MOLECULAR DETECTION OF *DIOSCOREA ALATA BACILLIFORM* VIRUS (DABV) ON YAM LEAF SAMPLES AND MEALY BUGS ON YAM FIELDS IN NORTH CENTRAL NIGERIA

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SUMMARY

A survey was conducted in August 2017, in five States of North Central Nigeria, namely; Benue, Kwara, Kogi, Niger and Nassarawa States, to determine the presence of *Dioscorea alata bacilliform* Virus (DBaV) from yam leaf and mealy bug insect samples collected from yam fields in the study areas. Polymerase Chain Reaction (PCR) of DaBV was conducted on both the yam leaf samples and mealy bugs insect collected. PCR amplified products were analysed using sequence and Phylogeny. The Result showed that all the leaf samples tested positive for DaBV. While Sixty-one percent (61%) of the mealy bug samples tested positive for DaBV. Group one of the sequences showed 99% nucleotide identity to themselves while another group showed 88% nucleotide identity. These two (2) groups of sequences were closely related to *Planococcus ficus* and *P. minor*. Two of the sequences (BRK1 and K1) were clustered in the same group with *Rastrococcus invadens* and *P. marginatus* respectively. The two new sequences (ZM1 and GB1) appeared to be an out group of the *Planococcus* species and shared 99% nucleotide identity to themselves. The findings of this study proved that, there is the presence of DaBV in the study areas. Therefore, continue monitoring of mealy bug populations is also required, so as to reduce or maintain its population in the surveyed areas. Further studies should

be carried out to characterize the viral diversity by sequencing for thorough understanding of more existing virus strains and other mealy bug species that are likely infecting yam in the study areas; this is for increase yam productivity in the surveyed areas.

Keywords: *Dioscorea alata bacilliform* Virus, Polymerase Chain Reaction, Electrophoresis, Mealy bugs, Sequences

YAM (*Dioscorea species*) belongs to the family *Dioscoreaceae* in the genus *Dioscorea*. The genus consists of about 600 species of which 60 are cultivated for food or pharmaceutical purposes (18). White yam (*Dioscorea rotundata*), yellow yam (*Dioscorea cayenensis*) and water yam (*Dioscorea alata*) are among the edible and economically important yam species in the world (21). Water yam (*Dioscorea alata)* is the most widely distributed species globally while; white yam (*Dioscorea rotundata*) is the most preferred (13).Yam is the second most important food tuber crop in the world after cassava, in terms of production (8), and as a major staple food, which plays an important role in food security for the growing population of sub-Saharan Africa (SSA) (16; 5). Yam provides up to 500 kcal per day for millions of people and contributes to the income generation of smallholders in major yam producing areas of SSA (20).

Nigeria is the highest world producer of yam with more than 45.004 million metric tonnes (mmt) annually with a value equivalent to US$5.7 billion (7), this is contributing significantly to 40% of Nigeria's GDP which comes from Agricultural sector (15). Yam plays significant roles in the socio-cultural activities of Sub-Saharan Africa, especially in North central Part of Nigeria and is cultivated for food or pharmaceutical purposes (14; 6), Yam contributes more than 200 dietary calories per day for over 60 million people in Nigeria (16), it may be barbecued, roasted, fried in oil, grilled, boiled, baked, smoke, pounded into paste (fufu) or grated and made into a dessert. It may be cooked or fried with rice, beans, plantain, sweet potato, lamb, and chicken and butter nut as squash soup. It can be boiled, roasted and eaten with oil, vegetable or sauce. The tubers may be peeled and sliced into tiny pieces and dried to very low moisture contents and milled into yam flour and flakes. (16) Diseases caused by viruses, including members of the genus *Badnavirus* are seriously affecting yam production in Nigeria (1). This group of viruses exhibits high genetic as well as serological heterogeneity which has presented a

big challenge for developing reliable diagnostic tools for their detection (9; 7). Therefore, continuous detection of these viruses will help to determine their diversity for reliable diagnosis. Viruses are a major threat to sustainable yam production in Sub Saharan Africa (SSA) and are an impediment to the safe movement of clean planting materials (7). Estimates indicated that more than 30% of yams in SSA are lost due to viral diseases, resulting in annual losses of more than US$1.25 billion (10). These viruses are spread by sap-feeding mealy bugs *(Pseudococcidae)* and through perpetuation of virus infected planting materials by farmers (2). The sap-feeding insects of the family *Pseudococcidae* contain over 2, 200 species and many are active vectors of *Dioscorea alata bacilliform* Virus (DaBV) (18). Presently, there is no existing data on mealy bug species that are feeding on yam in north central Nigeria, although earlier researchers reported that in south western part of Nigeria, *Planococcus citri* can acquire and transmit *Dioscorea alata bacilliform* Virus (DaBV) between diseased and healthy yam plants (17). Furthermore, several mealy bug species were reported as vectors of so many members of the genus *Badnavirus* in different Agricultural crop products which include *Planococcus citri* (Risso or citrus mealy bug), *Saccharicoccus sacchari* (Cockerel or pink sugarcane mealy bug), *P. kenyae* (Lepelley or coffee mealy bug), *P. njalensis* (Laing or cacao mealy bug), and *Dysmicoccus boninsis* (Kuwana or gray sugarcane mealy bug) (8). The present research work provides the relevant information on the status of *Dioscorea alata bacilliform* virus (DaBV) and the mealy bug insects that are found in yam fields which serve as the potential vectors of DaBV in the study areas.

MATERIALS AND METHODS

The survey study was conducted in August, 2017 covering major yam producing regions of North Central Nigeria, including; Benue, Kogi, Kwara, Nassarawa and Niger State.

Yam Leaf Samples Collection

Leaf samples were collected based on criteria of disease severity ranking scale in which 1= symptomless, 2=mild, 3= moderate, 4 severe and 5= very severe. The samples were randomly collected from farmer's fields. A total of 200 yam leaf samples were collected from the surveyed areas; in a quantity of 45, 35, 40, 40, and 40 each from Benue, kwara, Kogi, Nassarawa and Niger States from a total number of 26 locations including 7,4,5,5, and 5 each from

the above-mentioned states. The samples were placed in Eppendorf tubes containing Cetyl-trimethyl ammonium bromide (CTAB) buffer and kept in a room temperature in the field and in the – 20 degrees refrigerator in the molecular laboratory of KSUSTA before DNA extractions, polymerase chain reaction (PCR), and gel electrophoresis.

Nucleic Acid (DNA) Extraction from Yam Leaf Samples

Total nucleic acid was extracted from all yam leaf samples using an adapted CTAB protocol (12) as follows: The yam leaf tissues (~100 mg per sample) were placed in individual polythene bags (10x15 cm Polybags, UK), followed by the addition of 1 ml extraction buffer (2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl) containing freshly added 2% (w/v) polyvinylpyrrolidone-40 (PVP-40) and 1% (w/v) sodium sulphite (Na_2SO_3). The leaf materials were then ground using a small hand roller. For each sample, crude extract (700 µl) was transferred into 1.5 ml micro centrifuge tubes, vortexed and incubated in a water bath (60^{o}C, 30 min) for cell lysis. The extract was then first clarified by the addition of 700 µl chloroform: isoamyl alcohol 24:1 (v/v), vortexed and centrifuged (13000 rpm, 10 min) in a micro centrifuge tube. The aqueous layer was removed carefully and transferred into a fresh micro centrifuge tube. A second clarification step using chloroform: isoamyl alcohol 24:1 (v/v) was performed as above. The aqueous layer was transferred carefully into a fresh micro centrifuge tube and then mixed well with 75 µl of 5 M NaCl and 450 µl of cold isopropanol. After incubation (-20^{o}C, 1 h), the mixture was centrifuged (4^{o}C, 13000rpm, 10 min) to pellet the nucleic acids. The supernatant was decanted off, and the pellet was washed twice by the addition of 500 µl of 70% (v/v) ethanol, and then recentrifuged (13,000rpm, 5 min). The ethanol was carefully decanted off and nucleic acid pellets then dried using a DNA vacuum drier (Thermo Scientific, UK). The dried pellets were re-suspended in 100 µl 1x TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at -20^{o}C until use. The extracted DNA samples were diluted 1:10 (v/v) in nuclease-free sterile distilled deionised water (SDW, Thermo Scientific, UK) prior to PCR.

PCR using primers set as Badna-forward (FP) 5′ATGCCITTYGGIITIAAR AAYGCICC3′and Badna-reverse

primer (RP) 5′CCAYTTRCAIACISCICCCCAIC C3′.

Mealy bug Samples Collection and Nucleic Acid Extraction

A total of 76 mealy bug samples were collected from the surveyed areas, in a ratio of 16,15,15,15, and 15 each from Benue, Kwara, Kogi, Nassarawa and Niger States, and the total DNAs were extracted from 76 mealy bugs samples using a non-destructive DNA extraction method with a Blood and Tissue Qiagen kit, following the manufacturer's protocol. In which Individual mealy bug were placed in 1.5-ml tube and 180-µl of ALT buffer was added, followed by the addition of 20-µl of proteinase K and incubated at 56°C overnight for cell lyses. Then 200-µl of AL buffer added and then incubated at 56°C overnight for 10 mins. 200 µl of ethanol was added, after then the mixture was then transferred in to column and the centrifuged 8000 rpm for 1 min. 500 µl of AW1 buffer was then added and the centrifuged 8000 rpm for 1 min, same quantity of AW2 buffer was added and centrifuged 14000 rpm for 3 mins, 30 µl of AE buffer was then added and centrifuged 8000 rpm for 1 min, this step was then repeated to obtain very qualitative DNAs but in this step 20-µl elution buffer was added.

PCR and Gel Electrophoresis of Mealy bug DNA

A 649-bp segment of the barcode region was amplified from each mealy bug sample using primer set Badna-forward (FP) 5′ATGCCITTYGGIITIAAR AAYGCICC3′and Badna-reverse primer (RP)5′CCAYTTRCAIACISCICCCC AICC3′ PCR thermos-cycling was done under the following conditions: Initial and Final Denaturation for 2 mins at 95°C and 94°C; Annealing for 30s, at 52°C, and Initial and final extension for 30s, at 72°C; 60s, 72°C; 5 min at 72°C; held at 4°C for about 30 cycles. PCR products were visualized in 1.5 % agarose gel stained with red safe. The gel was prepared by dissolving 1.5 grams of agarose powder in 100 ml of 0.5× TBE buffer (0.045 M Tris-borate, 0.5 mM EDTA, pH 8). The agarose-buffer solution was heated in a microwave oven for 3 minutes and was allowed to cool to 40 ^{o}C before pouring into a gel tray that was fitted with a gel comb. The gel was allowed to solidify for 20 mins before loading samples. 10 µl of the sample was loaded into separate wells on the gel, 5 µl of 5× orange G loading dye. 5 µl DNA markers (100 to 1000 base pair) were loaded into each end slots of the gel. Electrophoresis was performed at

80V for 1 h. The gel was observed under UV light. Analysis was done using PCR techniques, in which PCR amplified products were analyzed using sequence and phylogeny techniques.

RESULTS

PCR of *Dioscorea alata bacilliform* virus (DaBV) Extracted DNA

All symptomatic leaf samples showed positive bands of approximately 579 base pair (bp), indicating the presence or occurrence of DaBV in all the surveyed areas. The bands were graded from one plus (+) to three plus (+++) based on the intensity of the bands in 1.5% agarose gel electrophoresis, 64% showed weak bands and were scored one plus (+). The remaining 36% samples showed stronger bands and were scored three plus (+++). (Plate 1)

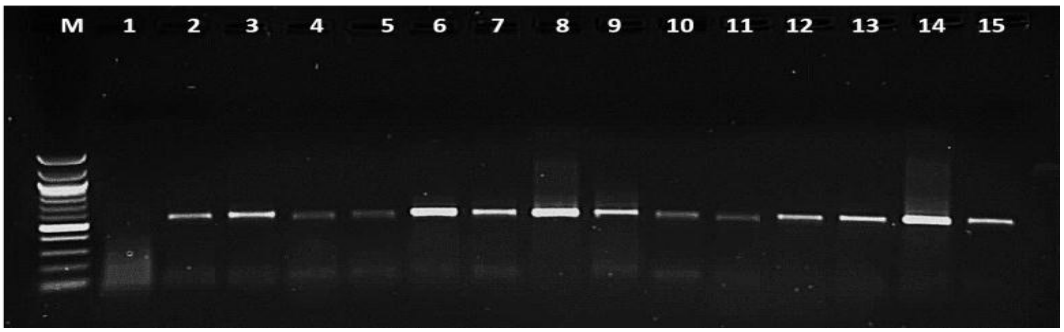

Plate 1: PCR Detection of DaBV sequences by PCR using Badna FP and Badna-RP primers. Lane M = 100bp ladder, Lane 1 = negative control (distilled water), Lane 2 = positive control and Lanes 3-15 = representative of the surveyed samples.

PCR and the Sequences of Mealy bugs Extracted DNA

PCR of 76 tested samples indicated that 56 samples showed positive bands of approximately A649 base pairs (bp). The total positive samples obtained indicated that 61% of the tested mealy bugs were able to acquire DaBV. The bands were graded from one plus (+) to three plus (+++) based on the intensity of the bands in 1.5% agarose gel electrophoresis. (Plate 2)

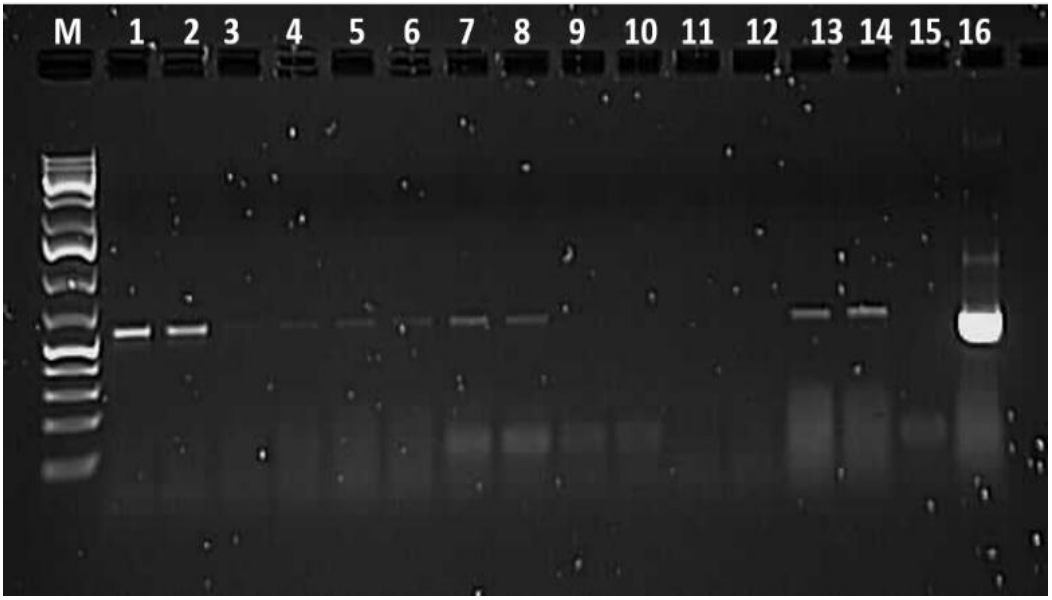

Plate 2: PCR of Mealy bugs on yam fields. Lane M = 1Kbp plus ladder, Lane 1-14 = representative of the surveyed samples. Sample 1 – 9, 13 and 14 were positive for the DaBV sequence whereas, samples 9 - 12 were negative of DaBV sequences. Lane 15 = negative control and Lane 16 = positive control

The PCR products of mealy bug insect vectors were sequenced and the sequences clustered into four main groups of the family *Pseudococcidae* namely: *Planococcus ficus* and *minor*, *Rastrococcuss invadens*, *P. marginatus* and a putative new group. Group one of the sequences showed 99% nucleotide identity to themselves while another group showed 88% nucleotide identity. These two groups of sequences were closely related to *P. ficus* and *minor*. The two sequences (BRK1 and K1) clustered in the same group with *R. invadens* and *P. marginatus* respectively. The other two new sequences (ZM1 and GB1) appeared to be an out group of the *Planacoccus* species and shared 99% nucleotide identity to themselves (Figure 1)

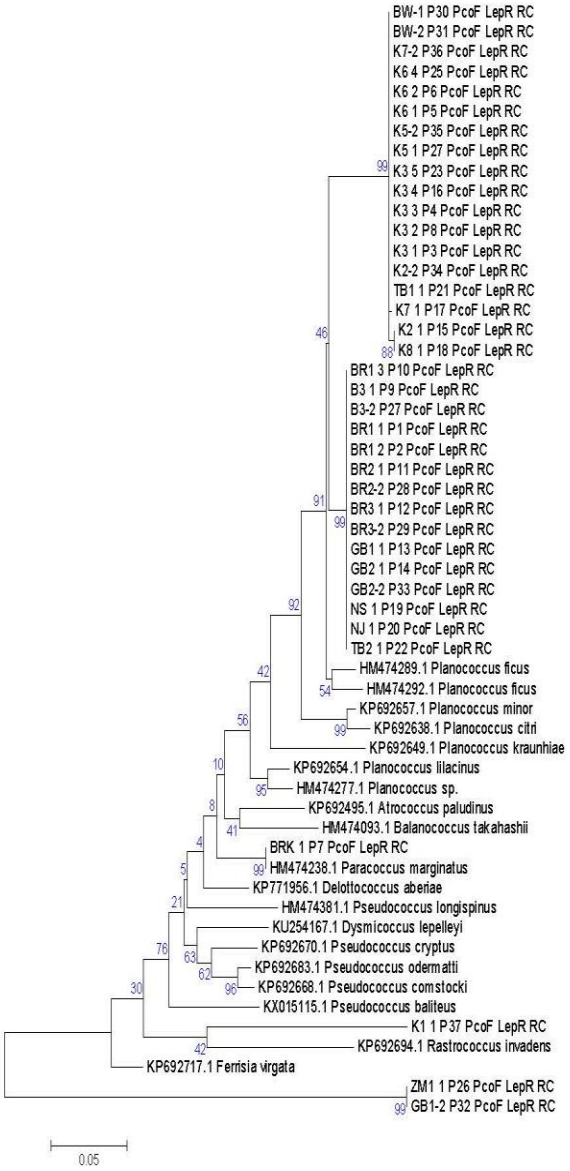

Figure 1: Phylogenetic analysis of CO1 mealybug sequences

DISCUSSION

Total extracted DNAs were screened for the presence of DaBV, all the samples tested positive bands of approximately 579 base pair. This result agrees with those reported by (19) for the detection of *Badnavirus* sequences in different tropical food crops such as Banana, Cacao, Pineapple, Sugarcane, Taro and Yam. Therefore, the analysis of the infected yam leaves sampled confirmed the presence of DaBV in the study areas. Many viruses have been identified in yam fields of sub-Saharan Africa (4, 3) and DaBV is among the most prevalent virus infecting yam cultivation in SSA. Similar result was also reported in Benin (4). PCR of 76 tested samples of mealy bug indicated that 56 samples showed positive bands of approximately 579bp. A segment of 649 of the barcode regions that was amplified from each mealy bug sample signified that mealy bugs are the potential vectors of DaBV in the study areas. This study agrees with the findings of (11), on the ability of Mealy bugs to acquire *Badnavirus* at all stage of their life using a single insect (*Planococcus citri)*. Phylogenetic analysis carried out on the mealy bug sequences amplified proved that DaBV are found in mealy bugs and could be the potential vectors of the former in the study areas. The study agrees with that of (14) which reported the ability of mealy bugs to acquire the *Badnavirus* at all stages of their life cycle.

CONCLUSION

This study proved that, there is the presence of DaBV in the study areas; therefore, there is need for the establishment of more working institutions that would be involved in the distribution of improved and clean planting materials in order to improve the level of yam productivity in the study areas. Further studies should be carried out to characterize the DaBV for understanding of the existing virus strains and other mealy bug species that are infecting yam in the study areas. This finding will help in adoption of management strategies against the DaBV in the study areas through effective control of mealy bug insect's populations, which will result in increase in income generation to the yam farmers in the surveyed states through increase in yam quantity production. Furthermore, result from this study will be of great benefit to plant virologists, entomologists, yam seeds multipliers, seed council and yam growers.

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