accessions chosen is a good representation of the population. Our analysis demonstrates that CBB resistance is present in genetically diverse cassava accessions (represented by groups of individuals, at different branches of the phenogram), as opposed to being concentrated in one or a few "lineages." This suggests that breeding

Table 2. Heterozygosity indices[a] for clusters, and total genetic diversity index. Clusters are numbered in accordance with Fig. 3.

Cluster	Hs	Accessions
0	0.165	CM4733–4, MBRA106, MBRA159, MBRA200, MTAI1
1	0.118	CG501–15, CG501–18, CG501–2, CM1585–13
2	0.199	CG501–1, CG501–16, CM1335–4, CM3306–19, CM3306–25, CM6438–14, MCOL2066, SM524–1, SM667–1
3	0.197	CG1141–1, CM3306–4, CM7389–9, SM853–7
4	0.169	CG402–11, CM2177–2, MCOL22, SM1053–12
5	0.200	CM507–37, CM523–7, CM6921–3, MBRA429, MBRA605, SM1031–2, SM627–5
6	0.218	MBRA579, MBRA685, MBRA784, MBRA872, MBRA902, MECU82, MNGA19, MNGA2, MPTR19, SG104–264, SG105–11
7	0.217	CM6855–3, MARG11, MBRA886, MCOL1505, MCOL1939, MCOL2041, MCUB5, MIND4, MVEN45, SG107–35, SG427–87, SM328–1
8	0.205	MBRA108, MBRA110, MBRA545, MBRA881, MBRA900, MBRA908, MBRA929, MBRA99, MCOL1438, MCOL1684, MCUB74, MMEX59, MPAR110
9	0.201	CM2623–1, CM2952–1, CM2952–2, CM2952–3, CM3171–8, CM3320–8, CM4013–1, CM4063–6, CM4772–4, CM5286–3, HMC1, MBRA12, MBRA383, MCOL2061, MIND48, MVEN25, SG536–1, SM494–2
A[b]	0.188	MCOL2261, MCOL647, MBOL3, MCOL1522, SG638–1, MCOL2215

Hst	Ht	Gst
0.066	0.263	0.249

Note: Hs represents the heterogeneity within clusters; Hst, heterogeneity among clusters; Ht, total heterogeneity; and Gst, the genetic differentiation coefficient.

[a]Accessions in each cluster are stated.

[b]A represents the group of accessions eliminated from the data set prior to conducting the second MCA.

for resistance, for example, with the goal of recombining possibly different favourable alleles, will not reduce the overall diversity of the breeding population. If resistant accessions are combined, sufficient variation will likely remain in the population to permit further improvement for additional traits in subsequent cycles of selection and recombination. Our results also reinforce the advantage of using data of a quantitative and additive nature, such as molecular-marker data, in selecting parents for development of breeding populations in an outcrossing species to maximise heterosis.

We also set out to define selection criteria to reduce the number of cassava varieties constituting a set of host differentials, and to establish suitable criteria for selecting or discarding material for further studies. In this analysis we did not observe a single band or a group of bands consistently associated with resistance, which could serve as resistance markers. Nevertheless, on the basis of the present study, it is possible to select a discrete number of accessions for further studies of the host–pathogen interaction. We suggest using the varieties MBra695, MBra881, MBra900, MNga19, and MVen45 in efforts to improve cassava for resistance to bacterial blight. These accessions have shown the highest resistance to both *Xam* strains. Interestingly, most of them are Brazilian landraces or derivatives, such as MNga19, which is an improved variety, obtained by crossing a third backcross derivative of *M. glaziovii* to African then to Brazilian landraces. Moreover, most of these varieties are located in different clusters throughout the phenogram. Combining accessions located throughout the phenogram would maximise the chance of maintaining higher diversity, and therefore, the possibility of getting more than one resistance allele in a breeding effort.

We further recommend using varieties CM2177-2, MNGA2, CM523-7, CM6438-14, and MCol22 that have shown medium resistance to both *Xam* strains, as well as accessions CM1335-4, MMEX59, and SM1031-2 which are resistant to one strain and highly susceptible to the other, to be tested as host differentials with a wider range of *Xam* strains. Indeed, with these cassava accessions and with more *Xam* strains, it would be possible to test more accurately the resistance response.

The use of the AFLP technology to map resistance loci, and their subsequent cloning, may help us better understand the genetic basis of resistance against pathogens (Cervera et al. 1996). Markers consistently associated with CBB resistance have not yet been found, perhaps because of the limited accessions used and the enzyme combination and primer sets tested. It is also likely that in this diverse set of cassava germplasm, several factors may contribute to the resistance observed, making it unlikely that any one genetic marker would be consistently associated with resistance across accessions. However, with a specific cross between a highly resistant parent and a susceptible one, it should be possible, not just to find specific markers associated with resistance, but also to map the resistance loci (Maugham et al. 1996). Our hypothesis of multiple resistance loci could be tested in this manner, by using a series of genetic stocks.

Further work on cassava bacterial blight could include marker-assisted selection based on the AFLP technique, molecular cloning, and characterisation of resistance gene(s). This may help elucidate the mechanisms and genetics of resistance. Mapping defined traits using selected crosses and the AFLP technique appears to be promising for future studies (Meksem et al. 1995).

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References

Beeching, J.R., Marmey, P., Gavada, M.C., Noirot, M., Haysom, H.R., Hughes, M.A., and Charrier, A. 1993. An assessment of genetic diversity within a collection of cassava (*Manihot esculenta* Crantz) germplasm using molecular markers. Ann. Bot. **72**: 515–520.

Boher, B., and Verdier, V. 1994. Cassava bacterial blight in Africa: The state of knowledge and implications for designing control strategies. Afr. Crop. Sci. J. **2**: 505–509.

Bonierbale, M.W., Maya, M.M., Claros, J.L., and Iglesias, C. 1994. Application of molecular markers to describing the genetic structure of cassava gene pools. *In* The cassava biotechnology network. Proceedings of the 2nd International Scientific Meeting, Bogor Indonesia, 22–26 August 1994, Vol. I (Working document No. 150). *Edited by* CBN, Central Research Institute for Food and Crops (CRIFC) of the Agency for Agricultural Research and Development (AARD) and the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia. pp. 106–112.

Cervera, M.T., Gusmao, J., Steenackers, M., Peleman, J., Storme, V., Vanden Boerck, A., Van Montagu, M., and Boerjan, W. 1996. Identification of AFLP molecular markers for resistance against *Melampsora larici-populina* in *Populus*. Theor. Appl. Genet. **93**: 733–737.

Gilbertson, R.L., Rojas, M.M., Russell, D.R., and Maxwell, D.P. 1991. Use of the asymetric polymerase chain reaction and DNA sequencing to determine genetic variability of bean golden mosaic geminivirus in the Dominican Republic. J. Gen. Virol. **72**: 2843–2848.

Hahn, S.K. 1978. Breeding of cassava for resistance to cassava mosaic disease and bacterial blight in Africa. *In* Diseases of tropical food crops. Proceedings of the International Symposium of the UCL, Louvain-La-Neuve, Belgium. *Edited by* H. Maraite and Y.A. Meyer. pp. 211–219.

Hahn, S.K., Howland, A.K., and Terry, E.R. 1980. Correlated resistance of cassava to mosaic and bacterial blight diseases. Euphytica, **29**: 305–311

Hill, M., Witsenboer, H., Zabeau, M., Vos, P., Kesseli, R., and Michelmore, R. 1996. PCR-based fingerprinting using AFLPs as tool for studying genetic relationships in *Lactuca* sp. Theor. Appl. Genet. **93**: 1202–1210.

Hongtrakul, V., Huestis, G.M., and Knapp, S.J. 1997. Amplified fragment length polymorphism as a tool for DNA fingerprinting of sunflower germplasm: genetic diversity among oilseed inbred lines. Theor. Appl. Genet. **95**: 400–407.

Kpémoua, K., Boher, B., Nicole, M., Calatayud, P., and Geiger, J.P. 1996. Cytochemistry of defense responses in cassava infected by *Xanthomonas campestris* pv. *manihotis*. Can. J. Microbiol. **42**: 1131–1143.

Lefevre, F., and Charrier, A. 1993. Heredity of seventeen isozyme loci in cassava (*Manihot esculenta* Crantz). Euphytica, **66**: 171–178.

Lozada, T. 1990. Cruzamientos dialéticos em mandioca (*Manihot esculenta* Crantz). PhD Thesis, Universidade de São Paulo, Brazil.

Lozano, J.C. 1975. Bacterial blight of cassava. PANS (Pest Artic News Samm), **21**: 38–43.

Lozano, J.C., and Sequeira, L. 1974. Bacterial blight of cassava in Colombia: Epidemiology and control. Phytopathology, **64**: 83–88.

Mackill, D.J., Zhang, Z., Redoña, E.D., and Colowit, P.M. 1996. Level of polymorphism and genetic mapping of AFLP markers on rice. Genome, **39**: 969–977.

Majer, D., Mithen, R., Lewis, B., Vos, P., and Oliver, R. 1996. The use of AFLP fingerprinting for the detection of genetic variation fungi. Mycol. Res. **100**: 1107–1111.

Maraite, H., and Meyer, J.A. 1975. *Xanthomonas manihotis* (Athaud-Berthet) Strarr, causal agent of bacterial wilt, blight and leaf spots of cassava in Zaire. PANS (Pest Artic News Samm), **21**: 27–37

Marmey, P., Beeching, J.R., Hamon, S., and Charrier, A. 1994. Evaluation of cassava (*Manihot esculenta* Crantz) germplasm collections using RAPD markers. Euphytica, **74**: 203–209.

Maugham, P.J., Saghai-Mafoof, M.A., Buss, G.R., and Huestis, G.M. 1996. Amplified fragment length polymorphism (AFLP) in soybean: Species diversity, inheritance and near-isogenic line analysis. Theor. Appl. Genet. **93**: 392–401.

Meksem, K., Leister, D., Peleman, J., Zabeau, S.F., and Gebhardt, C. 1995. A high-resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. Mol. Gen. Genet. **249**: 74–81.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. **70**: 3321–3323.

Nei, M., and Li, W.H. 1979. Mathematical models for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **76**: 5268–5273.

O'Neill, N., Van Berkum, P., Lin, H., Kou, J., Ude, G., Kenworthy W., and Saunders, J. 1997. Application of amplified restriction length polymorphisms for genetic characterization of *Colletotrichum* pathogens of alfalfa. Phytopathology, **87**: 745–750.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey S., and Rafalski, A. 1996. The utility of RFLP, RAPD, AFLP and SSRP (microsatellite) markers for germplasm analysis. Mol. Breed. **2**: 225–238.

Restrepo, S., and Verdier, V. 1997. Geographical differentiation of the population of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. Appl. Environ. Microbiol. **63**: 4427–4434.

Roa, A.C., Maya, M.M., Duque, M.C., Tohme, J., Allem A.C., and Bonierbale, M.W. 1997. AFLP analysis of relationships among cassava and other *Manihot* species. Theor. Appl. Genet. **95**: 741–750.

Rohlf, H.J. 1994. NTSYS-pc: Numerical taxonomy and multivariate analysis system. Applied Biostatistics Inc., New York.

SAS Institute, Inc. 1989. SAS/IML software: Usage and reference, v. 6, 1st ed. SAS Institute, Inc., Cary, N.C., U.S.A.

Sharma, S.K., Knox M.R., and Ellis, T.N.H. 1996. AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. Theor. Appl. Genet. **93**: 751–758.

Sneath, P.H.A., and Sokal, R.R. 1973. Numerical taxonomy: The principles and practice of numerical classification. W.H. Freeman Co., San Francisco, California.

Tohme, J., González, D.O., Beebe S., and Duque, M.C. 1996. AFLP analysis of gene pools of a wild bean core collection. Crop. Sci. **36**: 1375–1384.

Umemura, Y., and Kawano, K. 1983. Field assessment and inheritance of resistance to cassava bacterial blight. Crop. Sci. **23**: 1127–1132

Verdier V., Restrepo, S., Boher, B., Nicole, M., Geiger, J.P., Alvarez E., and Bonierbale, M. 1997. Cassava bacterial blight: Recent achievements in understanding the disease. Afri. J. Root. Tuber Crops, **2**: 64–68.

Verdier, V., Boher, B., Maraite, H., and Geiger, J.P. 1994. Pathological and molecular characterization of *Xanthomonas campestris* strains causing diseases of cassava (*Manihot esculenta*). Appl. Environ. Microbiol. **60**: 4478–4486.

Verdier, V., Dongo, P., and Boher, B., 1993. Assessment of genetic diversity among strains of *Xanthomonas campestris* pv. *manihotis*. J. Gen. Microbiol. 139: 2591–2601.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: New technique for DNA fingerprinting. Nucleic Acids Res. **23**: 4407–4414.