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RESEARCH ARTICLE

METAGENOMIC ASSESSMENT OF THE EFFECTS OF GENETICALLY MODIFIED CROPS ON PHAGES ECOLOGY OF SOIL

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Abstract

Genetically modified crops are already successfully grown worldwide in more than 18 countries on more than 67 million hectares which increases annually by more than 10%. Nigeria, in October 2018 joined the many other countries by approving Bacillus thuringiensis (Bt) cotton and maize, therefore, there was the need to carryout environmental risk assessment studies. A total of fifteen (15) four litter (4L) octagonal ceramic pots were filled with four kilograms (4Kg) of soil and placed on bench in two rows of ten pots each and a third row of five pots. First row pots were used to plant GM cotton seeds, while the second row pots were used for non GM cotton seeds and a third row of five pots served as control, all in the screen house. The GM cotton seeds were collected from National Biosafety Management Agency, Abuja while the non GM cotton seeds were collected from seed bank of Tissue Culture Unit of NABDA. Soil samples for metagenomic DNA extraction were collected at random and at monthly interval after planting at a distance of 2mm to 5mm from plant's root and at a depth of 5cm to 10cm using sterile spatula. The DNA was extracted using Zymobiomic soil DNA extraction kit, nano drop technique and gel electrophoresis were used to confirm the DNA before sequencing. Sample 1A (DNA from GM cotton Soil at first interval) gave the lowest sequence read with 0.853M while sample 2B (DNA from GM cotton Soil at second interval) gave the highest with 5.785M, others gave between 1.8M and 4.7M. The samples treatment were grouped into four, Group 1 (GM cotton soil from 1 to 3 intervals), Group 2 (non GM cotton soil from 1 to 3 intervals), Group 3 (control soil) and Group 4 (initial soil). The microbes observed were predominantly bacteria (including archaea), fungi, dark matter alongside protists and phages with focus on the phages community. The predominant phages were, Acinetobacter virus, Bacillus virus SPbeta, Hpunavirus_u_s, Staphylococcus phage, Paenibacillus phage, Enterobacteria phage and Stenotrophomonas phage. The comparative analysis between groups was done using JACCARD PERMANOVA beta diversity analysis at P – value not more than 0.68 and there was no significant pair found. The results suggest that, the GM crops have no significant effect on phage ecology of the soil and in turn no direct or indirect effects on human health.

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Introduction:-

Genetically modified (GM) crops/plants are already successful phenomenally; Eighteen countries worldwide grow GM crops on more than 67 million hectares (ha) and this amount increases by more than 10% annually (James, 2010; James, 2011; Gruissem, 2015). This remarkable growth occurred over 2 decades ago, when the Flavr Savr tomato being the first transgenic crop became available to farmers (Raman, 2017; Kamle et al., 2017), seven such crops are being grown currently – Cotton (Bromoxynil resistance), Canola (increased oil production), Maize, Papaya, potato, soya bean (Glyphosate resistance) and squash, with the world most bioengineered hectare being cotton (7 million ha), maize (10 million ha) and soyabean (33 million ha) (Bawa and Anilakumar, 2013; Raman, 2017). In 2009, more than 134 million hectares were cultivated with biotech crops in 25 different countries all over the world with prediction of further increase in the near future, especially in developing countries such as those in Africa (James, 2011).

Since they were commercialized, GM crops have been beneficial both environmentally and economically thereby increasing global food crop yield by >370 million tonnes over a relatively small acreage area (Raman, 2017).Two agronomic traits accounts for virtually all planted hectares - resistance to herbicides and resistance to insect pests (Huang et al, 2003; James, 2010; Brookes and Barfoot, 2015; Kamle et al., 2017).Currently, the GM crop pipeline has expanded to cover other fruits, vegetables and cereals such as lettuce, strawberries, eggplant, sugarcane, rice, wheat, carrot, etc, with planned uses to increase bioproduction of vaccine, animal feed nutrients as well as present salinity and drought resistant traits for plant growth in unfavourable environmental and climatic conditions (Bawa and Anilakumar, 2013; Zhang et al., 2016; Raman, 2017). GM crops are modified using recombinant DNA technology in three (3) different ways, which are; transgenic, cisgenic and intragenic. Transgenic which involves the insertion of foreign DNA from unrelated species or genus as seen in cotton, Cisgenic which involves insertion of one or more gene from related species or crossable donor as seen in potato and Intragenic which involves the use of genetic elements from other plant's sexually compatible gene pool which are then combined with promoters and terminators (Kamle et al., 2017).

GM crops are expected to be widely adopted for their great potential in agriculture (Huang et al., 2003; Kumar et al, 2008; Mocali, 2010), but since the release of GM crops there has been a great controversy over the unexpected potential effects on the environment and human health(Thompson, 1998; Bownas, 2008; Mocali, 2010). After all, with GM technology, traits can be obtained that were previously not present in crops, these new traits may have direct or indirect effect on the environment due to different methods of cultivating the new crops (Keese, 2008; Sanvido et al., 2012). Furthermore, the rapid development of agricultural biotechnology and the release of new transgenic varieties have made ecological risk assessment of GM crops on the environment extremely important and also an urgent task (Ammann, 2005; Mocali, 2010).

Soil microorganisms play a vital role in the biogeochemical cycling of organic matters and nutrients which is mainly caused by organic matter decomposition in the soil that sustains agricultural ecosystem (Tian et al., 2020). The plant grows in association with the bacterial community that thrives in the soil around the surface and/or inside their roots closely (Hiraoka et al., 2016; Perez-cobas et al., 2020; Tian et al., 2020). Most plants share symbiotic relationship with soil microorganisms especially fungi and bacteria during their growth and development among which bacteria are the most abundant and are distributed widely in the soil, while the phages share a parasitic relationship with other microbes (Perez-cobas et al., 2020; Tian et al., 2020). Plants are particularly known to define the composition of their rhizobiome, therefore, structure and composition of soil microbe's alteration is reflected in soil quality deterioration and thus plant health (Hiraoka et al., 2016; Perez-cobas et al., 2020; Tian et al., 2020). The rhizosphere is defined as the zone around the root where microorganisms and processes important for plant growth and health are located (Tian et al., 2020). Distribution of crops and morphology of root affect the structure of the soil microbiome while the abundance is directly affected by root exudates and the growth pattern and quality of the plant reflects indirectly the soil quality. Hence, it is of great importance to investigate the effect of GM crops on the structure and diversity of soil microbiome (Hiraoka et al., 2016; Perez-cobas et al., 2020; Tian et al., 2020).

Metagenomics is the study of microorganisms in their natural environment involving the complex microbial communities in which they exist normally, it examines the genomic make up of an entire organism including the various microorganisms that are present within it (Coughlan et al., 2015; Tian et al., 2020). Metagenomic DNA extraction and sequencing can be used to obtain detailed information about the structure, function and diversity of soil microbiome as well as for the in-depth assessment of the changes in the soil microbiome over time (Hiraoka et al., 2016; Perez-cobas et al., 2020; Tian et al., 2020).

Several studies have been carried out in order to evaluate the potential unintended effects of genetically modified plants on the environment and non-target organisms (Bruinsma et al., 2003; Saxena et al, 2004; Icoz and Stotzky, 2008). Although the issue is still controversial in many countries, especially in Europe where there is a level of continuing debate and public concerns (Drobnik, 2008), insect-resistant varieties including the 'stacked crops' with multiple traits occupied around 36% of the biotech area in 2009 (James, 2015). GM crops is only allowed in the field after undergoing an in depth environmental risk assessment among which is whether the GM crops have a different effect from the non GM crop on the soil microbiome, insects and neighboring plants of which have been the subject of scientific study for over 30 years (Nicolia et al., 2014). However, there are still concerns relating to this potential unknown effects of GM crops on the environment and a well defined risk assessment is still required (Wolfenbarger and Phifer, 2000; Bruinsma et al, 2003; Liu, 2009).

In Nigeria, some GM crops have been approved after series of field trials in October, 2018 which are Bt cotton and Bt maize though not yet released for commercial purpose as at the time of this research.

Literature Review:-

Microbes in Soil Cultivated with Genetically Modified Crops

Soil microorganisms play a vital role in the biogeochemical cycling of organic matters and nutrients which is mainly caused by organic matter decomposition in the soil that sustains agricultural ecosystem (Tian et al., 2020). The plant grows in association with the microbial community that thrives in the soil around the surface and/or inside their roots closely (Hiraoka et al., 2016; Perez-cobas et al., 2020; Tian et al., 2020).

Several years of studies on genetically modified crops grown on soil has shown that there is no significant effect on the rhizosphere functional microbial population as well as on the numbers of culturable bacteria, actinomycetes and fungi (Tarafdar et al., 2012; Swilla et al., 2016; Velmourougane and Blaise, 2017). However, some researchers observed minor to significant effects of Cry proteins and Bt crops on soil microbial community structure stating that Cry proteins may serve as substrate for the soil microbes, therefore, causing minor increase in the populations of the microbes which includes, bacteria such as Arthrobacter, Sphingomonas (especially in GM cotton), Azospirilum, Bacillus and Pseudomonas, actinomycetes and fungi such as Fusarium, Aspergillus, Rhizopus and Alternarica (Pindi and Sultana, 2013; Lehman et al., 2015; Gunal et al., 2015; Swilla et al., 2016; Velmourougane and Blaise, 2017; Winsome et al., 2017).

Environmental Benefits of Bt cotton

Substantially, Bt cotton can reduce the amount of sprayings of pesticides which in turn can provide benefits to the environment significantly (Raman, 2017). A number of studies have shown that spraying of insecticides are reduced by the use of Bt cotton (Edge et al, 2001; Carpenter et al., 2002; Purcell and Perlak, 2004)). Bt cotton farmers in the United States reduced use of insecticide by 1,870,000 pounds of active ingredient (AI) per year in 2001 (Gianssi et al., 2002; Purcell and Perlak, 2004). In China, application of insecticides were reduced by an average of 67% and the Kilograms of active ingredients by 80% (Huang et al., 2003, Purcell and Perlak, 2004), while in South Africa, farmers reduced insecticide sprays by 66% (Ismael et al., 2002a; Purcell and Perlak, 2004). The use of Bt cotton in place of conventional cotton systems can positively impact non-target organisms (NTOs) and beneficial organisms by preserving populations (Purcell and Perlak, 2004) and is compatible with the integrated pest management initiatives (Benedict and Altman, 2001).

In addition, the adoption of Bt cotton can provide secondary positive environmental impacts such as; Saving on raw materials needed to manufacture chemical insecticides;

Conserving fuel oil required to manufacture, distribute and apply such insecticides and
Eliminating the need to use and dispose off insecticide containers (Leonard and Smith, 2001; Purcell and Perlak, 2004).

Bt Crops and Soil

There are several considerations on how Bt crops could affect soil microbes; they include both direct and indirect effects (Icoz and Stotzky, 2008; Mocali, 2010). The direct effects depend on the impacts of the DNA or proteins released from the modified crops on soil microflora. In contrast, indirect effects are mediated by changes in plant tissues and root exudates composition that could determine alterations of soil organic matter and microbial diversity with unpredictable consequences on soil quality and sustainability. Microbial communities represent more than 80% of the total soil biomass, excluding plant roots (Kowalchuk et al., 2003; Mocali, 2010), and perform many essential functions in the soil system such as organic matter decomposition and humification, redox reactions, Nitrogen fixation and solubilization, nutrient mineralization and immobilization (Nannipieri et al., 2003; Mocali, 2010; Tian et al., 2020). Therefore, any change in microbial functional or genetic diversity could lead to unknown consequences for the soil ecosystem (Lynch et al., 2004; Mocali, 2010; Tian et al., 2020).

It is a fact that the plant root exudates have a direct interaction with soil microbes in the rhizosphere (Kent and Triplett, 2002; Kowalchuk et al., 2003; Blais et al., 2006; Mocali, 2010; Tian et al., 2020) and that the rhizosphere is a critical spot for the interaction between Bt crops and soil Microbes. Unintended changes in the Microbiome cannot be excluded and should therefore be assessed (Mocali, 2010; Tian et al., 2020).

Metagenomics and Research Technigues

Metagenomics is the study of microorganisms in their natural environment involving the complex microbial communities in which they exist normally (Coughlan et al., 2015; Stewart et al., 2018, Tian et al., 2020), it examines the genomic make up of an entire organism including the various microorganisms that are present within it. Metagenomic approaches are increasingly becoming popular in large scale applications of genomics as a tool to studying functional and taxonomic composition of microbial communities from clinical, agricultural and environmental origin (Coughlan et al., 2015; Stewart et al., 2018, Tian et al., 2020; Perez-cobas et al., 2020), metagenomics unlike traditional single-genome approaches does not rely on singularizing individual bacterial clones from complex microbial community but catalogs at once by sequencing all genes and genomes from a mixed community (Hiraoka et al., 2016; Stewart et al., 2018, Tian et al., 2020; Perez-Cobas et al., 2020).

In metagenomics, whole genome DNA is extracted from samples irrespective of its microbial composition and it is characterized by whole genome sequencing. Sophisticated bioinformatic tools are then used to assign resulting DNA fragments, individual reads or assembled sequence contigs to individual taxonomic groups or known genome sequences (Stewart et al., 2018, Tian et al., 2020; Perez-Cobas et al., 2020). Metagenomics is aimed at making advancements in clinical and environmental microbiology, irrespective of the significant barriers such as the genomic diversity of microorganisms and difficulty in culture making (Coughlan et al., 2015; Tian et al., 2020), it is expected that increased understanding of microbes and their nature in the environment will have a significant impact on research areas of other sciences such as Ecology, Biotechnology, Medicine and Biology (Coughlan et al., 2015; Hiraoka et al., 2016; Stewart et al., 2018, Tian et al., 2020; Perez-Cobas et al., 2020).

Materials and Methods:-

Study Area

The study was carried out at Genetically Modified Crop Screen House of Plant Improvement Unit, Agricultural Biotechnology Department, National Biotechnology Development Agency (NABDA), Abuja, the capital and eight most populous city of Nigeria with estimated population of 1,235,880 in 2011 and Federal Capital City and Municipality area of 1,769km^2 on $9^0 4^1 0^{11}$N $7^0 29^1 0^{11}$E coordinates (Lat. 9.08^0 and Long. 7.49^0), the indigenous inhabitants are the Gbagyis and their major occupation is farming. Abuja is bordered by the states of Niger to the west and northwest, Kaduna to the northeast, Nassarawa to the east and south and Kogi to the southwest.

Collection and Processing of Samples

The genetically modified (GM) cotton seeds MRC 7361 BG11 were obtained from National Bioseafety Management Agency (NBMA), Abuja, Nigeria as it has not been commercialized as at the time of this research while the non GM cotton seeds were obtained from the seed bank of Tissue Culture Unit of NABDA.

The seeds were planted (GM and Non-GM cotton) in twenty (20) four litter (4L) octagonal pots (one seed per pot) without added manure in the screen house due to the sensitivity of the GM cotton seeds. The pots were filled with four kilograms (4kg) of soil dug from the Tissue Culture Laboratory Unit of National Biotechnology

Development Agency (NABDA), Abuja soil deposit and placed on bench in two rows of ten pots each and a third row of five pots (total of twenty-five pots), first row pots were used to plant GM cotton seeds, while the second row pots were used for non GM cotton seeds and a third row of five pots served as control, all in the screen house.

Soil samples were collected at random and at 1 month interval after planting at a distance of 2mm to 5mm from the roots of the plants (rhizosphere) and at a depth of 5cm to 10cm using a sterile spatula (the spatula was sterilized with 90% ethanol and damped with sterile paper after each sample collection) then sealed in a sterile falcon tube and taken to the tissue culture laboratory of National Biotechnology Development Agency (NABDA), Abuja where the metagenomic DNA extraction was immediately carried out (Amorim et al., 2008; Tarafdar et al., 2012).

Metagenomic Analysis

The Metagenomic DNA extraction of the soil samples from the GM cotton, Non-GM Cotton and initial soil (in the screen house) which served as control was done by weighing 2g of each soil sample from the falcon tubes after which the Zymo biomics (ZB) soil DNA extraction kit protocols were followed strictly. Nano drop and electrophoresis techniques were then used to quantify and ascertain the extracted DNA samples.

Sequencing of DNA samples

Whole genome shortgun sequencing was used to fragment all the microbial DNA in the samples into small pieces for next generation sequencing, the algorithms then identified phages based on the entire genomes of the microorganisms that are in the database. The taxonomic identification number obtained was linked to the NCBI taxonomic ID for the organism's name, abundance score (makes the metric suitable for downstream comparative analysis), relative abundance, unique matches and the frequency (Mardis, 2008; Metzker, 2010). This was done at CosmosID INC., USA.

Statistical Analysis

The bioinformatics for sequenced samples was done using JACCARD PERMANOVA's beta diversity analysis so as to compare the samples and ascertain either or not significant differences existed in the phages composition between samples.

Results and Discussions:-

Bioinformatics and Statistical Analysis

Bioinformatics

DNA samples were analysed using Whole Genome Sequencing (Tab. 1). Samples 1A and 3E gave lower reads of 0.853M and 0.912M respectively and samples 1C, 1D, 1E, 1F, 2A, 2D, 2E and 3C gave average reads but lower than the

Tab. 1:- Whole Genome Sequence (WGS) reads of DNA samples.

Sample name	Analysis type	Reads(M)
1A	**WGS**	**0.853**
1C	**WGS**	**1.867**
1D	**WGS**	**1.363**
1E	**WGS**	**1.377**
1F	**WGS**	**2.049**
2A	**WGS**	**2.672**
2B	**WGS**	**5.785**
2C	**WGS**	**4.169**
2D	**WGS**	**2.373**
2E	**WGS**	**2.480**
2F	**WGS**	**3.619**
3A	**WGS**	**3.124**
3B	**WGS**	**4.759**
3C	**WGS**	**2.465**
3E	**WGS**	**0.912**
G	**WGS**	**4.401**

H	**WGS**	**3.740**

average required read of 3M while samples 2B, 2C, 2F, 3A, 3B, G and H gave reads above the average required value with sample 2B giving the highest read of 5.785M.

ABBREVIATIONS	**INTERPRETATION**
A and B	**GM cotton soil**
C and D	**NON GM cotton soil**
E and F	**Control soil**
G and H	**Initial soil**
1, 2, 3 and 4	**Monthly intervals**

Bar chart representation of phages community present in the samples

This bar chart representation shows the phages community present in the DNA samples and the rate at which they occur (fig. 1).

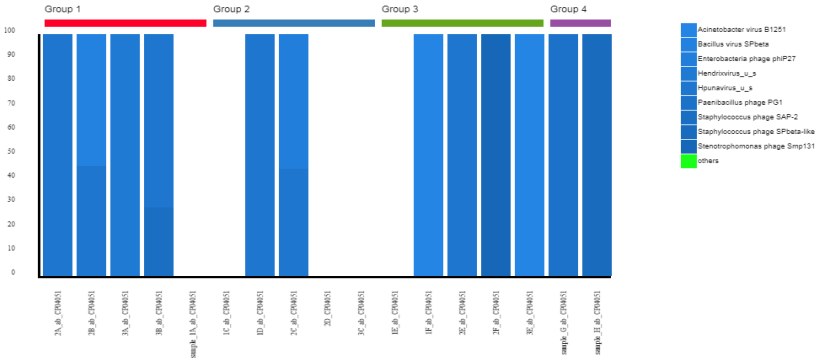

Fig. 1:- Bar chart for Phages present in the DNA samples.

KEY

Group 1 – GM cotton soil samples
Group 2 – NON GM cotton soil samples
Group 3 –Control soil samples
Group 4 – Initial soil samples

Principal component analysis for Phages

The Principal Component Analysis was used to reduce the number of variables of data set for easy visualization and understanding. Figure 2 below shows there is close association between the phages community present in groups 3 and 4 and within group 1.

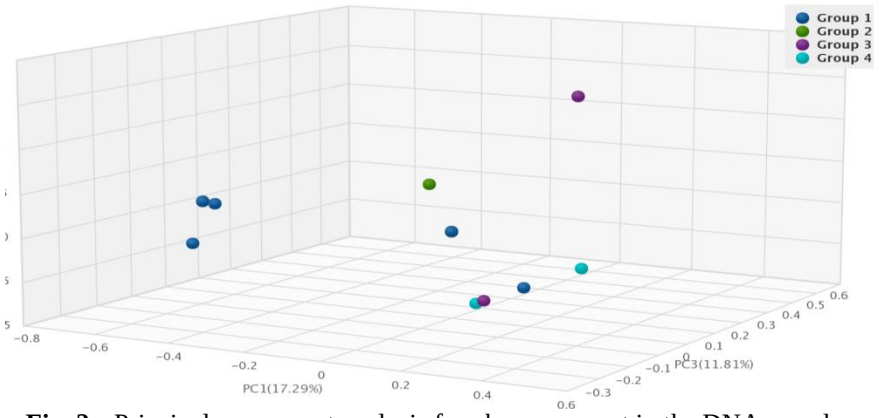

Fig. 2:- Principal component analysis for phages present in the DNA samples.

Principal coordinate analysis for Phages

The Principal Coordinate Analysis shows the map-based visualization of the distances between the sample groups. Figure 3 shows that there are no close association between or within groups based on the species of phages present in the samples.

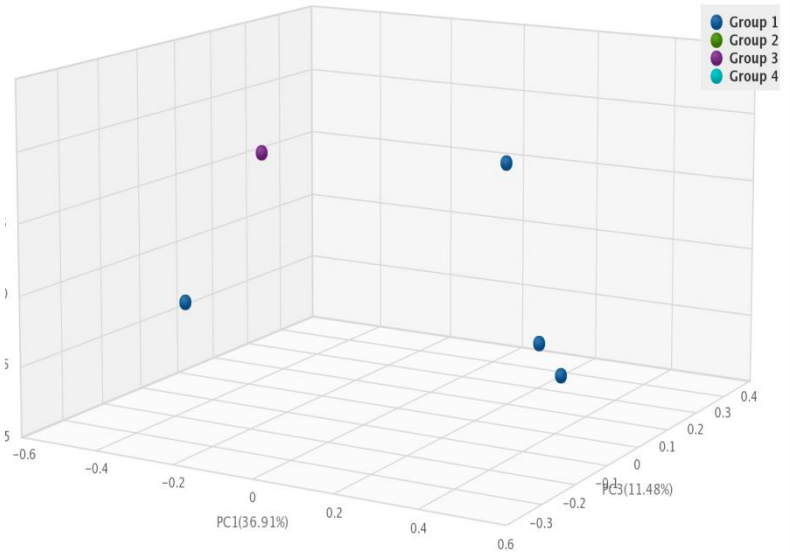

Fig.3:- Principal coordinate analysis for phages present in the DNA samples

Discussion

Crops modified with herbicides tolerance, disease resistance, insect/pest resistance, drought tolerance and salt tolerance genes gives superior agronomic traits and improved product quality (Nalluri and Karri, 2020; Tian et al., 2020). Assessing the effects of GM crops on microbial ecology of soil is important as there may be unexpected potential effects on the environment and human health. After all, with GM technology, traits that were not initially present in crops may now be obtained which may have direct or indirect effects on the environment due to different methods of cultivation (Mocali, 2010; Sanvido et al., 2012; Tian et al., 2020).

Based on the studies conducted at Central Institute for Cotton Research, Nagpur and College of Life Sciences, Shihezi University, Shihezi, it was observed that Bt cotton cultivation does not have effect on the soil biological properties (Velmourougane and Blaise, 2017; Tian et al., 2020). In this study, the effects of GM cotton on phages community of soil was assessed using metagenomic soil DNA extraction process and analysed with Whole genome short gun sequencing technique, the extracted DNA from GM cotton, Non GM cotton, control and initial soil samples at monthly intervals from germination to maturation (a month after planting to the fourth month before harvest) was quantified using nano drop technique and confirmed with Gel electrophoresis successfully. The sequence reads of not less than 0.853M and as high as 5.785M (Tab. 1) further confirmed the quality and purity of the extracted DNA, the microorganisms observed in the samples belong to different groups (database name) which are Dark matter, Phages, Fungi, Protists, Bacteria and a Virus at various hits with major concentration on the phages groups, though they are not numerous but their role in controlling microbial population in any environment cannot be over emphasised. The predominant phages were, Acinetobacter virus, Bacillus virus SPbeta, Hpunavirus_u_s, Staphylococcus phage, Paenibacillus phage, Enterobacteria phage and Stenotrophomonas phage (fig. 1).

The results clearly showed that there were numerous microorganisms in the DNA from the soil samples which may not have been observed in culture-dependent techniques due to the inability of researchers to supply the required growth nutrients but are now detectable by using culture-independent techniques such as metagenomic DNA extraction and whole genome sequencing. According to the JACCARD PERMANOVA Beta diversity's principal coordinate and component analysis at not more than P-value of 0.68 there was no significant difference between the phages (Fig. 2) composition of GM cotton, non GM cotton, control and initial soil sample groups. It can then be deduced that the genetically modified crops had no apparent effect on the phages ecology of soil and thus may not have any adverse effect on the environment and in turn no direct or indirect effect on human health. These assertions are is in agreement with the results of Shahmoradi et al. (2019) and Tian et al. (2020). Similarly, many other previous studies had revealed that the effects of GM crops were minor or not significant on microbial

ecology of rhizosphere soil (Victorov, 2008; Liu, 2009; Velmourougane and Blaise, 2017). However, the effects of GM crops on soil microbial ecology can be direct or indirect (Liu et al., 2005). Direct effect depends on the accumulation of GM protein, that is, GM proteins for pest and disease resistance can lead to the production of chemical substances that are toxic to non – target soil microbes while indirect effects are caused by changes in crop protein and composition of root exudates that arise as a result of modification of the metabolic pathways in the plant's tissue (Tian et al., 2020). Therefore, there is still need to assess the potential effect of GM crops on the soil environment.

Conclusion

Nigeria has joined the list of countries growing GM crops by approving Bt cotton and Bt maize after several field trials in October, 2018. The predominant phages were, Acinetobacter virus, Bacillus virus SPbeta, Hpunavirus_u_s, Staphylococcus phage, Paenibacillus phage, Enterobacteria phage and Stenotrophomonas phage, though not the largest group of microbes but the role they play in controlling the microbial population in any community cannot be overlooked. JACCARD PERMANOVA beta diversity analysis at P – value not more than 0.68 showed there was no significant pair between groups of samples.

Though many other studies suggested that BT plants cause minor changes in the microbial ecology of soil, this research has proved otherwise since there is no apparent effects on the phages community in the soil. This suggests that there are no significant effects on the environment as well as direct or indirect effects on human health.

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