OCCURRENCE OF BACTERIAL LEAF BLIGHT OF COCOYAM AND CHARACTERISATION OF THE CAUSAL ORGANISM IN NORTHERN NIGERIA

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SUMMARY

Field surveys were conducted in Kaduna and Kano States of northern Nigeria to determine occurrence of bacterial leaf blight of cocoyam. On farm sampling and assessment were done using a 2 x 2 square meter quadrat to determine the incidence and severity of the disease. Bacterial isolates obtained from the diseased samples were used for pathogenicity and hypersensitive reaction (HR) tests. Biochemical tests such as Gram, catalase, oxidase, pectolytic, amylolytic and production of acid from glycerol were carried out on the isolates. A nearly full length of the 16S rDNA gene of selected isolates was PCR amplified using 16S rRNA primers. The resulting amplicons were sequenced using Sanger sequencing. The 16S rDNA gene sequences were aligned along with other *Xanthomonas* sequences imported from the NCBI database using muscle tool from MEGA6.The results showed Kaduna State had higher incidence and severity (50.2 %, 13.5 %) than Kano State (20.5%, 5.1%) and that bacterial isolates induced blight symptoms on cocoyam and elicited HR reactions on tobacco. Isolates were Gram negative, catalase positive, oxidase negative, amylolytic, pectolytic and produced no acids from glycerol. BLASTn search of sequenced genes showed 98-100% homology to *Xanthomonas axonopodis* pv. *dieffenbachiae.* Maximum likelihood phylogenetic trees constructed for the 16S rDNA gene sequences revealed isolates were identical to the *Xad* reference strains KP247494, KM576803 and EU203153.

Generally, all the isolates obtained were *Xad.* Bacterial leaf blight of cocoyam occurred in Kaduna and Kano States and the bacterial isolates were identical to *Xanthomonas axonopodis* pv. *dieffenbachiae*.

Keywords: Incidence; severity; Kaduna; Kano; biochemical; molecular; 16S rDNA.

COCOYAM (*Colocasia* and *Xanthosoma*s pecies) which belongs to the family Araceae is one of the oldest crops grown mostly in the tropics for its edible corms and leaves and as an ornamental plant [1]. Cocoyam is one of the basic food crops of major economic importance and ranks third in importance after cassava and yam among the root and tuber crops cultivated and consumed in Nigeria [2]. It is superior to yam and cassava nutritionally, with higher protein, mineral and vitamin contents and the starch more readily digested [3]. Nigeria is the world's leading producer of cocoyam, with annual production of 3.27 million metric tonnes, accounting for about 36 percent of total world output of cocoyam [4]. The yield and quality of this crop are threatened by various abiotic and biotic factors. Diseases caused by fungi [5; 6; 7] and bacteria [8; 9] are among biotic factors militating against the production of cocoyam in Nigeria.

Bacterial leaf blight (BLB) was first reported as an important disease on cocoyam in Nigeria [9]. The causal agent was identified as *Xanthomonas axonopodis* pv. *dieffenbachiae* using pathogenicity and biochemical tests. The status of this disease in Northern Nigeria, another cocoyam producing region has not been reported. Molecular characterisation using polymerase chain reaction (PCR) based technique has proven to be a fast, sensitive and reliable method for determining genetic relationships among pathogenic organisms [10]. However, genetic profile of *X. axonopodis* pv. *dieffenbachiae* on cocoyam using this technique has also not been reported anywhere in Nigeria.

There is the need to assess the status of this disease in Northern Nigeria where the crop occupies a very important position in the farming system. This research is aimed at providing valuable information that will form the basis for cocoyam bacterial leaf blight disease management decisions and breeding cocoyam cultivars with resistance to the pathogen. Molecular characterisation of the pathogen in the region will also provide information that could be used to map the pathogen in the region, a tool that could serve quarantine purposes.

MATERIALS AND METHODS

Incidence and Severity of Bacterial Leaf Blight of Cocoyam

Incidence and severity of bacterial leaf blight of cocoyam were determined between June and July November, 2015 in Kaduna and Kano States. In each State, three Local Government Areas (LGAs) and three farming communities per LGA were visited. Three fields were surveyed in each community. In each field, cocoyam leaves were examined for symptoms of bacterial leaf blight, thereafter, a 2 by 2 m^2 quadrat was set each at four corners and at the centre of the field. The total number of plants in the quadrat was recorded. Disease incidence was assessed by counting the number of plants showing bacterial leaf blight symptoms in each quadrat and the total number of plants in that quadrat. The disease incidence was calculated using the formula:

$$Disease incidence = \frac{Number\ of\ plants\ infected}{Total\ number\ of\ plants\ examined} x\ 100$$

Disease Severity was recorded per plot using a disease scale of 0-5 developed by Opara *et al.* (9):

0 = no disease symptom visible on the plant;
1 = less than 10% of plant affected;
2 =10-30 % of plant affected;
3 = 31-50% of plant affected;
4 = 51 - 70% of plant affected;
5 = above (>) 70% of plant affected;

Disease Severity (DS) was evaluated using the following formula [11]:

$$DS = \frac{Sum\ of individual\ disease\ scores}{Total\ number\ of plants\ scored\ x maximums\ core)} x\ 100$$

Leaf samples were collected from each field, wrapped in paper bags, labeled and taken to the Bacteriology Laboratory of the Department of Crop Protection, Ahmadu Bello University, Zaria for isolation. Global position system (GPS) parameters for each location were recorded using a Handheld GPS Navigator manufactured by Garmin, USA. Unstructured questionnaire was used to obtain some cropping information from the surveyed areas.

Characterisation of *Xanthomonas* spp. Isolation of organism, hypersensitive reaction (HR) and pathogenicity tests

Sampled leaves were surface-sterilised with 3.5 % w/v sodium hypochlorite and washed thoroughly with sterile water for 3 minutes before isolation. Small pieces (3 mm) of advancing lesions on leaves were excised from the boundary between diseased and the healthy tissues using sterile scalpel. Excised tissue was rinsed with sterile distilled water (SDW) for 1 minute before it was placed in a Petri dish containing 3.0 ml of SDW, shredded with sterile needles and allowed to stand for 10 minutes. The suspension was then streaked on nutrient agar (Difco) plates, placed upside down in an

incubator set at 27 ^{0}C for 48-72 hours. Pure cultures were obtained after sub-culturing twice. The pure cultures were then stored in SDW in McCartney bottles at 4 ^{o}C. Twenty-four-hour old cultures of the bacterial colony obtained were adjusted with spectrophotometer to an optical density of 0.3 at 600 nm of light, corresponding to a bacterial concentration of 10^8cfu/ml. Inoculum concentration of 10^8cfu/ml was used for HR test on tobacco leaves according to Wick [12]. Cocoyam corms were planted into 30 cm diameter pots filled with heat sterilised soil at the rate of one corm per pot. The bacteria isolate was tested for pathogenicity on 15 day-old cocoyam plants (*Colocasia esculenta*).

Biochemical characterization

Biochemical characterization was done by subjecting the bacterial isolates to the following tests [13].

Test for gram reaction

For Gram reaction, the alternative potassium hydroxide (KOH) test was done for all samples. Twenty four-hour old culture was added to 3% KOH solution on clean glass slide using flame-sterilized needle. The bacterial suspension was stirred against the KOH solution while occasionally lifting the loop for slimy and thickened slurry.

Test for catalase

A drop of 3% hydrogen peroxide (H_2O_2) was placed on glass slide, onto which a loopful of bacteria from a 24-hour old culture was suspended and observed for bubbling and production of gases.

Oxidase reaction test

One ml 1 % aq. w/v solution of N'-tetramethyl-p-phenylene-diamine dihydrochloride solution was prepared and 100 µl of the solution were placed on a new piece of Whatman No. 1 filter paper with a clean Pasteur pipette. Part of a colony was removed with a sterile toothpick and smeared onto the moistened paper. This was observed for colour changes which occurred 30 seconds later.

Test for pectolytic reaction

Bacterial isolates were grown on sodium pectate gel medium which contained pectate substrate and incubated at 28 ^{o}C for three days. Production of degrading enzymes was detected by observing depressions in the gel around the colony where the substrate has been degraded.

Test for amylolytic reaction

Twenty four-hour old cultures of *Xanthomonas* spp. isolates were transferred to nutrient agar plates amended with 0.2% (w/v) soluble starch. Cultures that emerged were incubated at 30 ^{0}C for 72 hours. Plates were flooded with Lugol's iodine solution and observed for any change of colour which signifies starch hydrolysis.

Acid production

Solutions of Dye's medium C and carbohydrate sources were prepared in tubes and inoculated with the isolates. This was incubated at 28 ^{o}C for 2-10 days and observed for colour changes. Yellow colour formation indicates acid production from carbohydrate. Substrates that were tested included glucose, fructose, mannose, lactose, galactose, sucrose, glycerol and arabinose.

DNA extraction, genomic finger printing and agarose gel electrophoresis

Genetic profiling was conducted at the Department of Biochemistry, Entomology, Molecular Biology and Plant Pathology, Mississippi State University, USA. Bacteria cultures grown at 28°C on NBY medium (8 g liter^{-1} nutrient broth, 2 g liter^{-1} yeast extract, 2 g liter^{-1} K_2HPO_4, 0.5 g liter^{-1} KH_2PO_4, 15 g agar, 974 ml distilled water, 25 ml 20 % glucose, 1 ml 1 M $MgSO_4$; pH 7.2) for 24 hours were cultivated in NBY liquid medium (NBY Medium above without Agar).16S rDNA was extracted using Wizard Genomic DNA Purification Kit [14]. Genomic DNA thus extracted was stored at -20 ^{o}C. PCR protocol was used to amplify the purified bacterial genomic DNA. I6S rRNA primers were used for the amplification, forward: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse: 1492R (5′-GGYTACCTTGTTACGACTT-3′) [15]. The component of PCR master mix included buffer (10 µl), $dNTP_s$ (1.5 µl), $MgCl_2$ (5 µl), forward primer (1.5 µl), reverse primer (1.5 µl), Taq polymerase (0.5 µl) and sterile water (30 µl). Two microlitre of the purified DNA of each sample was pippeted into a separate PCR tube. Fifty microlitre of the master mix was added to each of the DNA samples, vortexed and centrifuged before placing in the thermocycler. The amplification program included initial denaturationat 94°C for 3 min, 32 cycles consisting of denaturation at 94°C for 20 s, annealing at 56°C for 30 s, andextensionat72°C for 1 min 10 s, and an extra extension step at 72°C for 5 min.

Amplification of the DNA was confirmed by gel electrophoresis. Agarose was prepared at the desired concentration (w/v) in 1xTris-Acetate-EDTA (TAE) buffer (1.0 % gel). 1.6 µl of loading buffer (15% Ficoll^{R}400, 0.03 % bromophenol blue, 0.03 % Xylene cyanol FF, 0.4 % orange G, 10 mM Tris-HCl (pH 7.5)) was mixed with 8 µl of PCR product and loaded slowly into the wells. A known *Xanthomonas* strain (MSCTI) and sterile distilled water were also loaded to serve as positive and negative controls respectively. DNA molecular weight marker (1 kb) was also loaded. The order of sample loading in the gel was recorded. Ethidium bromide solution (0.5 µg/ml) was added to the buffer before running the gel.

Electrophoresis unit was then connected to the power pack and power supply turned on until the bromophenol blue dye reached the bottom of the gel (approximately 30 min at 100 V, for DNA to migrate 7 cm from the wells in a 1.0 % gel). The gel was removed and observed under UV Transilluminator. Photograph of the gel was taken using an orange filter fitted camera. The resulting amplicons were sequenced at the Eurofins Genomics Company, Eurofins MWG Operon LLC, Huntsville, Alaska, USA using Sanger sequencing. 16S rDNA gene sequences from the *Xanthomonas* spp. isolates were blasted against sequences of related xanthomonads from the NCBI online database (www.ncbi.nlm.nih.gov) to determine genomic relatedness.

Statistical Analysis

Data from the survey were analyzed using descriptive statistics. The *Xanthomonas* spp. isolates were analysed for their evolutionary history using the maximum likelihood method. The analysis was done following the Tamura-Nei model [16]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [17].

RESULTS

Incidence and Severity of Bacterial Leaf Blight

Mean bacterial blight incidence and severity observed in the Local Government Areas (LGAs) of the States are shown in Figs. 1 and 2. In Kaduna State, the incidence in Kudan (93.7 %) was highest, followed by Sabon Gari (40.4 %) and the least disease incidence (16.6 %) was recorded in Giwa. For Kano State, Kiru LGA (30.0 %) had the highest disease incidence, followed by Bebeji (10.4 %) and there was no incidence of the disease in Rogo LGA. The disease severity in Kaduna State was highest in Kudan LGA (45.3 %), followed by Sabo Gari LGA (14.4 %), while Giwa LGA (6.3 %) had the least. In Kano State, Kiru LGA (12.7 %) had the highest disease severity, followed by Bebeji LGA (2.7 %). The mean bacterial blight incidence and severity for Kaduna and Kano States are shown in Fig. 3, with Kaduna State having higher disease incidence and disease severity index than Kano State, respectively. The different symptoms observed in the locations were mostly spots and blight, consisting of small, water-soaked lesions, usually surrounded by prominent chlorotic halo (Plate I).

Characterisation of *Xanthomonas* Spp.

Tobacco hypersensitive and pathogenicity tests

The isolated bacteria formed yellow, convex, mucoid colonies on nutrient agar medium. Isolates corresponding to different locations were designated numerically with the prefix 'CE' to indicate cocoyam genus (*Colocasia esculenta*) origin. Four isolates namely CE2, CE3, CE4 and CE6 designating isolates from Tudun Sarki, Jaja, Yelwan Paki and Hunkuyi, respectively elicited hypersensitive reaction (HR) on tobacco and were found to be pathogenic on cocoyam. On tobacco, a clear HR was observed 48 hours after suspension of the inoculum was infiltrated into leaf tissues (Plate II). Control leaves did not show similar reaction. On cocoyam, symptoms appeared 2-5 days after inoculation on the leaves. Prominent chlorotic halos developed around the lesions which were similar to those observed in the field (Plate III).

Biochemical characterization

All the isolates were Gram negative, oxidase negative, catalase, amylolytic, pectolytic and, producing acid from glucose, fructose, mannose, lactose, galactose, sucrose, arabinose and not from glycerol (Table 1).

Gel electrophoresis of amplified 16S rDNA

The 16S rDNA of four out of the six isolates were amplified using 16S rRNA primers (Plate IV) and the expected band size of 1465 bp was obtained

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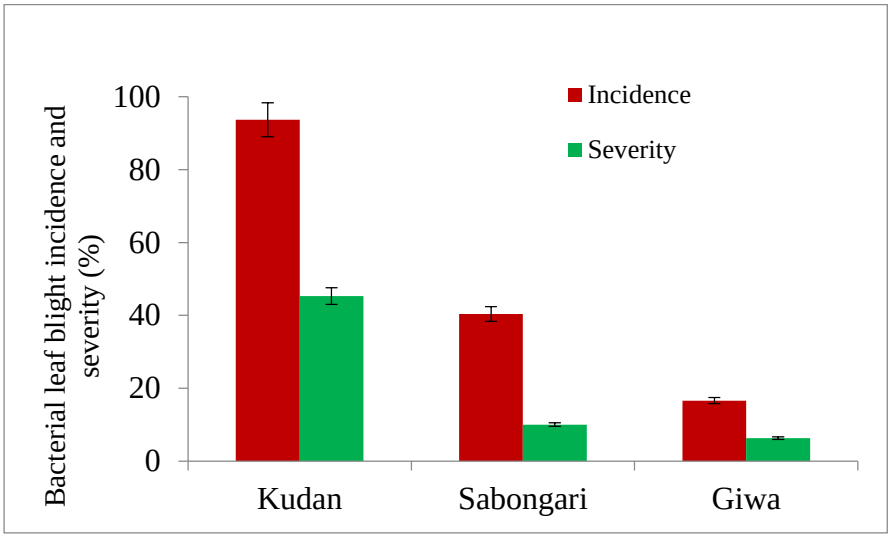

Figure 1: Incidence and severity of bacterial leaf blight on cocoyam leaves in three local government areas of Kaduna State, Nigeria during 2015 wet season

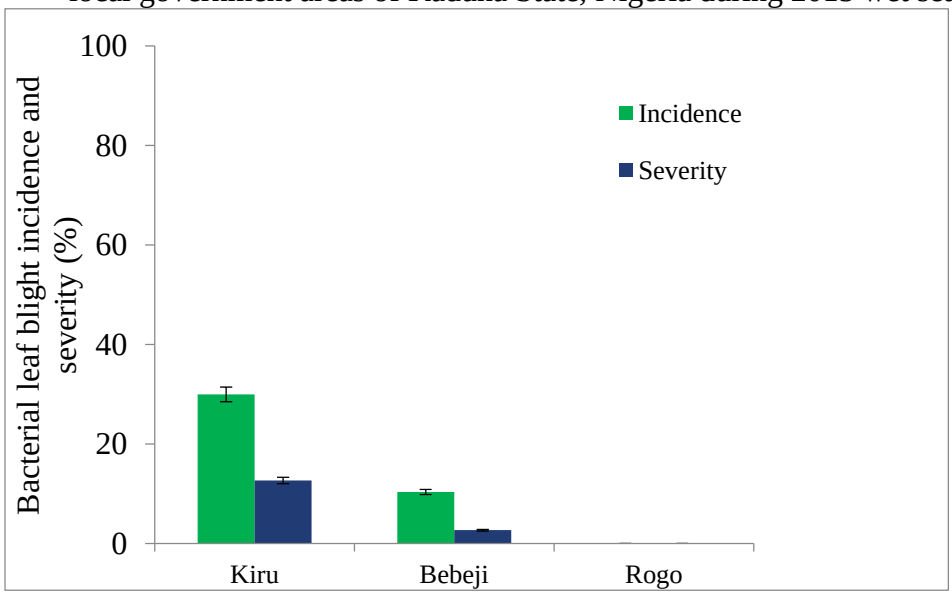

Figure 2: Incidence and severity of bacterial blight on cocoyam leaves in three local government areas of Kano State, Nigeria during 2015 wet season

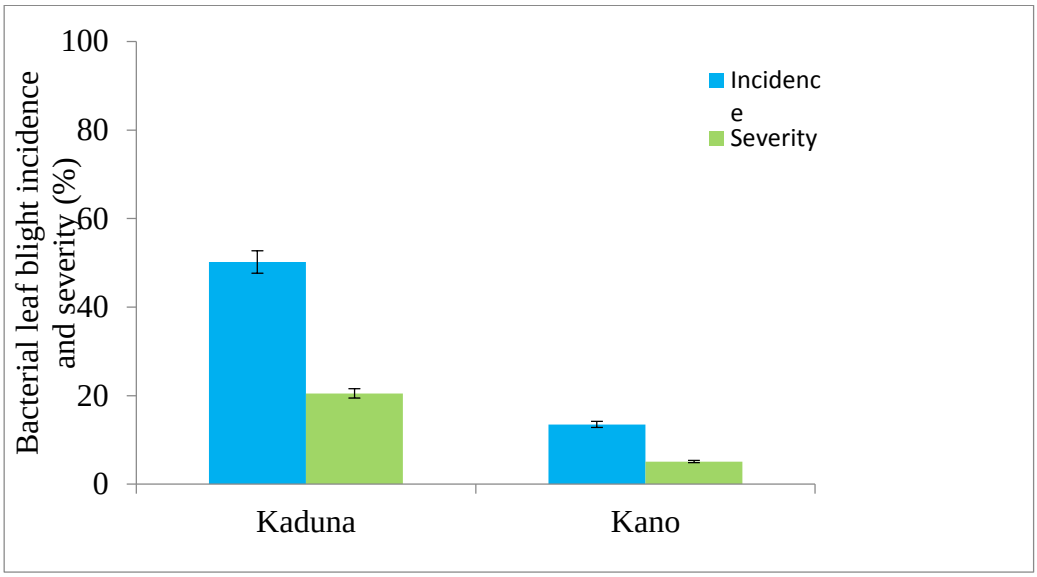

Figure 3: Incidence and severity of bacterial blight on cocoyam leaves in Kaduna and Kano States, Nigeria during 2015 wet season

Phylogenetic analysis

The sequences of the isolates were aligned and phylogenetic trees of sequenced isolates were constructed. The isolates obtained from Northern Nigeria form the same clade with three *Xanthomonas axonopodis* pv. *dieffenbachiae* strains KP247494, KM576803 and EU203153 on the dendogram (Fig. 4).

DISCUSSION

The bacterial leaf blight of cocoyam was recorded in the two States surveyed, with Kaduna State recording a significantly higher incidence and severity of the disease than Kano State. The high incidence and severity in Kaduna State could be as a result of higher rainfall and relative humidity of the areas surveyed in Kaduna State than in Kano State (Appendix 1). Most of the fields surveyed in Kaduna State, such as those in Kudan and Giwa LGAs were wetlands, a condition which favours the pathogen. *Xanthomonas axonopodis* pv. *Dieffenbachiae* is favoured by warm and moist conditions. Hot, humid, and rainy weather conditions are ideal for disease development and the spread of *Xad* [18]. The disease incidence recorded in some Local Government Areas in Kaduna and Kano States was in line with the earlier report of Opara *et al*. [9]**,** who reported over 65-68% incidence of BLB at Umudike, Abia State, South East Nigeria. A 74-100% incidence of BLB was also reported from a survey of commercial cocoyam fields in Florida, U. S. A. [19]. However, the disease severity generally was low, averaged 2 or less, indicating that less than 30 % of the foliar area was damaged by *Xad*. For example, Pohronezny*et al*. [19] reported a less than 10 % disease severity of BLB in their survey of commercial cocoyam fields infected by *Xad* in Florida.

Plate I: Typical symptoms of bacterial leaf blight on cocoyam leaf in a field in Jaja

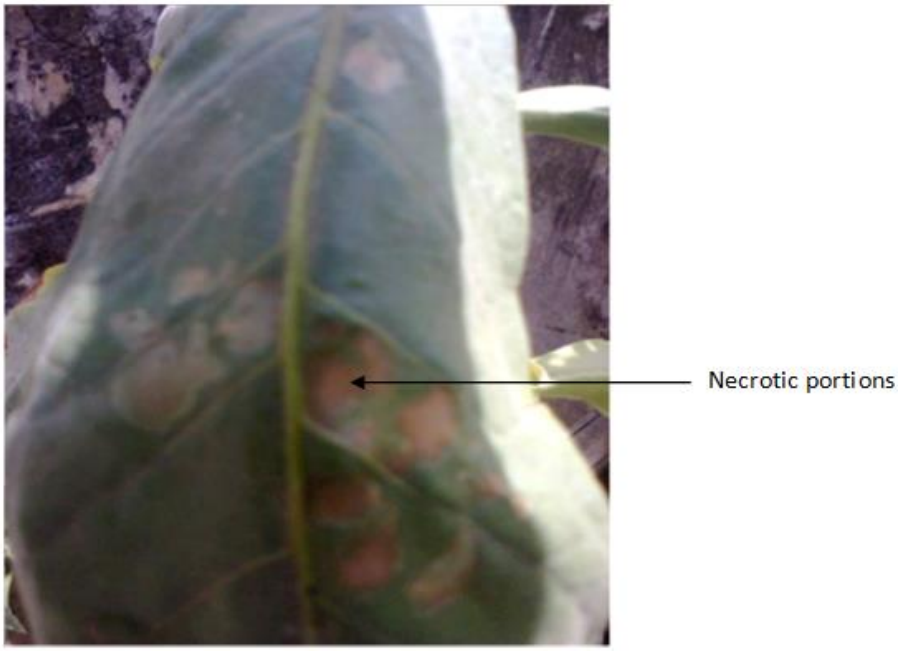

Plate II: Necrotic portions on tobacco leaf inoculated with *Xanthomonas axonopodis* pv. *dieffenbachiae* showing hypersensitive reaction

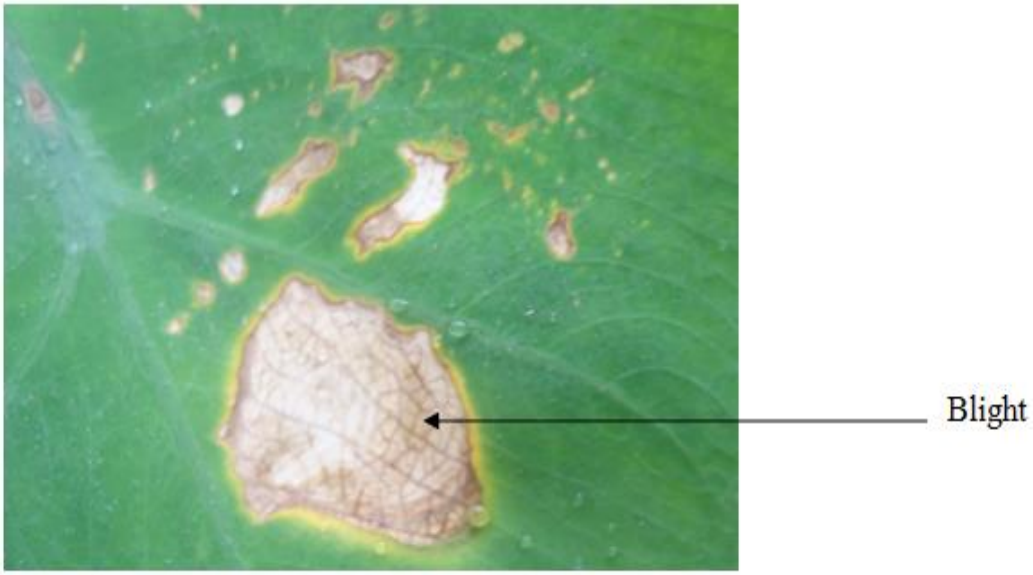

Plate III: Leaf blight symptoms on cocoyam inoculated with *Xanthomonas axonopodis* pv. *dieffenbachiae* in the screenhouse in Zaria

Table 1. Biochemical reaction of the isolates

Biochemical tests	Isolates			
	CE2	CE3	CE4	CE6
Gram's reaction	-	-	-	-
Oxidase activity	-	-	-	-
Catalase Activity	+	+	+	+
Amylolytic Activity	+	+	+	+
Pectolytic Activity	+	+	+	+
Acid production from:				
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannose	+	+	+	+
Lactose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	+	+
Arabinose	+	+	+	+
Glycerol	-	-	-	-

+and - indicate positive and negative reactions, respectively to the test indicated
CE2 = Tudun Sarki isolate, CE3 = Jaja isolate,
CE4 = Yelwan Paki isolate, CE6 = Hunkuyi isolate

Bacterial organisms isolated from diseased leaf of cocoyam collected from Northern Nigeria fit biochemical descriptions of *Xanthomonas axonopodis*. Biochemical tests showed that the isolates are pectolytic, confirming Chase *et al*. [20] report which stated that strains from *Colocasia*, *Dieffenbachia* and *Philodendron* were highly pectolytic, while those obtained from *Xanthosoma* and *Syngonium* are non-pectolytic. Acids were not produced from glycerol by isolates obtained from various locations in Northern Nigeria which confirms the findings of Pohronezny *et al*. [19] and Opara *et al*. [9] who reported that strains from cocoyam did not produce acid from glycerol.

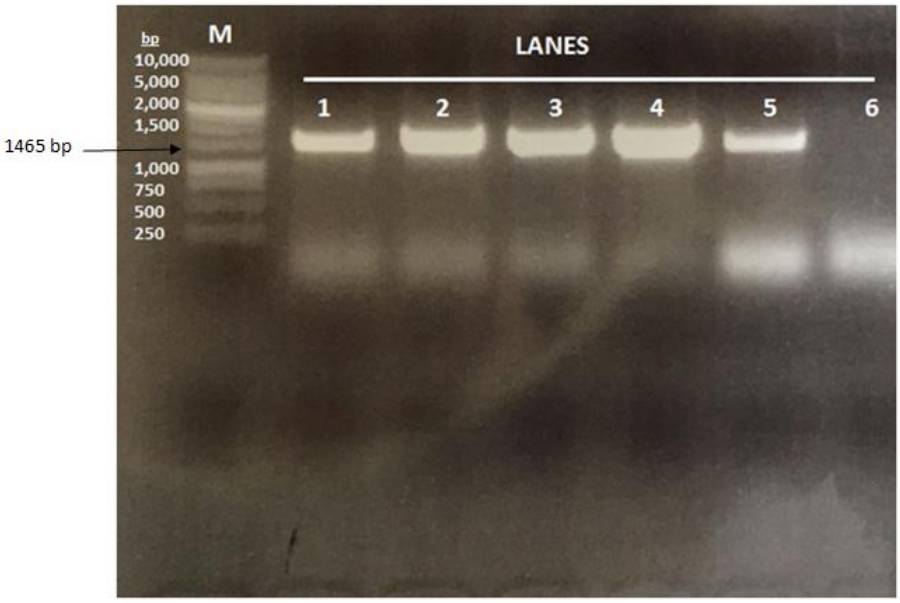

Plate IV: Electrograph of amplified 16S rDNA genes of *X. axonopodis* pv. *dieffenbachiae* isolates from Northern Nigeria. M = molecular marker, Lane 1= CE2 (Tudun Sarki isolate), Lane 2 =CE3 (Jaja isolate), Lane 3=CE4 (Yelwan Paki isolate), Lane 4=CE6 (Hunkuyi isolate), Lane 5=MSCTI (positive control), Lane 6=sterile distilled water (negative control)

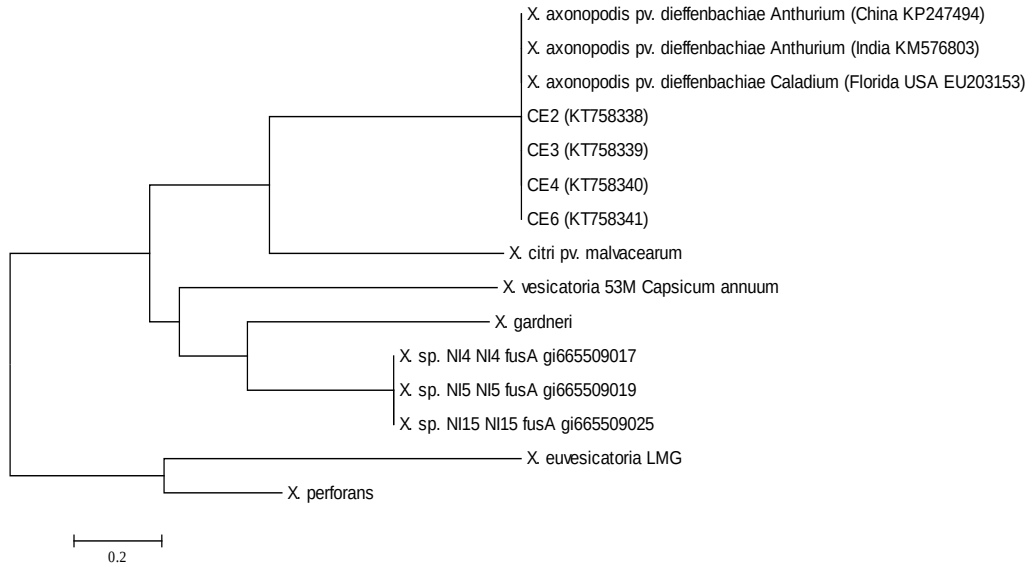

Figure 4: Phylogenetic tree showing relatedness of gene sequences of *Xanthomonas* isolates from Northern Nigeria to other *Xanthomonas* spp. CE2 = Tudun Sarki isolate, CE3 = Jaja isolate, CE4 = Yelwan Paki isolate, CE6 = Hunkuyi isolate.

Gel electrophoresis plates showed that DNA of all isolates were amplified with 16S rRNA primers used. BLAST results for the isolates show 98-100 % genetic relatedness to *Xanthomonas axonopodis* pv. *dieffenbachiae* strains in the Genbank. On the phylogenetic trees, it is observed that all the strains form same clade with *XAD*reference strains KP247494, KM576803 and EU203153. This result re-affirms that isolates from Northern Nigeria are *Xanthomonas axonopodis* pv. *dieffenbachiae.* 16S rDNA profiling used, though reported by Vauterin *et al*. [21] to be inconsistent was able to distinguish the XAD as evidence from the PCR results and phylogenic tree in this work.

CONCLUSION

Bacterial leaf blight of cocoyam occurred in Kaduna and Kano States and the disease is more prevalent in Kaduna State. The nucleotide sequences of the isolates have been deposited in the Genbank (Accession numbers: KT758338.1, KT758339.1, KT758340.1 and KT758341.1) and can be accessed on http://www.ncbi.nlm.nih.gov/nuccore. The sequences represent the first submission of *Xanthomonas axonopodis* pv. *dieffenbachiae* (causal organism of bacterial leaf

blight of cocoyam) on any plant in West Africa and on cocoyam in Africa.

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