African Journal of Biotechnology Vol. 6 (3), pp. 179-183, 5 February 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Rep-PCR reveals a high genetic homogeneity among Ugandan isolates of *Xanthomonas campestris* pv *musacearum*

Aritua, V.*, Nanyonjo, A., Kumakech, F. and Tushemereirwe W.

National Agricultural Biotechnology Center, Kawanda Agricultural Research Institute, P.O Box 7065, Kampala, Uganda

Accepted 12 June, 2006

Wilting of plants incited by a bacterium, *Xanthomonas campestris* pv *musacearum* (Xcm), was first described on Ensete (*Ensete ventricosum*) and later bananas (Musa species) in the highlands of Ethiopia in 1968. Although the spread outside Ethiopia remained unreported for several decades, an epidemic of the disease on banana in Uganda was observed in 2001, in the districts of Mukono and Kayunga. Since then, the disease has spread into almost three quarters of the major banana growing areas in Uganda. It has also been confirmed affecting banana plantations in the Democratic Republic of Congo (DRC) and Rwanda. Repetitive sequence based genomic fingerprinting that uses a PCR-mediated amplification of DNA sequences located between specific interspersed sequences of highly conserved elements in prokaryotic genomes was used to characterize a collection of Xcm isolates from banana in Uganda. Fingerprints of bacterial isolates collected from Xcm symptom bearing banana plants grown in production fields from 10 districts including Kayunga, Masindi, Luwero, Kampala, Kiboga, Lira, Wakiso, Kibale and Nakasongola revealed similar patterns. Cluster analysis of pair wise similarity values performed using unweighted pair group method with arithmetic averages clustering technique did not generate any differences in the fingerprint patterns either. The implications of this genetic homogeneity on the origin and management of Xcm is discussed here.

Key words: Musa, finger printing, banana xanthomonas wilt.

INTRODUCTION

Wilting of plants, often known as Banana Xanthomonas Wilt, is incited by a gram-negative, flagellated, xylem limited bacterium, *Xanthomonas campestris pv musacearum* (Xcm), that belongs to the family *Xanthomonadaceae*. It was first described on Ensete (*Ensete ventricosum*, Musaceae) in 1968 (Yirgou and Bradbury, 1968) and later on bananas (*Musa* species) in 1974 (Yirgou and Bradbury, 1968, 1974) in the highlands of Ethiopia. Although spread outside Ethiopia remained unreported for about 4 decades, an epidemic of the disease on banana in Mukono and Kayunga districts in Uganda was observed in 2001 (Tushemereirwe et al., 2001). Surveys that followed since then have revealed that the disease is widespread, affecting all types of bananas, in over half of the major banana growing areas in Uganda. The disease has also been confirmed affecting banana plantations in the Democratic Republic of Congo (DRC) and Rwanda (Ndungo et al., 2005). Characteristic symptoms induced on affected banana by Xcm are several and include progressive yellowing and complete wilting of entire plant, premature and uneven

*Corresponding authors E-mail: arituavalentine@yahoo.com or v.aritua@csl.gov.uk
*Current Address: Central Science Laboratory, Sand Hutton, YO41 1LZ, York, UK. Phone: (+256)-41-566102. Fax: (+256)-41-566381.

yellowing, ripening and rotting of the bunch. Affected fingers are characterized by brown internal colorations. When excised, pockets of pale yellow bacterial ooze appears within 5-15 minutes from cut stems. There are also yellow or brown vascular streaks throughout the interior of infected plants. Other symptoms include wilting of bracts, shriveling and rotting of the male buds while the flower stalks turn yellow-brown (Tushemereirwe et al., 2001, 2004).

The development and implementation of an integrated disease management program against this epidemic would include the use of host-specific resistance in different production regions. However, the identification and deployment of such a resistance would clearly depend on a detailed understanding of the genetic diversity of the pathogen. Moreover, due to the preventive nature of such a control, sensitive and rapid methods of detection and discrimination are needed. The commonly used methods for diagnosis and identification of pathogens include biochemical and physiological characteristics, pathogenicity tests as well as serological and polymerase chain reaction based techniques. Repetitive sequence based genomic fingerprinting (Rep-PCR) that uses primers corresponding to endogenous interspersed repetitive sequences is one the PCR-based techniques used in bacterial identification and classification. The technique is based on a PCR-mediated amplification of DNA sequences located between specific interspersed sequences of highly conserved elements in prokaryotic genomes. Sequences of these elements have been extensively characterised in several prokaryotic microorganisms and are known to be families of short intergenic repeated sequences that contain highly conserved sequence occurring in the genome singly or as multiple adjacent copies (Versalovic et al., 1991). The sequences have been divided in three classes. Class I consists of 35-40 bp repetitive extragenic palindromic elements (REPs) (Stern et al., 1984), also known as palindromic units (Higgins et al., 1988). Class II consists of 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991), otherwise known as intergenic repeat units (Sharples and Lloyd, 1990). Class III consists of the 154 bp BOX elements (Versalovic et al., 1994).

Nowadays, specific oligonucleotides primers to the REP, BOX and ERIC elements are commonly used in detecting a large variety of genera of prokaryotic microorganisms (Versalovic et al., 1994). In addition, the selective amplification of distinct genomic regions located between the REP, ERIC or BOX sequences generates an array of differently sized DNA fragments from the genomes of individual strains. The separation of these fragments on agarose gels yields highly specific DNA fingerprints that are commonly used in fingerprinting including subspecies differentiation of some pathovars of *Xathomonas* species (Louws et al., 1994; Vauterin et al., 2000). Since the genetic diversity among strains of Xcm isolates causing disease on banana in Uganda is yet unknown, this study was initiated to characterize a collection of banana *Xanthomonas* isolates from multiple geographic locations by the rep-PCR approach. Our results revealed a high genetic homogeneity among *Xanthomonas* isolates currently infecting banana in Uganda. The implications of this genetic homogeneity on the origin and management of is also discussed.

MATERIALS AND METHODS

Source of bacterial isolates

Xcm samples were collected from symptomatic plants grown in production fields from 10 districts in Uganda. The districts that represented a geographically diverse regions and their corresponding isolates included Kayunga (KY44), Mbale (MBL01), Masindi (043/MSD/05), Luwero (014/LUW/05), Kiboga 038/KBA/05), Kampala (045/KLA/05), Lira (036/LRA/05; 034/LRA/05), Wakiso (007/WKS/05; 005/WKS/05), Kibale (031/KIB/05; 033/KIB/05) and Nakasongola (041/NSA/05). Bacteria was isolated by initially surface sterilizing cut pseudostems of symptomatic plants using 76% ethanol, followed by cutting of small portions (approx. 0.5 cm^2) from inner most part and suspending in 2 ml of sterile distilled water. Loopfuls of the resulting suspension was streaked onto yeast peptone glucose agar (YPGA) and the plates incubated for 3 days at 25°C. Single yellow colonies were purified by repeated streaking on YPGA and confirmed as *Xanthomonas* by catalase, urase and hydrogen sulphide production tests as described previously (Verniere et al., 1993). Isolated bacteria were stored in YPG containing 50% glycerol at -20°C and re-grown on YPGA plates prior to analysis.

Pathogenicity test

To ensure that only pathogenic bacterial isolates were compared, a pathogenicity test was performed prior to DNA analysis. All isolates were tested for pathogenicity on banana cv. Mpologoma. Before inoculation, single colonies were surface streaked on YPGA and incubated at 25°C for 1-2 days. The resulting bacterial growth was suspended in sterile distilled water to give a visibly cloudy suspension, 1 ml of which was injected onto leaf petiole of young tissue culture plants. At least four plants were inoculated with each isolate. Control plants were inoculated with sterile distilled water. Inoculated plants were observed daily for five weeks for symptom development and the bacteria re-isolated from symptomatic plants on YPGA and confirmed as *Xanthomonas* as described before.

DNA isolation

DNA was isolated from bacterial cells following the method previously described by Mahuku (2004) with slight modifications. Briefly, bacterial cells were either harvested from a 3-day old culture grown on YPG broth or from YPGA plates and suspended in 1 M NaCl by vigorous vortexing. The tubes were centrifuged to reduce and separate the cells from the polysaccharide xantham gum. To reduce salt concentration, the cells were twice washed in sterile-distilled water. Cells were then lysed by suspending in TES extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS) and macerating in acid-washed, sterilized sea sand. The resulting lysate was treated with 50 mg/mL proteinase K at 65°C for 30 min to inactivate bacterial protein. DNA was finally purified using 7.5 M ammonium acetate on ice for 10 min and

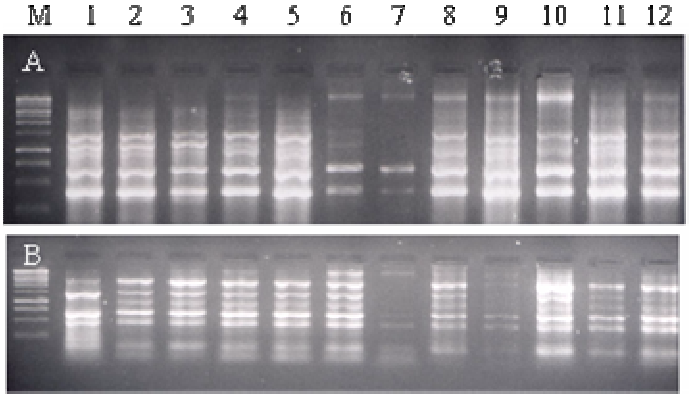

Figure 1. Rep-PCR patterns of Ugandan isolates of *Xanthomonas campestris* pv *musacearum* isolates. A and B are ERIC- and Box-primer pair profiles; M is 100 bp DNA Ladder delimiting size of the products. Lane 1, isolate KY44; lane 2, 043/MSD/05; lane 3, 014/LUW/05; lane 4, 038/KBA/05; lane 5, 045/KLA/05; lane 6, 036/LRA/05; lane 7, 034/LRA/05; lane 8, 007/WKS/05; lane 9, 005/WKS/05; lane 10, 031/KIB/05; lane 11, 033/KIB/05; lane 12, 041/NSA/05 (isolate details given in the text).

precipitated by using isopropanol at 20°C for 1h or overnight. The precipitated DNA was eluted with twice-repeated extractions with 250 µL of 1×TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). To remove RNA, the eluted DNA solution was treated with 20 mg/mL RNase A at 37°C for 60 min and the DNA recovered using isopropanol as described before.

Genomic fingerprinting analysis

A total of 12 isolates pathogenic to banana were subjected to Rep-PCR genomic fingerprinting using primer sets corresponding to BOX, ERIC, and REP elements (Versalovic et al., 1994). The 18-mer primer pair REP 1R (5'-IIIICGICGICATCIGGC-3') and REP 2 (5'-ICGICTTATCIGGCCTAC-3') (where I is Inosine); ERIC 1R (5'-ATGTAAGCTCCTGGGGAT-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGGT GAGC-3') and BOX1A (5'-CTACGGCAAGGCGACGCTGACG-3') were used to amplify putative REP-, ERIC- and BOX-like elements in bacterial DNA, respectively. The Rep-PCR and electrophoresis conditions followed were as described previously (Versalovic et al., 1994). Briefly, approximately 50 ng of purified DNA was used as a template in a 25 µl reaction mixture containing 600 pM of each primer, 200 µM deoxynucleoside triphosphates, and 1 unit of DNA polymerase in 2.5 µl of a reaction buffer (Promega, Madison WI, USA). A negative control to detect reagent contamination was included in each PCR, containing all components except the DNA extract, which was replaced by 2 µl of sterile distilled H_2O. PCR amplification reactions were performed with a Ependorff Mastercycler (Ependorf AG, Hamburg, Germany) using the following conditions: an initial denaturation at 95°C for 7 min; 30 cycles consisting of 94°C for 1 min and annealing at 40, 52 or 53°C for 1 min with either REP, ERIC or Box primers, respectively; extension at 65°C for 8 min; and a sing1e final polymerisation at 65°C for 15 min before cooling at 4°C. To ensure consistency in results, PCR was repeated for each isolate for at least three times.

A 12 µl aliquot of amplified PCR products were separated by gel electrophoresis on ethidium bromide stained 1.5% agarose gels in 1% Tris-borate-EDTA for 1 h at 100 V. The molecular sizes of fragments generated by electrophoresis were judged by comparison with concurrently run 100 bp DNA Ladder (Bioline Ltd, UK). For photographic documentation, the Rep-PCR fingerprints were exposed to UV light, photographed on a UV transilluminator using Polaroid Type 55 film (Polaroid Corp., Cambridge, MA) and compared visually. To allow a direct comparison, the Rep-PCR profiles were electrophoresed in the same lane on each gel and compared by visual inspection. For each isolate bands were scored as plus (+) and minus (-) depending on whether or not the individual did or did not have each band. Fingerprints were considered to be highly similar when all visible bands had the same apparent migration distance. Variations in the intensity or shape of bands were not taken into account. Cluster analysis was attempted using Unweighted Pair Group Method with Arithmetic averages (UPGMA) was performed with PHYLIP soft-ware (version 3.5c).

RESULTS AND DISCUSSION

The pathogenicity of the Xcm isolates on banana cv. Mpologoma was assessed by symptom observation. With exception of isolate MBL01 from Mbale, all isolates used in this study were pathogenic on the banana cv Mpologoma. In addition, for any given pathogenic isolate, disease severity and symptom type were not significantly different among the 12 pathogenic isolates and between replicate inoculated plants. Characteristic symptoms induced on affected banana included progressive yellowing and wilting of leaves, appearing within two weeks following inoculation and which eventually resulted into wilting of entire plant by the forth week. There were also yellow or brown vascular streaks throughout the interior of infected pseudostems. From the symptomatic plants, bacteria was successfully re-isolated on YPGA and confirmed as *Xanthomonas* by catalase, urase and hydrogen sulphide production tests as described previously (Verniere et al., 1993). Twelve isolates pathogenic to banana were subsequently selected for DNA analysis using the Rep-PCR.

All the isolates were typeable by the Rep-PCR and the same fingerprints were observed even when the PCR was repeated at least three times, demonstrating the reproducibility of this technique for genetic studies in Xcm (Figure 1). Analysis of the 12 isolates with REP and ERIC primers yielded four major amplification bands with all isolates. The size of these bands ranged from 250 to 600 bp for ERIC (Figure 1a) and from 350 to 1000 bp for REP (data not shown). Although a lower number of clearly scorable bands were observed in some isolates presented in Figure 1, the three repeats of REP-PCR amplifications revealed that the absent bands are reproducible. Some minor light amplification bands were also not reproducible, suggesting none specific binding and such bands were not taken into account. Figure 1b shows the genetic fingerprints of the 12 Xcm isolates pathogenic to banana as determined by BOX-PCR. As with ERIC-and REP primers, each isolate gave a BOX-PCR profile that is similar to other isolates and in total, up to five BOX bands clearly scorable in the same size range as that of ERIC profiles were produced within the

range 300–600 bp. Cluster analysis of the similarity values among the different Rep-PCR profiles with the UPGMA did not allow us to identify any major genomic clusters since the majority of the isolates were genetically similar (data not shown).

Since Xcm was first reported on Ensete and later on banana in Ethiopia over 3 decades ago, no genetic information was obtained on this pathogen. Analyses done using Rep-PCR reported here provides the first data on the molecular characteristics of Xcm infecting *Musa* spp. in Eastern and Central Africa, and perhaps not yet unreported in other parts of the world. The Rep-PCR generated similar fingerprints for 12 Ugandan isolates from the districts of Kayunga, Masindi, Luwero, Kampala, Kiboga, Lira, Wakiso, Kibale and Nakasongola compared here. Cluster analysis of the pair wise similarity values performed using UPMA did not generate any differences in fingerprint patterns either, all suggesting a considerable level of genetic homogeneity among the isolates infecting banana in Uganda. The very low genetic diversity among the isolates suggests that, except for the original big invasion events, there has been almost no genetic exchange (mutation) since 2001 and over the 200–300 km coverage (from Kayunga to Kibale). It may thus be speculated that there has not been any unfavorable abiotic or biotic factors which could trigger differential genetic or epiphytotics effects.

In a related restriction fragment length polymorphism (RFLP) analysis of DNA of Xanthomonas spp, *X. campestris* pv *vasculorum* isolates from the Southern African mainland were found to be similar, but different from isolates found on Mauritius and Reunion in the Indian Ocean plus two Australian isolates (Qhobela and Claflin, 1992). In addition, isolates from mainland Africa could not hydrolyse starch, as could those from the Indian Ocean islands and Australia. The possible independent development of the pathogen in Southern Africa and the Indian Ocean was hypothesized (Qhobela and Claflin, 1992). The genetic closeness among the Xcm isolates observed in this study highly supported the induction of the same scale epiphytotic effects in our laboratory based experiment, therefore, suggests that the isolates have originated from the same previous invasion event. However, the exact source of the prevailing epidemic in Uganda still remains unknown. In order to postulate the possible origin of current Xcm epidemic in Uganda, an ongoing study is comparing isolates from Ethiopia, and DRC and Rwanda. This, is hoped, will give an insight into whether the epidemic in Uganda is a spread from Ethiopia or an independent development of the pathogen within Uganda.

Over the last few decades use of DNA based techniques led to reclassification of Xanthonomas species. Whereas some pathovars were elevated to species level, others were given new pathovar status. For instance, some isolates of *X. campestris* pv. *vasculorum* has been given a species status as *X. axonopodis* pv. *vasculorum*; *X. campestris* pv *holcicola* became *X. vasicola* pv *holcicola*; *X. campestris* pv *Oryzae* was changed to *X. oryzae* pv. *Oryzae*; *X. campestris* pv. *Citri* to *X. axonopodis* pv *Citri; X.* camp*estris* pv *melonis to X. melonis*; *X. campestris* pv. *Carotae to X. hortorum* pv *Carotae*; *X. campestris* pv *phaseoli* to X. *axonopodis* pv. *Phaseoli* while *X. campestris* pv *vesicatoria* became *X. vesicatoria* (Yang et al., 1993; Vauterin et al., 1995, 2000; Gonçalves and Rosato, 2002). Future studies on Xcm should, therefore, be aimed at clarifying its current taxonomic position as well as investigating the genetic relatedness to isolates from Ethiopia, DRC and Rwanda. In conclusion, the results of this work show that Rep-PCR is a suitable typing method for Xcm. All the three primer pairs involved in Rep-PCR generated good patterns that can be used as a rapid means of identifying Xcm isolates for epidemiological investigation. The results also demonstrated a very high genetic relatedness within Xcm isolates that were isolated on banana from Uganda. From this, one can speculate that the current Xcm populations in Uganda have evolved from the same origin, and this, may prove useful in future breeding programmes

ACKNOWLEDGEMENTS

This research was supported by the Gatsby Charitable Foundation (UK) and the Government of Uganda. The authors would like to express their sincere gratitude to Dr. Geofrey Tussime (Department of Crop Science, Makerere University, Uganda) and Dr. Africano Kangire (Coffee Research Institute, Kituza, Uganda) for helpful discussion.

REFERENCES

Gonçalves RE, Rosato BY (2002). A phylogenetic analysis of *Xanthomonas* species based upon 16S–23S rDNA intergenic spacer sequences. Int. J. Syst. Evol. Micro. 52: 355-361

Higgins CF, McLaren RS, Newbury SF (1988). Repetitive extragenic palindronic sequences, mRNA stability and gene expression: evolution by gene conversion? A Rev. Gene 72: 3-14.

Hulton CSJ, Higgins CF, Sharp PM (1991). ERIC sequences : a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. *Mol. Micro.* 5: 825-834.

Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Envir. Micro 60: 2286-2295.

Mahuku GS (2004). A simple extraction method for PCR-based analysis of plant, fungal and bacterial DNA. Plant Mol. Biol. Rep. 22: 71-81.

Ndungo V, Eden-Green S, Blomme G, Crozier J, Smith J (2005). Presence of banana xanthomonas wilt (*Xanthomonas campestris* pv. *musacearum*) in the Democratic Republic of Congo (DRC). Plant Path. 55: 294.

Qhobela M, Claflin LE (1992). Eastern and southern African strains of *Xanthomonas campestris* pv. *vasculorum* are distinguishable by restriction fragment length polymorphism of DNA and polyacrylamide gel electrophoresis of membrane proteins. Plant Pathol. 41: 113-121.

Schroth AMN, Hildebrand OC, (1983). Towards a sensible taxonomy of bacterial plant pathogens. Plant Dis. 67: 128.

Sharples GJ, Lloyd (1990). A novel repeated DNA sequence located in the intergenic regions of bacterial chromosome. Nucl. Acid. Res. 18: 6503-6508.
Stern MJ, Ames GFL, Smith NH, Robinson EC, Higgins CF (1984). Repetitive extragenic palindromic sequences: a major component of the bacterial genome. Cell 37: 1015–1026.
Tushemereirwe WK, Kangire A, Smith J, Nakyanzi M, Karyeija R, Kataama D, Musiitwa C (2001). An out break of Banana bacterial wilt in Mukono and Kayunga districts: A new and devastating disease. NARO /KARI.
Tushemereirwe W, Kangire A, Ssekiwoko F, Offord LC, Crozier J, Boa E, Rutherford M, Smith JJ (2004). First report of Xanthomonas campestris pv. musacearum on banana in Uganda. Plant Path. 53: 802.
Vauterin L., Hoste B, Kersters K, Swings J (1995). Reclassification of *Xanthomonas*. Int. J. Syst. Bact. 45: 472-489.
Vauterin L, Rademaker J, Swings J (2000). Synopsis of the taxonomy of the genus *Xanthomonas*. Phytopathol. 90: 677-682.
Verniere C, Pruvost O, Civerolo EL, Gambin O, Jacquemoud-Collet JP, Luisetti J (1993). Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. Citri. Appl. Envir. Micro. 59: 243-249.
Versalovic J, Koeuth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acid. Res*. 19: 6823–6831.
Versalovic J, Schneider M, De Bruijn FJ, Lupski JR (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction Meth. Mol. Cell. Biol. 5: 25-40.
Yang P, Vauterin L, Vancanneyt M, Swings J, Kersters K (1993). Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. Syst. Appl. Micro. 16: 47-71.
Yirgou D, Bradbury JF, (1968). Bacterial wilt of Enset (Enset ventricosa) incited by *Xanthomonas* pv *mussearum*. Phytopathol. 58: 11-112.
Yirgou D, Bradbury JF (1974). A note on wilt of banana caused by the enset wilt organism *Xanthomonas musacearum*. East Afric. Agric. For. J. 40: 111-114.