Comparative analysis of genetic variation among *Xanthomonas axonopodis* pv *manihotis* isolated from the western states of Nigeria using RAPD and AFLP

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Abstract *Xanthomonas axonopodis* pv *manihotis* is the causal agent of cassava bacterial blight (CBB) worldwide. CBB disease is a major constraint to cassava cultivation, and losses can be extremely severe in regions where highly susceptible cultivars are grown. To develop an efficient disease management policy, the genetic diversity of the pathogens population must be known. There is dearth of information on the genetic diversity of *X. axonopodis* pv *manihotis* population in Nigeria. We used RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism), a PCR-based technique, to characterize the *X. axonopodis* pv *manihotis* isolates from the western States of Nigeria. Thirteen strains *Xam* and 2 reference strains were tested with eight primers combination of AFLP and 4 RAPD primers. RAPD amplified DNA fragment data showed four major clusters at 80 % similarity coefficient level and two strains were not clustered by this analysis. Strains Kwa76A and Ond48A were also separated in the principal component analysis of the same data. Numerical analysis differentiated the AFLP patterns into four distinct clusters and grouped two strains separately at 66 % similarity. PCA assembly grouped the bacterial strains into 4 and one of the strains was singled out from the others. The two DNA analyses techniques seem to be complimentary to one another and informative on the genomic structure of *Xam* population in Western Nigeria. The genetic analysis

presented here contributes to understanding of the *Xam* population structure in Western Nigeria.

Keywords RAPD · AFLP · DNA · Genetic diversity · Dedrogram analysis and PCA

Introduction

Cassava (*Manihot esculenta*) is a starchy root and a major tropical food crop in Africa, Latin America and Asia. Cassava, as important as it is however is susceptible to many pathogens that decrease yields. More than thirty bacterial, fungal, viral, virus-like, mycoplasma and nematode agents had been reported to attack cassava [1].

Xanthomonas axonopodis pv *manihotis* (*Xam*) is a causative agent of cassava bacterial blight (CBB) which is considered as a major biotic constraint for cassava production in all cassava-cultivating regions of the world [2]. Effective breeding for resistance to this disease requires information on the diversity and geographical distribution of the pathogen.

Molecular markers, such as restriction fragment length polymorphisms (RFLPs), revealed that a high genetic diversity exists among *Xam* strains in Latin America, the center of origin for cassava [3–5]. Recent studies have shown that there is a high potential of *Xam* populations to continually evolve, thereby, permitting an assessment of the role of host selection in pathogen population structure [6, 7]. In Africa, *Xam* populations are highly homogenous [3], although, recently, some genetic variation has been detected [8, 9]. These results highlight the importance of developing cassava varieties with adequate resistance to the *Xam* strains specific to each ecological area.

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According to Dixon *et al.* (2002) [10] Ibadan and Ubiaja were identified as locations with high level of disease pressure in Nigeria. These two areas are located in the western part of the country, hence, the need to give more attention to the pathogen population in this location. There is dearth of information on the genetic diversity of the population structure of *Xam*, the causative organism of CBB, in Nigeria. Nigeria is the world largest producer of cassava [11] and the most destructive disease of cassava world wide is CBB. To establish a strategy for the control and management of the cassava bacterial blight population in the area where the virulence of the bacteria is pronounced the bacterial population structure needs to be ascertained. Hence, the essence of carrying out this study on the location where the bulk of cassava is produce in Nigeria and where the virulence action of the disease is most experienced.

Genetic relationships among 13 isolates of *X. axonopodis* pv. *manihotis* from the same geographic regions were determined by two PCR methods that rely on different amplification priming strategies: Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP).

Materials and methods

Bacterial Strains and DNA Isolation. We used 13 field isolates collected in Western Nigeria in year 2000 and two reference strains from German Collection of Microorganisms and Cell Cultures (DSMZ). The states, year of collection and towns of collection for each bacterial strain are described in table 1. The Bacteria were isolated from the leaves and stems of diseased cassava plants collected from the field survey of the western States of Nigeria. They were identified and evaluated for virulence characteristic as previously described by [12, 13]. The bacteria were cultured on yeast extract dextrose peptone agar (YDPA) containing 5 g yeast extract, 10 g dextrose, 5 g peptone and 15 g agar per litre of distilled water (pH 7.2).

Extraction of genomic DNA of bacteria: The cells of the bacteria were harvested from 1.5ml suspension of cell by low speed centrifugation at 1200xg for 2 minutes. The pellet was washed in 1 ml of 5M NaCl and once in 1 ml TE buffer (10 mM Tris-HCl, 1mM EDTA (pH8)). Genomic DNA was extracted by the method of [6, 14]. The DNA quality was checked on agarose and quantified with DNA fluorometer (model TD-700).

Polymerase Chain Reaction (PCR) amplification All PCR reactions were performed in a total volume of 25 µl using 20 – 50 ng DNA. In RAPD amplification, the primers OPAC 5,

OPAC 19, OPAC 20 and OPAG 20 (Operon Technoligise Inc Alameda CA. USA) were used. The reaction mixture per PCR tube contained the following: Thermo buffer, 2.5 µl; MgCl₂, 2.5 µl; 5% Tween 20, 2.5 µl; Deoxynucleotide triphosphate (dNTP) made up of 2.5 mM/µl each of dATP dCTP dGTP and dTTP, 2.0 µl; 2 µM RAPD Primer, 1.0 µl; *Thermus aquaticus*(Taq)polymerase, 0.2 µl (2 unit); DNA, 5.0 µl; Distilled H₂O, 12.3 µl. The reaction mixture was put in the PCR tube and run in PCR machine (Model PTC-200 Peltier Thermal Cycler) for 32 cycles of 94°C for 30 sec; 56°C for 30 sec; 72°C for 60 sec in a total time of 3. Thereafter, the products were run on 2% agarose gel and stained in ethidium bromide. The pictures were captured with Polaroid film for documentation and scoring.

Amplified Fragment Length Polymorphism (AFLP) restriction endonuclease digestion: The Genomic DNA was quantified and diluted to 50 ng/µl. AFLP marker was assayed as previously described by [6], with some modification. About 15 µl of the diluted DNA was digested with EcoR1 and Mse1 restriction endonuclease simultaneously. EcoR1 has a 6-bp recognition site while Mse1 has a 4-bp recognition site. When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1kb) for separation on denaturing polyacrylamide gels. The digested samples were then ligated at 20°C for 2 hours to the respective adapters. Ten microlitre of the ligated mixture was added to 90 µl TE buffer and mixed well. Five microlitre of the ligation reaction dilution was pre-amplified for 20 cycles at 94°C for 30 sec; 56°C for 60 sec; 72°C for 60 sec and soaked temperature was 4°C. The primers used were screened with the bacterial DNA.

Table 1 Description and source of the bacterial isolates

Isolates	The states	Location	Year of collection
Edo1A	Edo	Uzebba	2000
Eki45B	Ekiti	Iyin	2000
Kog52a	Kogi	Ajaokuta	2000
Osu80	Osun	Osunleja	2000
Lag83	Lagos	Aja/Epe	2000
Ogu88	Ogun	Abeokuta	2000
Ond41B	Ondo	Isua	2000
Ond48A	Ondo	Ogbese	2000
Kwa76A	Kwara	Ilorin	2000
Oyo130A	Oyo	Sango/U.I	2000
Oyo130B	Oyo	Sango/U.I	2000
Oyo131A	Oyo	IITA/Moniya	2000
Oyo131B	Oyo	IITA/Moniya	2000
RefB	DSM no 3849	Germany	1985
RefC	DSM no 1220	Germany	1980

The primers selected include primers E-G/M-A; E-T/M-T; E-AA/M-G; E-AC/M-T; E-AA/M-C; E-G/M-T; E-C/M-G and E-A/M-C. In the first reaction, genomic DNA was pre-amplified with AFLP primers with no selective nucleotides (Primers E+0; M+0) [GIBCO BRL products]. The PCR products of the pre-amplification reaction were diluted in ratio 1:50 and 5 μ l of this was used as the template for the second amplification using two AFLP primers containing either zero, one or two selective nucleotides. This reaction mixture was introduced into PCR machine and run 23 cycles of 94°C for 30 sec; 56°C for 30 sec; 72°C for 60 sec. After the selective amplification the PCR products were mixed with equal volume of loading buffer (formamide dye; 98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol). This was heated in the PCR machine to denatured DNA at 95°C for 3min and immediately placed on ice to chill.

Six percent polyacrylamide gel (20: 1 acrylamide: bis; 7.5 M Urea; 0.5X TBE buffer) with 0.4 mm spacers and sharks-tooth combs was prepared on glass plate gel. The gel was pre-electrophoresed at constant power (Bio RAD, Model: 3000Xi computer controlled electrophoresis power supply) for about 20–30 minutes. About 5 μ l of each sample were loaded on the gel and electrophoresed at constant power until xylene cyanol (slower dye) is two-thirds down the length of the gel which takes about two hours. Thereafter the gel was stained in silver staining solution and de-

stained in double distilled H₂O. The plates were thereafter dry at room temperature overnight. The gel was thereafter scored for polymorphism on fluorescence light pack and scanned for record purposes.

Both bands from the RAPD and AFLP gels were scored usually for the presence or absence. A band was considered polymorphic if it was absent in one or more of the isolates studied. The data generated was analysis using the Jaccard coefficient of similarity present in NTSYS-PC Version 2.0 [15] and SAS package Version 8.0 [16].

Results

Four primers were selected to assess the diversity among the 13 *X. axonopodis* pv *manihotis* strains and two reference strains of *X. campestris* pv *malvacearum* after primer screening. The PCR reaction product was run at least twice for each primer. The primer OPAC 19 yielded fragments ranging from 0.2 bp to 2.0 kb and nine fragments were considered for analysis. Primer OPAC 20 amplified up to seven fragments per strain (0.5–2.0 kb) and six bands were included in the cluster analyses. The dendrogram constructed using 30 bands scored from the RAPD amplified DNA fragment data (Fig. 1) showed four major clusters at 80 % similarity. The first cluster comprised about 33 % of the bacteria while the second cluster contained

Fig. 1 The dendrogram generated from the RAPD data analysis showing the relationship of the pathogen population from the western region of Nigeria.

two of the bacteria, which is 13 % of the Xam population. Two Strains were not cluster by RAPD data analysis. The principal component analysis (PCA) showed the relatedness of the strains in clear scattered plots (Fig. 2). The PCA also assembled the bacterial strains into three groups with a major one containing 60% of the bacterial isolates and the other two containing two strains each. The reference strains were grouped together and separated from the others isolates. Strains Kwa76A and Ond48A were also separated in the principal component analysis.

AFLP analysis with restriction endonucleases *EcoRI* and *MseI* revealed a considerable degree of genomic heterogeneity among the 15 *Xanthomonas* strains tested. Analysis of 13 *X. axonopodis* pv *manihotis* and 2 *X. campestris* pv *malvacarum* strains obtained a total of 257 AFLP bands from the eight primers combinations, only clearly recognized and visible bands were considered. Bands obtained per strain on a glass plate polyacrylamide gel were between 30 and 70 fragments. However a high number of bands per primer combinations were monomorphic and therefore were not considered in the analysis. Only 141 bands (54.9%) that were polymorphic fragments were considered for the Principal component and dedogram analysis.

When the 141 polymorphic AFLP bands were used for the cluster analysis using the un-weighted pair group arithmetic means analysis (UPGMA) program of NTSYS-PC

Fig. 2 The Principal component analysis showing similarity of the pathogen population from the western region of Nigeria.

Fig. 3 The dendrogram generated from the AFLP data showing the relationship of the pathogen population from the western region of Nigeria.

(version 2.0), a high level of polymorphism was shown in the pathogen population. The dendrogram in Fig. 3 shows

Fig. 4 The Principal component analysis showing the relationship of the pathogen population from the western region of Nigeria.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 5 RAPD Gel showing the PCR product of DNA fragment obtained for the 15 *Xanthomonas* using Primer OPAC 19. Lane M is 1 kb DNA marker.

the genotypic similarities among the AFLP banding patterns obtained, which contained clearly separated bands that varied in size from 30 to 360 bp. Numerical analysis differentiated the AFLP patterns into four distinct clusters, representing genogroups I to IV, and grouped two strains separately. The banding patterns of different clusters showed less than 66 % similarity. Cluster 1 is the largest containing 40 % of the entire bacterial strains. Strains Ond41B and Oyo131B

Fig. 6 AFLP Gel showing the PCR product of DNA fragment obtained for the 15 *Xanthomonas* using Primer E-A/MC. Lane M is 30–330 AFLP DNA ladder.

were separated in the dendrogram. The *X. campestris* pv *malvacearum* used as reference organisms were very closely related than the pathogen of cassava, having a similarity coefficient level of 95 % and were cluster separately. The PCA assembly grouped the bacterial strains into 4 clusters and one of the strains was singled out from the others (Fig. 4). Cluster 1 contained the reference organism and this appears to be more homogeneous than others in that they were separated in both analyses from the other clusters. Strain Edo1A was also separated in this PCA analysis. This was not detected with the RAPD data of the same set of organisms. The gels representing RAPD and AFLP of the DNA samples are shown in Figs. 5 and 6 respectively.

Discussion

Few studies have been conducted with *Xanthomonads* from cassava plants in Nigeria and here we present the first survey of this bacteria analyzed with RAPD and AFLP genomic fingerprinting. Genetic diversity was observed

among the *Xam* population and very few of the bacteria were considered to be identical. Four clusters were obtained in both molecular marker techniques used in this study. This signifies that the *Xam* population in Nigeria are not perfectly homogeneous as it has assumed earlier by some worker who reported that African population of the bacteria are homogeneous and heterogeneity was limited to Southern American countries where cassava was said to be indigenous [3, 5]. The first clusters of both dendrogram from the RAPD and AFLP contained the majority of the bacterial isolates.

Some level of relationship was found between AFLP and RAPD data and the two techniques provided complementary information on the genetic diversity of *Xam*. Although, AFLP analysis of the *Xam* population detected considerably more diversity in this species than was observed with RAPD analysis of the *Xam* species. This was in line with earlier report on the delineation ability of the two DNA marker techniques [6, 9]. This is observed in the levels of similarity coefficient obtained in both analyses. The genetic analysis presented here contributes to the understanding of the *Xam* population structure in Western Nigeria. This will help in selecting strains of the pathogen from different clusters for screening cassava germplasms for resistance to the disease in an approach to develop a strategy for the control of the disease. The high disease pressure reported [10] in this ecological zone of the country may be related to the existence of different strains of the bacteria. The genetic diversity observed in this location, however did not correlate with the pathogenic characteristic of the bacterial strains [12]. Pathogenicity of this bacterial strain may not be chromosome related or determined [17].

Based on the study of the pathological diversity of *Xam* in Nigeria [12], an overall strategy for the identification and further characterization of pathological types and resistant cultivars in different ecological zones at a country level is proposed. Although, it may be difficult to inoculate a large collection of bacterial isolates with all the cassava cultivars and also because of the long crop cycle of cassava and the problems of the availability of cassava cuttings for planting, it may not be feasible to practice the intensive screening of all the cultivars of cassava presently in cultivation in Nigeria with all the virulent group of the bacteria identified. Nevertheless, few cassava cultivars that are known for their good characteristics could be screened to start with. The two Molecular markers techniques for DNA analyses used in this study seem to be complimentary one to another and informative on the genomic structure of *Xam* population in Western Nigeria. Also, similarities exist in the clustering of the strains by the two techniques. If characterization of the bacteria population with different methods will yield similar results, it is

reasonable to conclude that there is variability in the genetic composition of the pathogen population in Nigeria.

Although, AFLP genomic fingerprinting analysis has been shown to be an accurate approach for determining bacterial taxonomy [18] and the phylogenetic structure of bacteria but no general threshold levels for delineation of bacterial species have been established with these two techniques.

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