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Unravelling the Endophytic Virome Inhabiting Maize Plant

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Abstract: Endophytes are well-known for their symbiotic interaction with plants and their ability to promote plant growth by producing various metabolites. The most well-studied endophytes are bacteria and fungi. For generations, viruses were misnamed, and their symbiotic associations were ambiguous. Recent advances in omics techniques, particularly next-generation sequencing, have given rise to novel developments in the mutualistic relationships that exist between plants and viruses. Endogenous viruses have received a lot of attention in the animal world, but limited information exists on their functions and importance to plants. Therefore, endophytic viral populations inhabiting the root of a maize plant were assessed in this study for the first time using shotgun metagenomics. Complete DNA was extracted and sequenced using shotgun metagenomics from the maize roots in farming sites where organic fertilization (FZ), inorganic fertilization (CZ), and maize planted with no fertilization (NZ) are being practised in an experimental field. Our results identified 2 orders namely: Caudovirales (67.5%) and Herpesvirales (28.5%) which dominated the FZ site, although they do not show any significant difference ($p > 0.05$) across the sites. At the class level *Microviridae*, *Phycodnaviridae*, *Podoviridae*, *Phycodnaviridae*, and *Poxviridae* dominated the FZ site. *Myoviridae* and *Podoviridae* were more abundant in the CZ site, while only Siphoviridae predominated the inorganic fertiliser site (NZ). Diversity analysis revealed that viral populations were more abundant in organic fertilization (FZ). Taken together, this research adds to our understanding of the symbiotic integration of endophytic viruses with maize plants and that their abundance is affected by farming practices. In addition, their potential can be exploited to solve a variety of agronomic issues.

Keywords: agricultural sustainability; farming practices; endophytes; metagenomics; viruses

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1. Introduction

In the natural environment, plant health relies on an overabundance of interconnection between micro and macro-organisms. Endophytes are the diverse microbial group that lives in plant tissues in a mutualistic approach. It has been scientifically proven that endophytes can benefit their host through the mitigation of various agroclimatic conditions like broad abiotic and biotic stresses. Endophytic microbes are commonly acknowledged for the promotion of the growth of plants by metabolite creation, which enhances soil nutrients [1–3]. The root endophytes are of great significance because of their immediate imminent relationship with soil [4–6]. Various information exists on the interaction between the root of plants and endophytic micro-organisms [7–10]. Nonetheless, some viruses have been reported to be pathogenic in studies involving animals, plants, and humans. This discovery has presented a general negative image of viruses until recently. The introduction of next-generation sequencing and omic tools has helped improve our interest in understanding the symbiotic viral–host associations. The coexistence of viruses with the host plants and genomic association in an asymptomatic approach were accentuated by scientific studies [11,12]. Viral diverseness and symbiotic relationships require more scientific observation because most of them are unknown.

Viruses are ample and important biological individuals on earth. New research found that they are abundant in soil, desert, plant ecosystems, ocean, and the mammalian gut.

The broad misunderstanding linked with viral associations was highlighted by ecological surveys [11,12]. Viral interaction may also be mutualistic, but it cannot be symptomatic at all times. Interconnection between viruses and their hosts is conditional and dynamic in some instances. Symbiogenesis may easily be led by viral symbiosis through the genomic fusion of two individuals; this is where the evolution of new species takes place. The immense plethora of viral sequences in extant genomes can aid coevolution and symbiogenesis [13,14]. They play a crucial role in the diverseness of living things on the earth, including the coevolution of viruses and hosts. Based on studies on the diversity of plant virus biodiversity, viruses discovered in a larger number of plants were found to have an asymptomatic existence [15]. The influence of biotic and abiotic stresses is improved by viruses. The ability to survive under extreme temperatures as well as the disease-suppressive potentials of plants are some of the contributions of viruses to plant health. In Yellowstone National Park, the plant's ability to acclimatize to geothermal sectors with the capability to endure the increased level of temperature was discovered to be correlated with a novel fungus which consequently was affected by the virus [16]. The development of a nitrogen-fixing nodule, which is a situation where the quantity of nitrogen is sufficient in the soil as a means of energy conservation, can be restricted by *White Clover cryptic virus* [12].

The ribosomal RNA gene, which lacks a universal coding sequence, is discovered in all biological life making it difficult to analyse the diverseness of viruses. To unveil the enormous wealth of viral details from dissimilar environmental tests, metagenomic research using shotgun sequencing serves as a promising approach [15,17]. Sequences of viral endophyte were also contracted with the indigenous viral group of the soil to detect the particular relationship or straight transference coming to the root of the plant from the soil. A broad analysis was given to propose the likely useful impact of such a symbiotic relationship. This present study is crucial to discovering the wider expectation of an internal viral relationship. Having a better understanding of the viral interactions with plants and their abundance will help in addressing disease emergence in plants and identifying beneficial integration that might be used to treat a variety of agricultural concerns.

The majority of maize producers in South Africa utilise conventional farming practices and inorganic fertilisers to increase plant production. In addition to having negative environmental consequences, excessive usage of inorganic fertiliser also has negative effects on the seed quality, microbial populations, and increased lodging in the plant [18]. Similarly, chemical fertilisers are not economical and a non-renewable nutrient source for the plant [19]. Examining microbiological sources and organic farming, which have excellent properties for stimulating plant development and productivity, is urgently necessary.

Furthermore, it is uncommon to find a well-organised study on how various agricultural practices affect endophytic virome in maize roots. According to reports, the biggest population of endophytes is found in a plant's roots [20,21], which is why maize roots were chosen in this investigation. To the best of our knowledge, there has not been any research on how agricultural practices affect the composition and variety of endophytic viromes inhabiting the roots of maize plants using the shotgun metagenomic technique.

To acknowledge the enormous wealth of viral details from dissimilar environmental tests, metagenomic research employing shotgun sequencing has presented itself as one of the best approaches [15,17,22,23]. Therefore, we present the first study assessing the community structure of endophytic viromes in the roots of maize plants using the shotgun metagenomic techniques.

2. Materials and Methods

2.1. Seed Sourcing

The drought resistant WE 3127 maize seed used in this experiment was collected from North-West University School Farm, Molelwane, Mafikeng, North West Province, South Africa.

2.2. Root Sampling

Because of the experimental farmland's triangular form, each agricultural site was separated into 3 divisions for sampling purposes. The roots of the ten fresh plants in each division were selected randomly from the farming site and pooled to represent biological replicates with a total of 30 plants for each site. The maize roots were then uprooted for the experimental purpose at the fruiting stage of growth [24]. A total of ninety samples of the plant were assessed, with 3 replicates for the individual sampling site, indicating three regions. The collected samples were stored in ice, and then promptly taken to the laboratory for subsequent analysis.

2.3. Description of the Study Site and Experimental Design

Organic and inorganic experimental fields (approximately 500 acres) had been established for 15 years at the University School in Molelwane, North West of South Africa (25°47′25.24056″ S, 25°37′8.17464″ E). Shrubs and trees dominate this province. The average province temperature varies from 3–21 °C and 17–31 °C during winter and summer, respectively. The annual province's rainfall is about 360 mm. For a long period, the main crops planted at this experimental site were sorghum, maize, and soybean (maize-soybean-sorghum), with sorghum sown in 2019. The physicochemical parameters of the soil samples from the sampling sites were identical (66% silt, 22%, 12% clay, pH 6; 0.15% total N, 0.48% organic C, 101.5 ppm *p*, and 0.962 ppm) (Supplementary Table S1).

The two regimes of fertilization that were employed are organic fertilization (FZ) and inorganic fertilization (NZ) and have been existing for more than fifteen years, along with control with no fertiliser application (CZ). The quantity of the inorganic fertiliser that had been in use is 75 P_2O_5, 75 K_2O, and 150 N in kg ha^{-1}, while the organic fertiliser site had been applying cow manure with a 10,625 kg ha^{-1} dosage for more than 15 years following the international best practices [25], and the last site has never experienced the application of fertiliser. The WE 3127 seeds were planted on 3 sites, respectively, using a farming space measuring up to 10 m × 4 m, and was terminated during the summer of the year 2019. To avoid drought stress, all of the sites were irrigated as needed. Manual weed control was employed.

2.4. Surface Sterilization of Maize Roots

Soil particles that came with the roots of the plants from the experimental field were removed via sieving, the procedure outlined by [26], was employed for surface washing of the new roots. The roots were first immersed in 70% ethanol for 3 min. After which, they were washed for 5 min with a 2.5% sodium hypochlorite solution. They were then washed again with 70% ethanol for 30 s before being washed with distilled water that had been sterilised. To make sure that epiphytes were perfectly taken out and that the sterilization process was successfully done, the washed roots were chopped into little pieces and cultured on a yeast extract-mannitol medium (YEM) [27]. After 72 h, the Petri dishes were incubated at 30 °C and were then inspected for the growth of bacteria. The roots of maize plants from uncontaminated plates were selected for DNA extraction [28,29].

2.5. Extraction of DNA and Shotgun Sequencing

Using a sterilised knife, the maize roots were sliced into minute pieces and macerated using a Qiagen TissueLyser. Qiagen DNeasy Plant Mini Kit (Dusseldorf, Germany) was used to extract completed metagenome DNA from the root of the plant samples. The extracted DNA samples were then sent to the Molecular Research LP in Shallowater, TX, USA, where shotgun metagenomic sequencing was performed. The Nextera DNA Flex kit (Illumina, San Diego, CA, USA) was used to prepare the libraries, and the typical protocol was followed. The Life Technologies Qubit®dsDNA HS Assay Kit was employed for the determination of the actual DNA concentration in all of the samples. After the formation of the library, its final concentration was determined by employing the Qubit®dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the Agilent 2100 Bioanalyzer.

The size of the library ranges from 683 to 877 bp, with an average of 731 bp. Library pooling was done with 0.6 nM ratios and paired-end sequencing was carried out using 300 cycles via the Illumina NovaSeq 6000 equipment.

2.6. Data Analysis

Sequences obtained for each metagenome were uploaded to the metagenomics rapid annotation online server (MG-RAST) [30], where QC of the raw sequences was performed, including the removal of adapter and low-quality reads using the Trimmomatic v 0.33 tool with default parameters [31]. Artificial sequences were removed, ambiguous bases ere filtered, a minimum read size was specified, and length filtering was all part of the quality control process. Following quality control, sequence annotation was performed using BLAT [32], against the M5NR database [33], which allows nonredundant database integration. The SEED database was used to categorise endophytic microbiomes, with characteristics including a 10^{-5} e-value cut-off and at least 60% similarity of the sequence to a subsystem. Sequences that failed that annotation test were not taken into consideration. However, because we focused on endophytic virome, we ignored sequences from bacteria, eukaryotes, archaea, and maize plants. The MG-RAST data normalization option was selected to reduce the impact of experimental error/noise. Each taxon's endophytic virome table was produced, and unclassified sequence reads were retained for statistical analysis. Furthermore, after an independent examination of the nine (9) sequences using MG-RAST, the relative abundance of the taxa in percentages was computed. For statistics, the mean values of the relative abundance of the three replicates for the experimental sites (CZ, FZ, and NZ) were employed. These standard sequences can be obtained in the PRJNA607664 NCBI SRA dataset.

2.7. Statistical Analyses

At the order level, the Shinyheatmap was used to plot a relative abundance graph of endophytic virome communities [34]. The Pielou evenness and Shannon diversity indices for all the sampling sites were analysed using PAST version 3.20 [35], and the indices across the farming sites were compared using the Kruskal–Wallis test. The beta diversity was defined using principal coordinate analysis (PCoA) based on a Kruskal–Wallis matrix, and the differences in community structure were assessed by employing the one-way analysis of similarities (ANOSIM) [35]. How the identified endophytic viral order was distributed among the maize plant fields was presented using Euclidean matrix-based principal component analysis (PCA).

3. Results

3.1. Metagenome Dataset and Quality Control

The sequence readings of samples were CZ (4839895527), FZ (2977205570), and NZ (48270695214), obtained for the three experimental sites. The sequenced reads for CZ were 334,259,767 with an average G + C content of 44%, FZ had 415,505,341 with an average G + C content of 44%, and NZ had 817,699,487 with an average G + C content of 49%, were obtained after quality-control analysis in MG-RAST. Sequences that mapped for identified proteins in the metagenome samples that passed the quality control assessment were 371,329, 325,439 and 643,141 for CZ, FZ, and NZ, respectively (Supplementary Table S2).

3.2. Community Structure and Abundance of Endophytic Virome Inhabiting Maize Root Samples

Two major viral orders identified in this experiment are the *Caudovirales* (67.5%) and *Herpesvirales* (68.5%) and are more abundant in the FZ site samples, although no significant difference ($p > 0.05$) exists across the sites (Figure 1). At the class level, Microviridae, *Phycodnaviridae*, *Podoviridae*, *Phycodnaviridae*, and *Poxviridae* dominated the FZ site (Figure 2). *Myoviridae* and *Podoviridae* were more abundant at the CZ site while *Siphoviridae* was found to be more abundant only in the site with the inorganic fertiliser (NZ), although, the difference across the experimental sites was found not to be significant ($p > 0.05$).

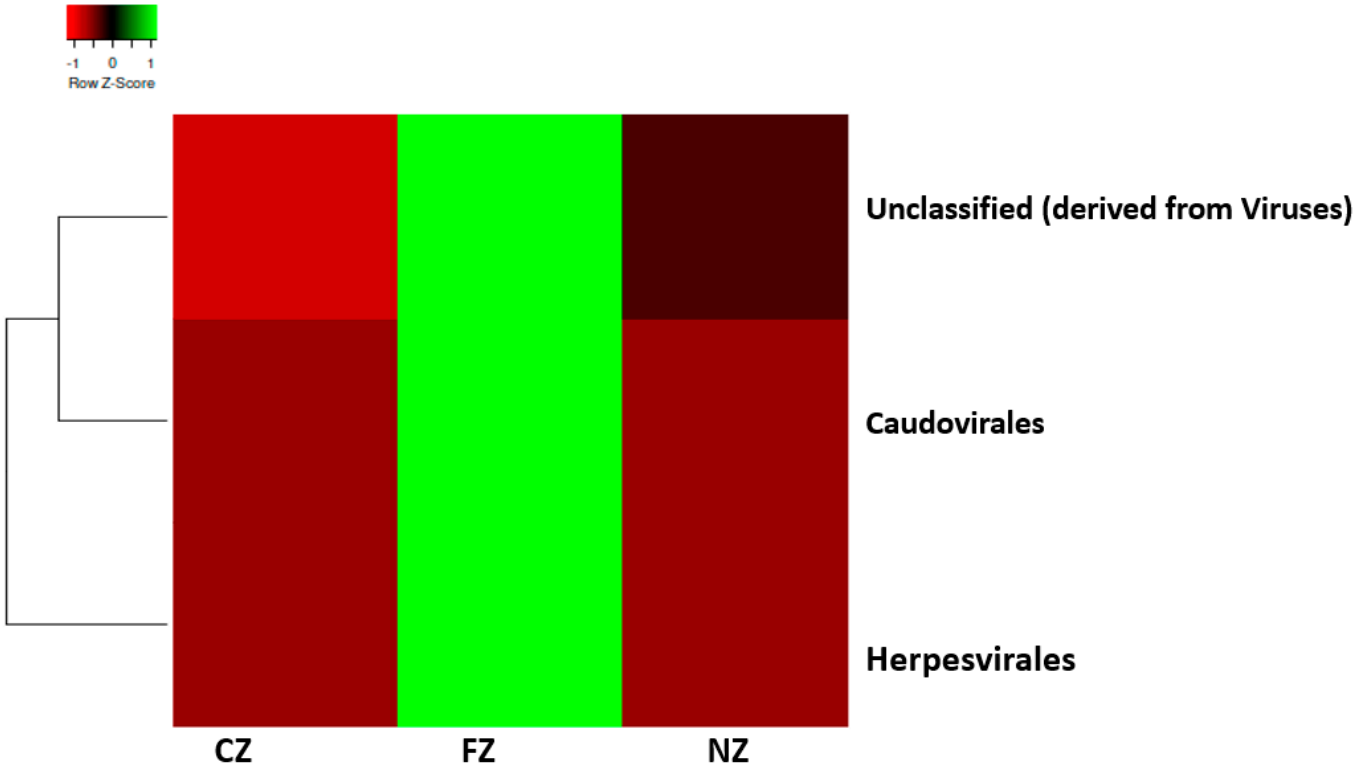

Figure 1. Heatmap of the order distribution of the notable endophytic virome from samples across the sites. The scale bar displays a colour-saturation gradient based on relative abundances that have been modified using the z-score for the endophytic virome.

Furthermore, at the genus level, unclassified Siphoviridae, Badnavirus, P2-like viruses, SPO1-like viruses, LUZ24-like viruses, N4-like viruses, Bpp 1-like viruses, Phi29-like viruses, T4-like viruses, T7-like viruses, L5-Like viruses, Lambda-like viruses and N15-like viruses dominated FZ sites. Chlamydiamicrovirus, unclassified Podoviridae, unclassified Myoviridae, and P22-like viruses were dominant in the CZ site, while unclassified Microviridae, Chlorovirus and T1-like viruses were found to be dominant in the NZ site (Figure 3). The PCA graph was employed in showing the virome distribution of the identified between the sites with the most abundant distribution observed in the organic farming site (FZ) (Figure 4).

3.3. Alpha (α) and Beta (β) Diversity of the Viral Endophytes across the Experimental Sites

The evenness and Shannon indexes derived for the order of the endophytic virome do not differ significantly ($p > 0.05$), while a significant difference ($p < 0.05$) was found at the genus level (Table 1). The virome community composition was analysed using PCoA with a Bray–Curtis dissimilarity matrix (Figure 5). The PCoA figure revealed that the FZ samples varied considerably from the CZ and NZ samples (Figure 5). ANOSIM revealed a significant difference (ANOSIM, R = 0.67, $p = 0.01$) in the diversity of the viral endophytes virome across the farming sites.

Table 1. Evenness and diversity assessment of endophytic virome across the sampling sites.

Level	Indices	CZ	FZ	NZ	*p*-Value
Endophytic Virome					
Order	Shannon_H	0.59 ± 0.03	0.68 ± 0.14	0.48 ± 0.11	0.42
	Evenness_eˆH/S	0.90 ± 0.03	0.66 ± 0.05	0.80 ± 0.14	
Genus	Shannon_H	1.77 ± 0.39	1.85 ± 0.22	1.60 ± 0.21	0.007
	Evenness_eˆH/S	0.73 ± 0.23	0.85 ± 0.17	0.82 ± 0.17	

Mean ± SD (n = 3). p-values based on Kruskal–Wallis matrix test. NZ = samples from the inorganic experimental site, FZ = samples from the organic experimental site, and CZ = no fertiliser site/control samples.

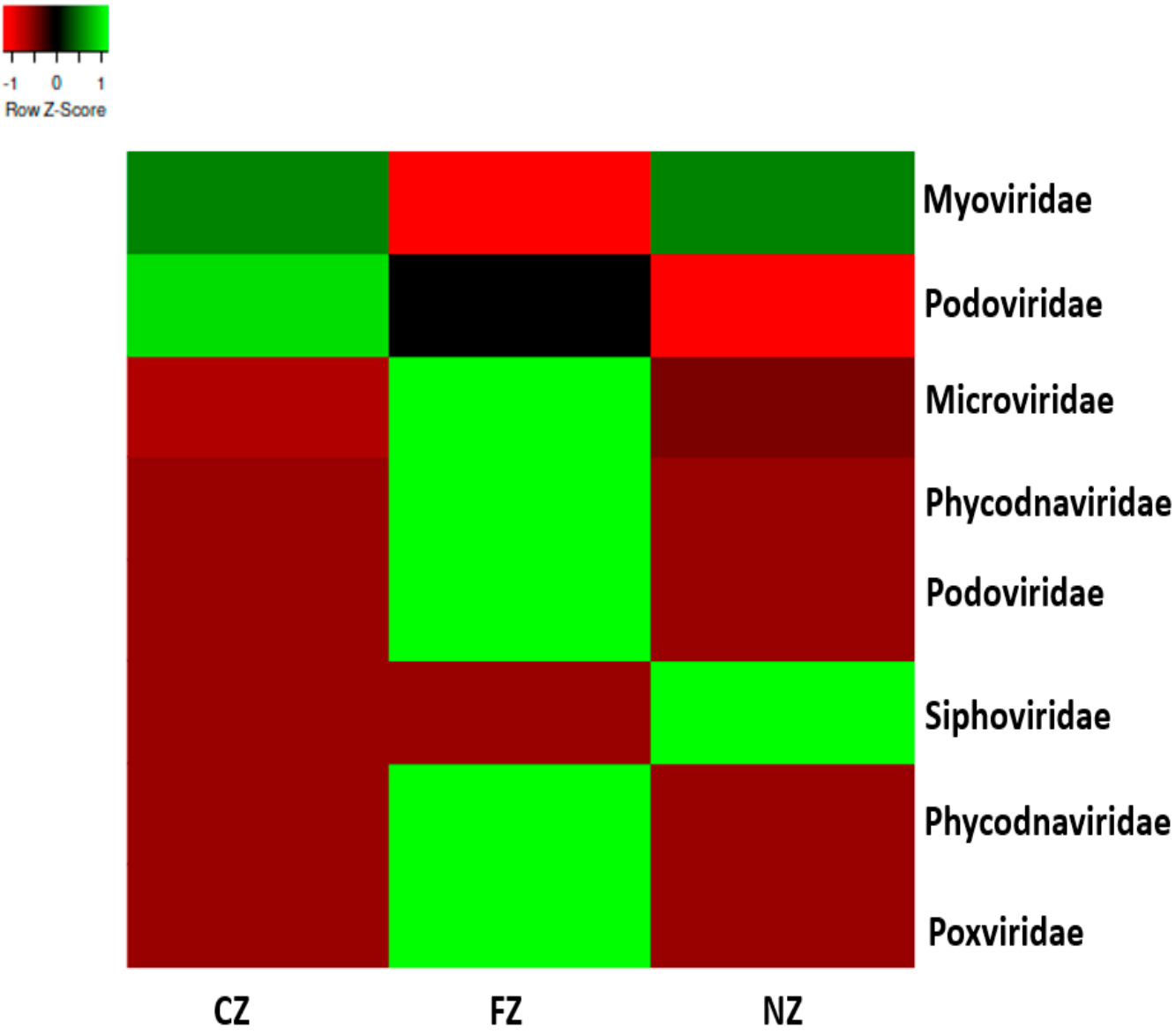

Figure 2. Heatmap of endophytic virome family. The scale bar displays a colour-saturation gradient based on relative abundances that have been modified using the z-score for the endophytic virome. NZ = samples from the inorganic experimental site, FZ = samples from the organic experimental site, and CZ = no fertiliser site/control samples.

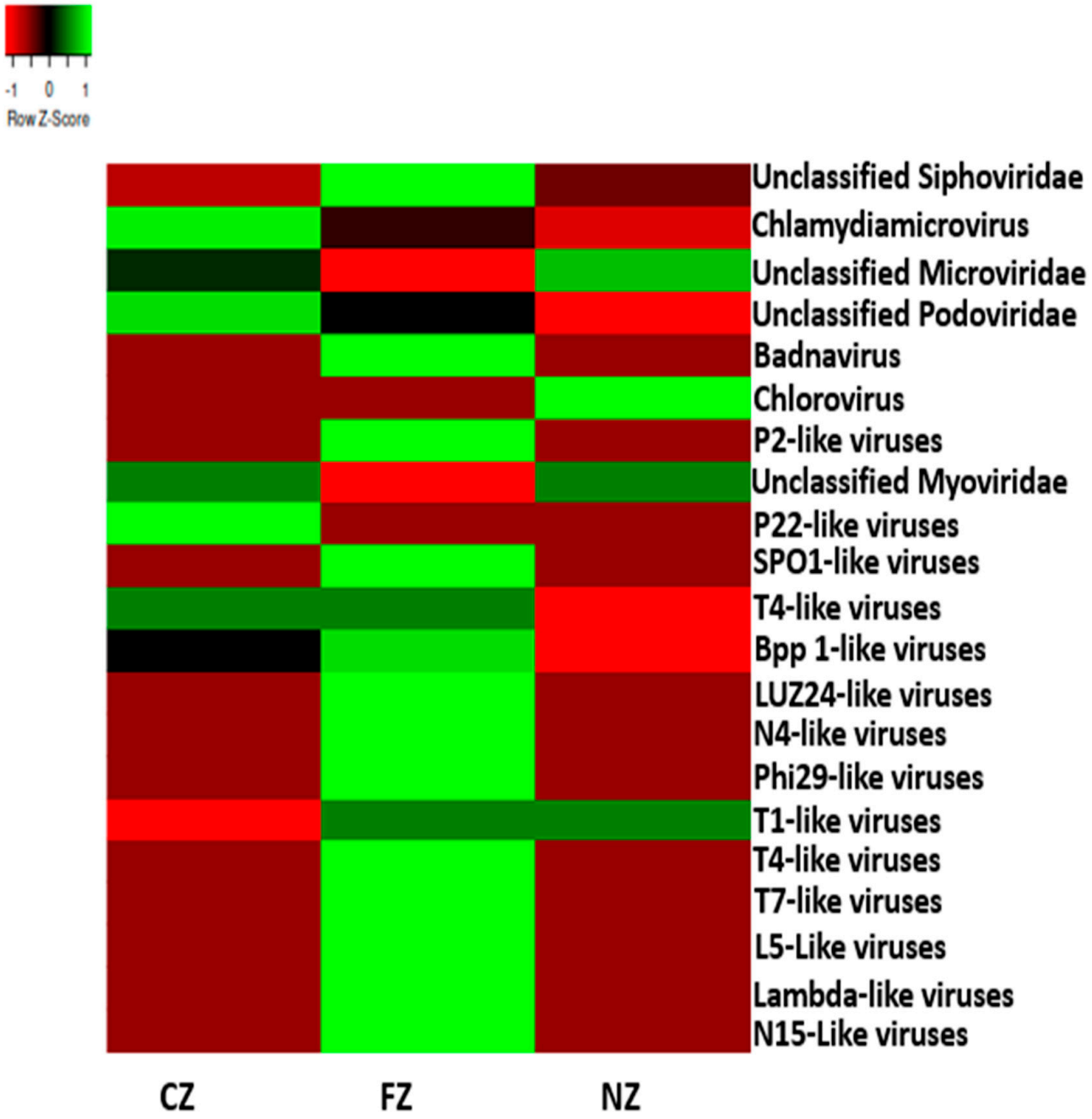

Figure 3. Heatmap of endophytic virome genus. The scale bar displays a colour-saturation gradient based on relative abundances that have been modified using the z-score for the endophytic virome. NZ = samples from the inorganic experimental site, FZ = samples from the organic experimental site, and CZ = no fertiliser site/control samples.

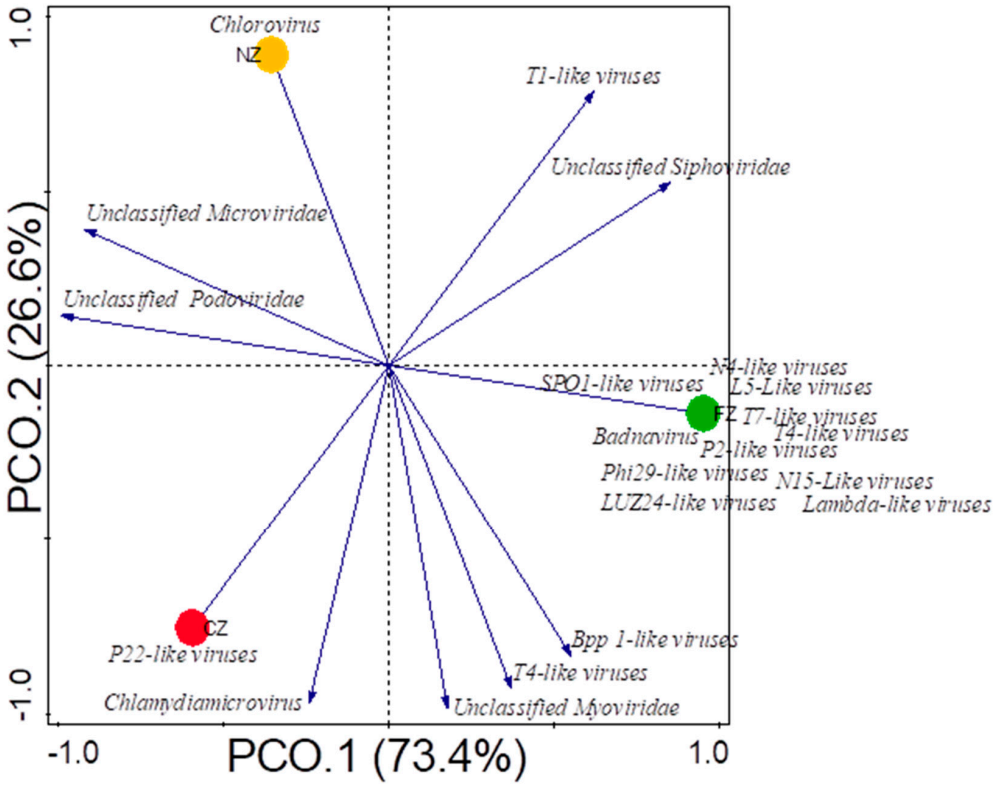

Figure 4. Principal component analysis graph of the mean metagenomes of endophytic virome. The effect of the metagenomes of the viral endophyte is shown by the vector arrow. Bray–Curtis dissimilarity matrix, axes 1 (73.4%), and 2 (26.6%) explained the variances.

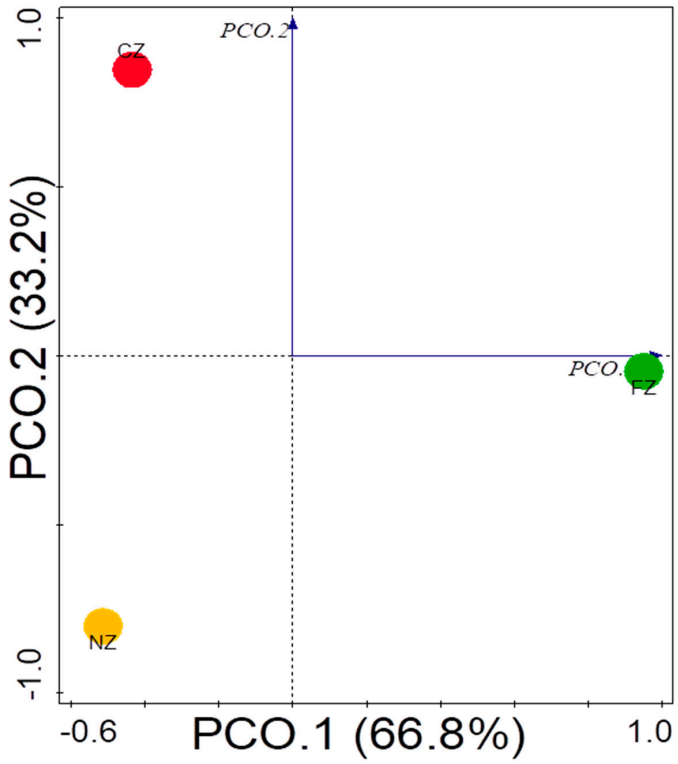

Figure 5. PCoA plot of the community composition of the endophytic virome in the experimental sites based on Bray–Curtis dissimilarities. FZ = samples from the organic experimental site, CZ = no fertiliser site/control samples, and NZ = samples from the inorganic experimental site.

4. Discussion

Farming techniques have a considerable effect on the abundance, diversity, and functions of microbial communities in the soil, and can thus be connected to increased crop output and development, as well as improve crop resistance to abiotic and biotic stress [36–38]. Using shotgun metagenomics, we investigated the effects of various farming practices on the community structure and abundance of endophytic virome inhabiting the root of maize grown under various fertiliser regimes. For years, viruses had a poor reputation and were mostly recognised for their capacity to spread disease. But more recently, symbiotic aspects using omics technologies have come into emphasis. The viral community discovered from maize root samples which are endogenous in origin were discussed in this study using the shotgun metagenomics. Interestingly, we also discovered phage virus genomes from the Caudovirales family, which may have spread from soil [39]. MG-RAST was used to examine the sequenced metagenome data collected. Genomes for the endophytic virome were identified, but plant-derived sequences were discarded.

Caudovirales and *Herpesvirales* were the major viral order in samples and the most predominant FZ site. This result agrees with an earlier study by Das, et al. [39], this may be a result of the application of organic fertiliser in the organic site, which might harbour more microbes. Caudovirales are a family of group-I viruses containing double-stranded DNA and an icosahedral head connected to a tail by a connector protein. The *Caulimoviridae* family provided the majority of unclassified sequences (Figure 1). *Caulimoviridiae* is a type of DNA virus with two strands of DNA. Their endogenous pararetroviral sequences have received a lot of attention (EPRVs). Natural integration into the host DNA has also been documented [40,41]. This natural interaction with the DNA of the host plant also points to a co-evolutionary relationship with the plant–virus pathosystem [42–44].

Also, at the class level *Siphoviridae*, *Microviridae*, *Phycodnaviridae*, *Podoviridae*, *Phycodnaviridae Poxviridae Myoviridae*, *Podoviridae*, and *Siphoviridae* were identified at the root of the maize plant. Most of the viral sequences discovered were comparable to those found in tea plants [39]. However, this study could imply that their relationship as an endogenous viral particle is well-known. Furthermore, at the genus level unclassified *Siphoviridae*, *Badnavirus*, P2-like viruses, SPO1-like viruses, Bpp 1-like viruses, LUZ24-like viruses, N4-like viruses, Phi29-like viruses, T4-like viruses, T7-like viruses, L5-like viruses, Lambda-like viruses, N15-Like viruses dominated the FZ site, *Chlamydiamicrovirus*, unclassified *Podoviridae*, unclassified *Myoviridae*, P22-like virueses dominated the CZ site, while unclassified *Microviridae*, *Chlorovirus*, and T1-like viruses were found to be dominant in the NZ site.

Badnavirus belongs to the *Caulimoviridae* family with the plant-associated bacilliform DNA virus. They have been reported to be a major pathogen of a variety of horticultural crops, including citrus, black pepper, cocoa, banana, taro, sugarcane, and yams [45,46]. Diseases of plants including root necrosis, leaf chlorosis, red vein banding in early leaves, tiny speckled pods, and the swelling of the stem/root followed by dieback are all caused by *Badnavirus* [39,45]. Several researchers have reported *Badnavirus* endogenous connection [47,48]. Endogenous recombination with the host genome, on the other hand, may not lead to infection in the host plant and can give protection against non-integrative counterparts [45]. Not many reports exist on its presence in maize plants. However, only one report of *Badnavirus* from the tea plant has been published, and it comes from Hao, et al. [49], who used metagenomic sequences of leaves and shoot samples.

The genus level was used for PCA due to the abundance of the virome at the genus level. The PCA graph revealed that each site has its unique viral genus, which accounts for 73.4% of the variance between all fertilization locations (Figure 4). The composition of sequences connected to each genus is reflected in the position of each endophytic virome; the vector arrows indicate the genus most heavily affected by the distribution. This information can be used to discover which viral genera are more prevalent at each sampling site when compared to others (Figure 4). In this investigation, viral genera were shown to be more prevalent in the FZ site than in other sites (Figure 4).

The Shannon and evenness indexes evaluated for each viral order revealed no significant differences ($p > 0.05$), while the result from the viral genus showed that they differ significantly ($p > 0.05$). Endophytic virome in maize grown in the organic farming site was more diverse and equally distributed than those in maize grown with inorganic or without fertiliser (Table 1). The result also agrees with the findings of Das et al. [39]. FZ's viral endophyte community structure differed from that of CZ and NZ, according to the PCoA plot (Figure 5). The endophytic virome in the root of maize plants differed significantly between sample sites, as seen by the Bray–Curtis dissimilarity matrix-based figure.

5. Conclusions

This is one of the foremost studies unravelling the diversity of endophytic virome inhabiting maize plants employing the shotgun metagenomics approach. This study gave a detailed taxonomic distribution of viral endophytes in maize roots and showed that farming practices have a significant effect on the abundance and diversity of these viromes. Endophytic viromes which were found to majorly dominate the roots of maize plants are *Caudovirales* and *Herpesvirales*. This report has added to our understanding of endogenous viruses, with a focus on the maize plant. There will be many more mutualistic viruses that need to be further studied to grasp their evolutionary significance. Understanding how viruses interact with plants and their diversity will help in communicating disease disclosure in plants and identifying the most favourable combination, which might be important in addressing a variety of agricultural issues. The findings of this study further add to our understanding of the virus–plant symbiotic connection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12081867/s1, Table S1: Physicochemical characteristics of the experimental field; Table S2: Analysis of sequenced data and diversity evaluation of the shotgun metagenomes of the maize plant from across the fertilizers sites.

Author Contributions: A.E.F. and O.G. handled the literature findings, carried out the laboratory and fieldwork, executed all necessary analyses, interpreted the results, and prepared the manuscript. O.O.B. initiated the next-generation sequence research, helped shape the research, verified the analytical methods, secured funds for the study, and commented on the manuscript at all stages. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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