Advances in Sciences and Arts

Journal homepage: https://asa.must.ac.mw/

RESEARCH ARTICLE
Vol. 1 Issue 1

Category
Science

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Citations
Mbewe et al. (2023) Banana bunchy top virus (*Babuvirus*; Nanoviridae) detected in all banana growing districts of Malawi. *Advances in Sciences and Arts.* 1(1), S011-0006, https://doi.org/10.37872/S001-0006

Supporting info
Please visit the journal's official website on
https://asa.must.ac.mw/

Received
12th Dec 2022

Accepted
18th Feb 2023

Published
13th Nov 2023

DOI
https://doi.org/10.37872/S001-0006

Banana bunchy top virus (*Babuvirus;* Nanoviridae) detected in all banana growing districts of Malawi

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Abstract Banana bunchy top disease, caused by Banana bunchy top virus (BBTV), is a serious disease that affects the productivity of bananas globally. It is responsible for the current reduction of banana production in Malawi, and there are ongoing efforts to resuscitate the local banana industry. Foliar disease scoring and aphid population counts were recorded in 130 randomly sampled farmer's fields across the country between 2020 and 2021 during a national survey. A total of 660 banana leaf samples were collected, and their total genomic DNA was extracted and amplified by polymerase chain reaction (PCR) using two gene-specific primers. The foliar disease incidences and severities varied across districts and variety types ($p<0001$). Disease foliar incidence was highest in Nkhata Bay district (93.33%) and lowest in Kasungu (0%). Molecular detection confirmed the distribution of the disease in all the sampled districts, including Chitipa, which has long been regarded as free from the disease and the sole source of clean planting materials for the country. Aphid populations were generally low with Karonga district having highest infestation score of 3 per field. The presence of the disease in Chitipa and subsequent increase in disease epidemiology pose serious threat to banana seed system and consequently food security in Malawi.

Keywords: *Banana bunchy top virus; disease surveillance; epidemiology; incidence; severity*.

1. Introduction

Banana (Musa spp.) is a major food and cash crop all over the world (Martínez-Solórzano & Rey-Brina, 2021). Banana cultivation is a significant socio-economic activity practiced on small farms in at least 80 countries (FAO, 2018). In Malawi, banana is predominantly grown by small-scale farmers both as a source of income and as a food security crop. Production of banana fruits for sale in local markets is among the few agricultural activities that provide households, especially in rural areas, with regular income throughout the year (Mshani et al., 2010). Unfortunately, Malawi has lately lost 30,000 hectares of banana crop stand representing a 90% percent loss due to poor agricultural practices, diseases and a lack of access to clean planting materials (Mikwamba et al., 2020). One of the most significant banana diseases is banana bunchy top disease (BBTD) (Blomme et al., 2013; Qazi, 2016; Sumi et al., 2022). The disease is caused by the banana bunchy top virus (BBTV), genus *Babuvirus*, family *Nanoviridae*, a multipartite circular single-stranded DNA virus (Qazi, 2016; Sumi et al., 2022).

BBTD symptoms include the development of morse code streaking of variable length in the leaf veins, midribs, and petioles, as well as progressive dwarfing of leaves and the development of marginal leaf chlorosis, upright and crowded leaves at the plant's apex, hence the name bunchy top disease. Plants infected early in their growth are unable to produce bunches, whereas those infected later in their growth produce bunches that are frequently of poor quality (Elayabalan et al., 2015; Kumar et al., 2011). The disease spreads into new fields primarily through infected suckers and within fields by the banana aphid, *Pentalonia nigronervosa* (Allen, 1978; Magee, 1927).

The disease was first discovered in the Fiji Islands in 1889 and has quickly spread throughout Asia, Africa, Australia, and the South Pacific covering more than 33 countries (Blomme et al., 2013; Kumar et al., 2011). In Africa, BBTD was first reported in 1901 in Egypt and has now been reported in 16 countries, including Angola, Bénin, Burundi, Cameroon, Gabon, the Central African Republic, the Democratic Republic of Congo, Equatorial Guinea, Nigeria, Rwanda, Zambia and just recently in Uganda, Tanzania, and Malawi (Jooste et al., 2016; Kumar et al., 2011; Oben et al., 2009; Ocimati et al., 2021; Qazi, 2016; Shimwela et al., 2022; Tongo Mukwa et al., 2014). In Malawi, the disease was first observed in 1994 around the Thiwi area in Nkhotakota district, but confirmed officially in 1997 (Kenyon et al., 1997). It has since spread all over the country in major banana producing areas except Chitipa district in the northern part of Malawi (Soko, personal communication).

Figure 1: Banana plants with symptoms of BBTD. The picture was captured in Lufita and Mwamkumbwa Extension Planning Areas (EPAs) in Chitipa district in 2021.

The spread of BBTD has negatively impacted the livelihoods of farm-dependent households in sub-Saharan Africa (SSA) (Blomme et al., 2013; Niyongere et al., 2012, 2013). For instance, banana production in Benin had dropped by 13% between 2013 and 2016 since BBTD was detected in 2012 (Géoffroy Dato et al., 2021). This alone poses a substantial threat to sustainable banana production in Africa, with a significant risk of destabilising food security and household income (Blomme et al., 2013). In Malawi, BBTD has not only caused a significant reduction in crop production levels but has also resulted in the loss of locally preferred germplasm. This is manifested by the significant variation in prevalence and distribution of banana varieties in most growing districts and areas of the country. To address the BBTD problem and subsequently restore banana production in the country, several initiatives have been carried out, including the distribution of clean planting materials. However, no formal study has ever been conducted to assess the impact of the clean planting materials distribution initiatives as a solution to curbing BBTV in Malawi. This study was therefore conducted to (i) assess the phytosanitary status and field performance of the distributed clean banana planting materials in Malawi and (ii) establish a molecular epidemiology of BBTV in all the major banana growing areas.

2. Materials and Methods

2.1 Survey area, sampling protocol and data collection

Surveys were conducted in 2020 in eleven administrative districts that represent the most important banana-growing areas of the country, viz; Salima, Nkhotakota, Nkhata Bay, Thyolo, Mulanje, Chiradzulu, Karonga, Chitipa, Mzimba North, Mzimba South, and Kasungu (in this manuscript, Mzimba was recorded as two administrative districts [North and South] as per Malawi's Ministry of Agriculture's Administrative Plan). In each district, 15 fields were targeted with four leaf samples collected from each field for laboratory virus assays. The samples were collected by cutting the top fresh leaf rolls from two symptomatic and two non-symptomatic plants in each field. In fields with no visually symptomatic plants, all four samples were collected from four

non-symptomatic plants. All the samples were wrapped in soft paper and stored in plastic bags containing silica gel. A total of 130 banana fields were surveyed and 660 symptomatic and asymptomatic leaf samples were collected for virus analysis in the laboratory.

BBTD foliar incidence was recorded as the percentage of symptomatic plants among 30 assessed plants per field. A mat that contained at-least one plant with clear and visible BBTD symptoms was regarded as infected. Severity was assessed using a scale of 1 to 5, where 1 = no BBTD symptoms; 2 = dark green streaks on the leaf veins, leaf midribs and petioles; 3 = marginal leaf chlorosis; 4 = dwarfing of leaves; and 5 = "bunchy top" an aspect of the plant showing upright, crowded, and brittle leaves. Aphid populations were also assessed using a scale of 1 to 5, where 1 = no aphid; 2 = a simple colony (no winged individuals); 3 = a large colony with one or more winged individuals; 4 = several colonies with winged individuals; 5 = generalised colonies at the level of the leaves and the pseudo stem of a banana plant with numerous winged individuals. The study adopted BBTD field data collection protocols developed by (Kumar et al., 2011).

2.2 In-district 2021 survey for Chitipa district

Chitipa district was the only district in Malawi with no reported cases of the disease until 2020 (Mikwamba et al., 2020). Molecular detection of the presence of BBTD in the district raised an alarm about the expanding presence of the disease in Malawi. The district had long been considered a BBTD-free district, from which, presumably, clean planting materials were being sourced countrywide. This prompted a second survey focusing solely on the district to understand the disease distribution and establish the actual disease hotspots. As such, a supplementary and detailed survey was conducted in the district in 2021 following the same procedures as described in this study. A total of 180 leaf samples were collected from six agricultural Extension Planning Areas (EPAs) (Lufita, Kavukuku, Chisenga, Misuku, Kameme, and Mwamkumbwa) representing all agricultural, geographical, and administrative areas of the district.

2.3 Data coding and analysis

Among the sampled banana varieties, var. *Zambia*, which was used as a reference for comparing other varieties, was the most sampled with up to 157 samples, followed by other common banana varieties that were found in almost all the sampled districts. During analysis, it was observed that some banana varieties were not common, sampled once or twice andarea-specific (cultivated in only one of the sampled districts). Those varieties that were not common and area specific were categorized as "minor local", including: *Chiteze, Ghana, Kalashya, Kamunowa, Kampeni, Ndyali, Ndoki, Wowolyo, Makumbuka,* and *Kamtumbiseni.*

A generalized linear model fitting the quasi-binomial family was done on the transformed banana bunchy top disease scores and foliar incidence percentage using R Statistical Package (V4.02). BBTD scores were transformed using min-max scaling (normalization) (Patro and Sahu 2015), as detailed in the equation [1];

$$\text{Transformed BBTD - Score} = \frac{(BBTD\ score - 1)}{4} \quad \ldots[1]$$

BBTD incidence percentage transformation involved dividing each BBTD incidence percentage by 100, as detailed in the equation [2].

$$\text{Transformed BBTD incidence} = \frac{(BBTD - incidence\ percentage)}{100} \quad \ldots.[2]$$

The model residuals were standardised in order to test for possible outliers and influential cases and were assessed using Cook's distance and hat values (leverage) which showed that there was no major influence on the biasness of the model. A significant estimate (coefficient) of logistic

regression, its associated probability, and R^2 measure of the model are presented.

2.4 Molecular detection of viruses

2.4.1 Total DNA extraction from leaf samples

The total DNA was isolated using the CTAB protocol (Hyder et al. 2007). Prior to extraction, 200 mg of the collected samples were weighed using a sensitive balance (Weinmann Technology, GMBH, Bulgaria). The weighed samples were manually pulverized in 750 µl of CTAB extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 20nM EDTA, 100 mM Tris-HCl pH = 8.0, and 0.2% (v/v) 2-mercaptoethanol] using mortar and pestles and incubated in 60ºC water bath for 30 minutes. Then, 750 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently. The contents were centrifuged for 10 mins at 13,000 rpm and 500 µl of the supernatant was transferred into new 1.5 ml Eppendorf tube. A sample of 300 µl of ice-cold isopropanol was added and the contents were incubated at -20ºC for 2 hours. Samples were then centrifuged at 13,000 rpm for 10 minutes at 4ºC to pellet the nucleic acids. The supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol by vortexing and centrifuging for 5 minutes at 13,000 rpm. Thereafter, the ethanol was discarded and the pellets were dissolved in 50 µl of molecular grade water and stored at -20ºC. The quality of the extracted DNA was checked using a Nanodrop spectrophotometer (Weinmann Technology, GMBH, Bulgaria).

2.4.2 Amplification of viruses using polymerase chain reaction

All DNA samples were tested for BBTV using specific primers that amplify the nuclear shuttle protein (NSP) genes of the virus. The primers, NSP F1 (5`CCTCGCAAGGTACTTCTTAG 3`) and NSP R1 (5`CCATGTCTCTGCTCCAATCT 3`), amplifies a product of 237 bp (Kumar et al., 2011; Mansoor et al., 2005; Oben et al., 2009). Reactions were performed in a final volume of 32 µl [2 µl of DNA template, 2.0 µl of 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2), 10 µl of 10 µM dNTPs, 0.5 µl of respective forward and reverse primers, 0.5 µl of Taq DNA polymerase (One Taq® DNA Polymerase, New England Biolabs) and 16.5 µl of sterilized double distilled water]. Amplification was performed using conventional PCR system (Prime Thermal Cycler, Bilbby Scientific Ltd, UK). The temperature profile used in the PCR for NSP gene was as follows: an initial denaturation for 3 min at 94 ºC, then 35 cycles of: 45 seconds at 94ºC, 45 seconds at 50.4ºC, 45 seconds at 72ºC, and then a final extension for 10 min at 72ºC.

2.4.3 Electrophoresis

Electrophoresis was carried out on agarose (1.5 % of w/v) gel at 100 Volt for 45 minutes. The DNA samples in the wells were stained using Ethidium Bromide and visualized using a Gel Documentation system (BioDoc-It™ 210 Imaging system, Cambridge, UK) and photographed.

3. Results

3.1 Foliar incidence and severity of Banana bunchy top virus disease

3.1.1 Banana bunchy top disease incidence percentage across sampled districts

Nation-wide, the mean BBTD incidence was 32.73% with Nkhata Bay district showing highest disease incidence of 93.33% followed by Mulanje (73.33%), Nkhotakota (40.00%) and Karonga (33.33%). Mzimba South and Chitipa districts recorded zero BBTD incidence (Table 1). Disease incidence varied significantly across the sampled districts (x^2=154, $p < 0.001$). Nevertheless, disease incidence was not significantly different among other districts including Chiradzulu, Kasungu, Mzimba North, Mzimba South, Salima and Thyolo.

Table 1. *Severity and incidence of Banana bunchy top disease in Malawi*

District	Mean BBTD severity	BBTD Incidence percentage
Chiradzulu	2.50	26.67
Chitipa	1.00	0.00
Karonga	2.30	33.33
Kasungu	1.00	0.00
Mulanje	2.27	73.33
Mzimba North	2.50	20.00
Mzimba South	1.00	0.00
NkhataBay	2.53	93.33
Nkhotakota	2.43	40.00
Salima	2.13	26.67
Thyolo	2.40	46.67
Mean	***2.38***	***32.73***

Although the BBTD scores were moderate (Table 1), a highest score of 5 was observed in one field in Mpata EPA, Bwiba section in Karonga district.

3.1.2 Banana bunchy top disease severity scores among sampled districts

There were significant variations (x^2 =134.66, p < 0.001), in banana bunchy top severity score among the districts (Table 1). Results show that Nkhata Bay, Nkhotakota and Mulanje districts recorded the highest BBTD-score followed by Karonga and Thyolo (Table 1, Figures 2 and 3). There was no significant difference in BBTD-score among the other districts (p<0.001).

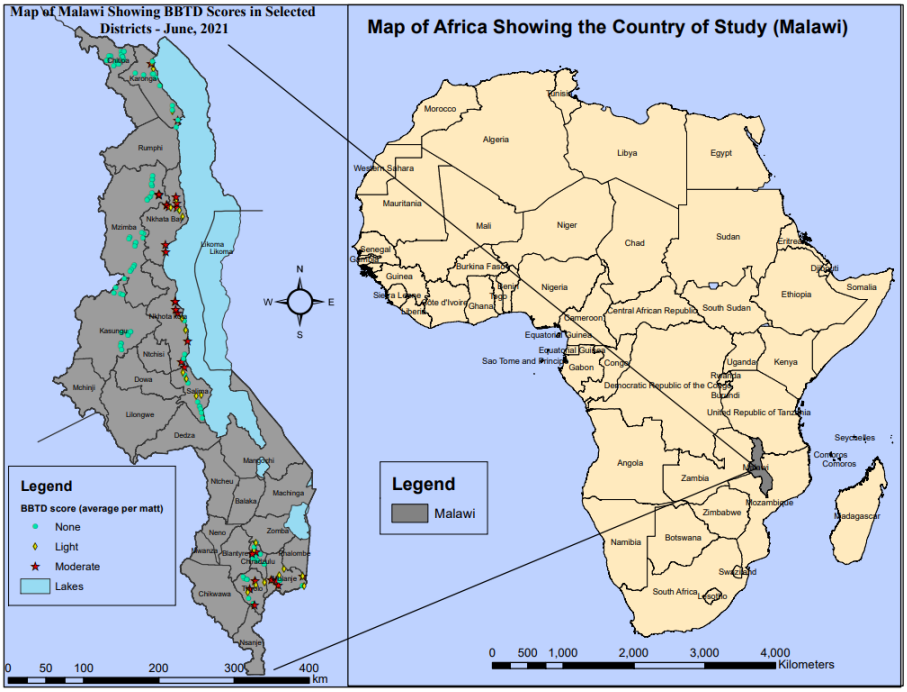

Figure 2: National distribution of BBTD in Malawi based on field disease scores.

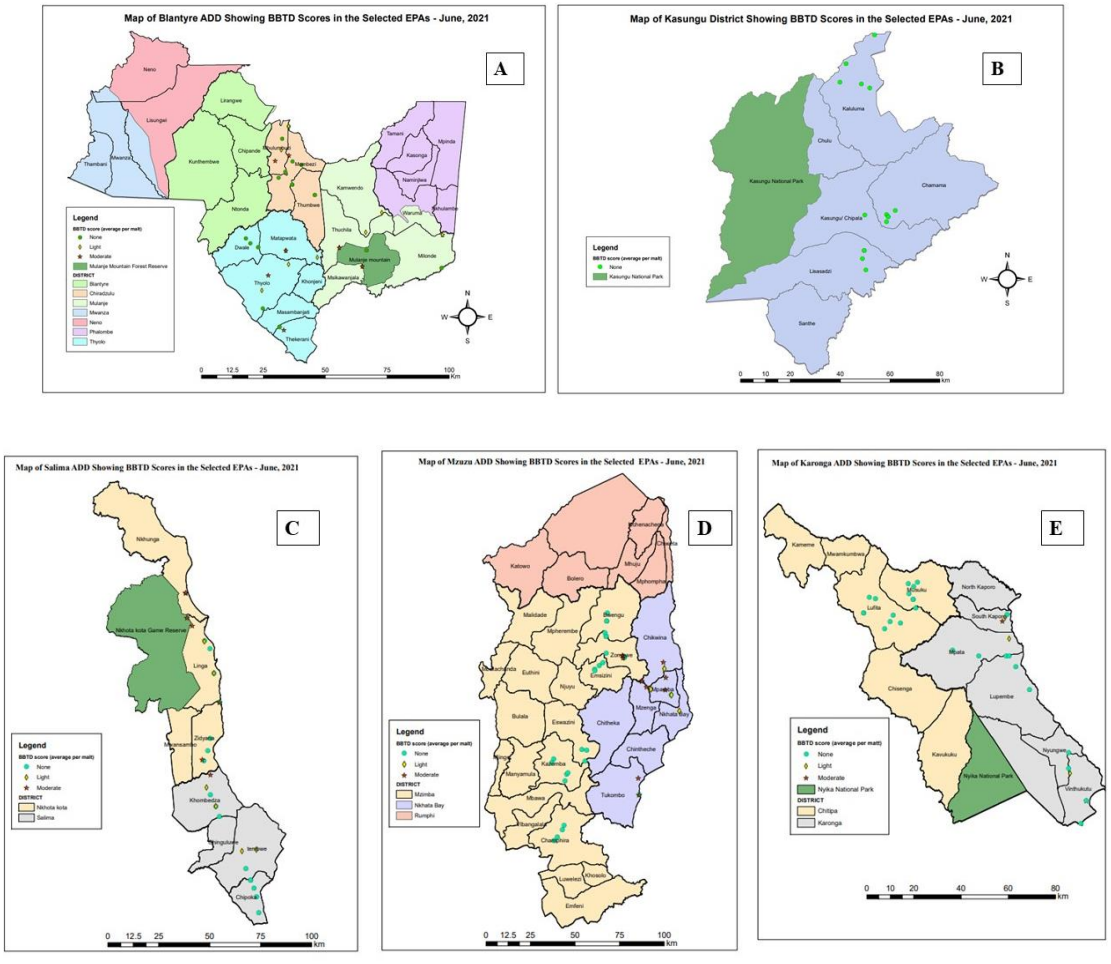

Figure 3. *Spatial distribution of Banana bunchy top disease in specific Agricultural Development Divisions in Malawi.*

3.1.3 Status of banana bunchy top disease among the sampled banana varieties

BBTD-severity scores among the sampled varieties were significantly different ($x^2 = 146.36$, $p < 0.001$. It was observed that variety *Sabowa* had the highest symptomatic mean score of 3.0 with disease foliar incidence of 100%. Varieties *Ndayima, Nzeru, Zanzibar, Mulanje* and *Zanda* had symptomatic mean scores of 3.0, 2.40, 2.76, 2.50, 2.74 with foliar incidence of 50%, 45.45%, 36.36%, 26.47%, 26.39%, respectively. BBTD scores in the other remaining varieties were not significantly different ($p > 0.05$) from the reference (*Zambia*). Within each sampled district and for all the districts, there was no significant variation in BBTD severity scores.

3.1.4 Correlation between altitudes and disease scores

A nonparametric Kendall's tau statistic correlation analysis showed that there was a significant negative correlation ($\tau = -0.20$, $p < 0.001$) between district-wide altitudes and BBTD score. This means that as the altitude increased the BBTD score decreased. The data showed that it is highly likely to find more BBTV free (clean) bananas on high altitude areas than in lower altitude areas.

3.2 Aphid colonisation

The banana aphid, *Pentalonia nigronervosa* (Hemiptera: Aphididae) populations were rarely observed in most fields. However, in the few fields where aphids were found, they were present in large numbers with an average score rating of 3, which is a large colony with one or two winged individuals (Figure 4).

Figure 4. *Aphid colonisation in Banana plants observed in a field in Nkhotakota district.*

3.3 Molecular detection of Banana bunchy top viruses

BBTV was detected using molecular analyses in all the sampled districts (Table 3). Nkhata Bay had the highest detection rate of 85% while Salima and Nkhotakota had the lowest (46.67% each). From the molecular analysis, it was observed that BBTV was also detected in non-symptomatic samples from nine out of eleven sampled districts.

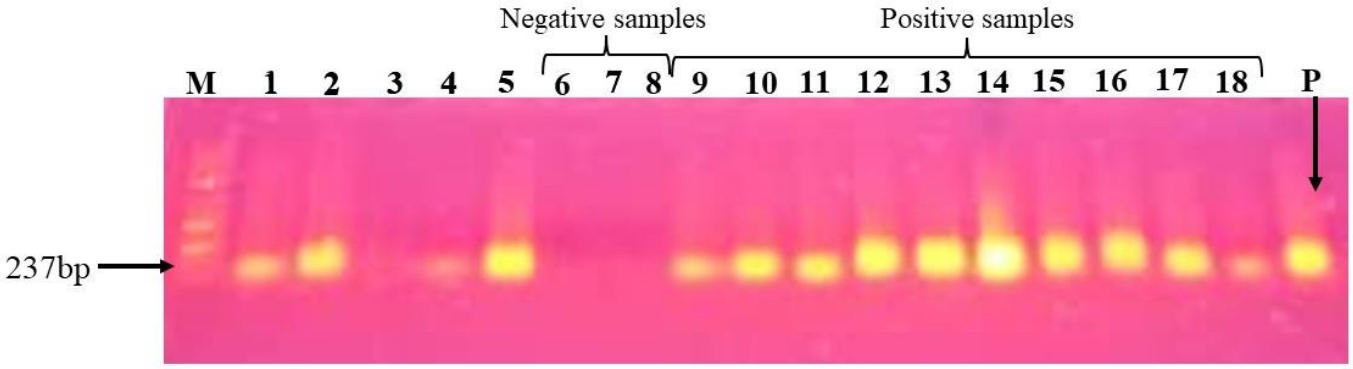

Figure 5: PCR Amplification of Banana bunchy top viruses in Malawi. Lane M is 100 Kbp DNA size molecular marker (Promega, Madison, USA) while Lane P is positive control.

3.4 In-district 2021 BBTD Survey of Chitipa district

3.4.1 Detection of BBTV in Chitipa district

From a total of 180 leaf samples that were collected in Chitipa, 52 samples tested positive to BBTV representing 28.89%. BBTV was detected in most samples (73.33%) that were collected from Chisenga EPA, while Misuku and Kameme were the least detected (6.67% for each).

3.4.2 Virus detection among different banana cultivars in Chitipa

The study revealed that vars. *Zambia*, *Harare*, and *William* were among the most sampled and most cultivated banana cultivars in Chitipa. BBTV was detected in all of these varieties (Table 5). Despite being among the predominant banana varieties *Kambani*, a local variety was among the least infected varieties with only 1 sample testing positive out of 22 tested samples, representing 4.55 %.

4. Discussion

The survey has reported an expanding epidemic of BBTD in Malawi. From earlier disease surveys, BBTD was reported in Malawi throughout all the banana growing districts except for Thyolo, Mulanje, Karonga and Chitipa districts (Kumar et al., 2011). Later it was reported that the disease had expanded to Mulanje, Thyolo and Karonga but not to Chitipa (Mikwamba et al., 2020). However, this study has documented molecular evidence for expansion of disease epidemic to the highlands of Chitipa for the first time. The study has also shown an increase in BBTD incidences and severities with Nkhata Bay recording the highest disease incidences of up to 93%. This means that BBTD has been expanding throughout the country over the last few years. This is a very serious occurrence as there have been several efforts to control the spread of the disease in the country. These results were also reported by Mikwamba *et al*., 2020 who documented that while there is so much talk about a shift in extension approaches to manage BBTD in the country, not much has changed over the years to co-innovation amongst actors (farmers, policy makers, government extension officers). Thus,

such top-down approaches have had limited success in the delivery of sustainable BBTD management in the country (Mikwamba et al., 2020).

The study has shown that there was a significant negative correlation between altitude and BBTD score. This means that as the altitude increased the BBTD score decreased. This is in line with findings by Niyongere et al., 2012 who indicated that it is very unlikely to find BBTD at altitudes more than 1300 metres above sea level (masl). This could explain why until 2020, there was no reported BBTD in Chitipa which lies at more than 1,200 meters above the sea level. Similarly, this explains the observation that within the district, the virus was rarely detected in EPAs lying at higher altitude such as Misuku which was not the case at lower altitudes like Chisenga and Kameme. Although populations of aphids were generally low, field transmission studies in Burundi have shown that aphids are able to acquire and transmit BBTV at altitudes as high as 2,090 masl. This means that, although not established in this study, the role of aphid transmission in Chitipa should not be underestimated.

Detection of viruses in asymptomatic samples is not a strange thing in plant virology. Although visual assessments are appropriate for many routine surveillance applications, PCR-based diagnostics are always more accurate where detection of early-stage infections or species-level determinations is required (Lacroix et al., 2016; Rubio et al., 2020; Wang et al., 2022). Furthermore, even though the reasons for the apparent undiagnosed infections were not investigated in this study, it is proposed that the most probable reasons are that either the plants were under some abiotic stress leading to expression of BBTD-like symptoms or that sequence variability of infecting BBTV led to primers failing to amplify target sequences.

Going forward, this study recommends sequencing of viral isolates for better understanding of the biology and molecular epidemiology of BBTVs in Malawi. It will be important to study the evolutionary relationship of the virus strains [and species] associated with disease etiology and the host susceptibility of the different varieties found within the country. Meanwhile, disease surveillance must continue and movement of banana suckers between districts should be closely monitored and better controlled.

5. Conclusions

This comprehensive field and molecular-based epidemiological survey has provided up-to-date data on the status and distribution of banana bunchy top virus disease in Malawi. Importantly, we have provided the first molecular evidence of the presence of BBTV in Chitipa district, which for the past many years has been declared a ‘disease-free district’. This provides critical insight into Malawi’s banana seed industry, as the district has for a long time been used as a source of planting material. Malawi continues to lose bananas due to the deadly BBTD and quick efforts are therefore needed urgently to arrest the problem which hampers directly on the country’s food and livelihood security.

Author Contributions: Conceptualization, W.M, A.M and PP; methodology, W.M, A.M, P.P, L.M; validation, All; formal analysis, W.M, A.M.; investigation, All; resources, PP..; data curation, W.M, A.N,; writing—original draft preparation, W.M, A.M; writing—review and editing, W.M, A.M, G.B; supervision, P.P, L.M, G.B; project administration, A.M, P.P; funding acquisition, P.P. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by Malawi government through a European Union funded program called Kutukula Ulimi m'Malawi (KULIMA).

Data Availability Statement: Data will be made available on request by the corresponding author.

Acknowledgments: We immensely thank all banana farmers in Malawi for giving us access to their fields. The support from Agricultural Extension Officers from all visited district is also greatly appreciated.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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