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Variation in Symptom Development and Infectivity of Banana Bunchy Top Disease among Four Cultivars of *Musa* sp.

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Abstract: Banana bunchy top disease (BBTD) is an invasive viral disease spreading in Africa. It is transmitted by banana aphids and infected planting material, causing production losses. Clean seeds and timely eradication of diseased plants are effective tools in BBTD management. These depend on timely disease detection. We assessed the relationship between symptom expression and infectivity of the virus in four cultivars of banana. Plantlets from four cultivars, 'FHIA 25'; '*Aloga*'; '*Ebenga*' and '*Sotoumon*', were exposed to viruliferous aphids and monitored for symptom expression. They were also tested as sources for virus transmission fortnightly by allowing non-viruliferous aphids acquisition access prior to transfer to healthy test plants. The time required to show symptoms and the symptom expression were compared, and infection tested by PCR. Disease expression varied from four weeks in 'FHIA 25' to fifteen in '*Sotoumon*'. Only the symptomatic leaves tested positive and could act as infection sources. Overall, 'FHIA 25' was the most susceptible cultivar, while '*Sotoumon*' was the least susceptible and most rapidly expressive of BBTD, yet there was no difference in the leaf emergence rate between the cultivars. These results present important aspects of BBTD control and the safety of planting materials that should be tested in the field.

Keywords: banana bunchy top disease; disease detection; diversity; infectiousness; eradication

Citation: Chabi, M.; Dassou, A.G.; Adoukonou-Sagbadja, H.; Thomas, J.; Omondi, A.B. Variation in Symptom Development and Infectivity of Banana Bunchy Top Disease among Four Cultivars of *Musa* sp. *Crops* **2023**, *3*, 158–169. https://doi.org/10.3390/crops3020016

Academic Editors: Munir Mawassi and Sergey Morozov

Received: 8 March 2023
Revised: 13 April 2023
Accepted: 17 April 2023
Published: 9 May 2023

1. Introduction

Early detection of transmissible diseases is a key component of their control and the prevention of outbreaks [1,2]. Reliable detection can greatly facilitate eliminating sources of inoculum and reducing the natural spread of diseases. The complete development and spread of a disease proceeds in four phases: infection, incubation, appearance (or not) of symptoms and transmission [3]. The length of each phase, as well as that of the total cycle, are characteristic of the parasite and host species and also depend on the external environment [4,5]. The genetic and physiological status of the host and the strain of the pathogen also play a role [6]. The removal of infected individuals (rouging—a common practice in disease management) minimizes contact between healthy individuals and inoculum sources by reducing the prevalence of infected individuals. However, if infectious individuals are not detectable early, the virus could spread, causing an epidemic [7]. The importance of effective detection and prevention of transmission are important for the sustainability of crop and animal disease management. Recently, this has been well illustrated in the campaign against the SARS-CoV2 pandemic.

Globally, *Musa* sp.(banana and plantain) is the fourth most important agricultural commodity in terms of production after rice, wheat and maize [8]. Since the 1970s, diseases in banana have become more common and frequent, resulting in epidemics with important economic losses [9]. Improving the agricultural production requires, among other things, a better management of pests in order to decrease the yield loss in crop production and safeguard food security. Africa is home to two centers of secondary diversity of banana (East African Highlands and West Africa). These centers hold a large diversity of farmer-selected cultivars. Banana bunchy top disease (BBTD), an invasive and devastating disease caused by the banana bunchy top virus (BBTV, *Babuvirus*, *Nanoviridae*), spread by seed and the banana aphid, *Pentalonia nigronervosa*. It threatens banana production and these secondary diversity centers of *Musa* [10–13]. BBTD is widespread in sub-Saharan Africa and is present in most major banana-producing regions of the world, except Central and South America [11].

BBTD causes a progressive production loss and simultaneous decrease in the availability of new healthy planting material for banana plantation establishment or rehabilitation [7,14]. The spread of BBTD in the main banana-producing areas of sub-Saharan Africa poses multiple constraints to banana production. It causes economic losses and threatens food security in outbreak areas, with losses ranging from 60 to 70% of the production within the first two years if not controlled [7]. Previous studies estimated that BBTD affects about 6–12 million small-scale farmers in Africa [15,16]. Ranked among the 100 most dangerous invasive species, the banana bunchy top virus (BBTV) presence on the continent threatens both banana production and diversity [11,13]. So far, the presence of BBTD has been reported in 18 African countries [14,17].

BBTD management involves an integration of multiple crop and vector management tools. These involve the destruction of infected plants in a field as soon as the first symptoms appear, the enforcement of a banana-free fallow of 3 months in target gardens leaving a buffer around them to minimize infective vector carry over between successive crops and from neighboring fields, the use of disease-free planting material, replanting in well-isolated fields, and the collaborative surveillance of the replanted banana plantations [15,16,18]. At the landscape level, inoculum reduction by the destruction of all infected banana mats on and off farm by social coordination and by legislation have been attempted [7,19]. These measures require accurate early recognition and destruction of the diseased plants to limit the landscape-scale spread of the disease.

BBTD expression may be influenced by environmental and genetic factors [20,21]. In the field, some cultivars apparently escape infection or show delayed disease expression, possibly due to a range of factors, including differences in susceptibility between cultivars, plant morphological properties, aphid attraction or tolerance to the virus, decreasing symptom expression [22]. Thus, the detection of BBTD and its management based on symptom expression could be context-dependent. The possibility of asymptomatic diseased plants to be sources of secondary infection has not been studied. We hypothesized that the infectiousness of BBTV in different cultivars at different times after infection would play an important role in the transmission and spread of the disease. Clear BBTD symptom expression facilitates the practitioners' ability to detect the disease, so its relationship with the onset of disease transmissibility is important in the rouging-based management of the disease. In the present study, we investigated the relationship between disease expression and virus transmission in four cultivars of banana and plantain commonly grown in Benin. These results will help in establishing the efficiency of rouging based on the visual detection of symptomatic plants in BBTD management in different cultivars.

2. Materials and Methods

2.1. Experimental Material

Four locally produced banana and plantain cultivars, including '*Aloga*' (AAB, Plantain), '*Ebenga*' (AAB, Plantain), '*Sotoumon*' (AAB—Apple banana group)' and a hybrid ('FHIA 25' AAAB) were used to study symptom development. The genotypes reported here

were inferred and not yet directly determined, save for FHIA 25 which is a known hybrid. Although '*Sotoumon*' is a dessert banana, we inferred its genotype from its similarity with the apple banana group including varieties from East Africa and South America such as 'Sukari *Ndizi*' from East Africa [23]. These cultivars are among the most commonly grown in southern Benin [24].

These studies were conducted in a greenhouse at ambient temperature (25–30 °C), and the exposed plantlets were maintained in an insect-proof screen house. Infectiousness of the leaves of the inoculated plants of all cultivars was assessed by aphid transmissions to plantlets. For these experiments, plantlets produced by macro-propagation from BBTV-free corms were used. The plantlets were transferred to plastic pots (15 × 15 × 15 cm) containing compost-enriched soil. The compost was prepared from a mixture of used chicken litter and cattle manure (1:1) and composted for six weeks with frequent mixing and occasional watering.

Non-viruliferous aphids were collected from the field and raised in a screen house on healthy 'FHIA 21' plantlets. Viruliferous aphids were collected from insectary colonies and maintained on BBTV-infected plants for the trial. The respective colonies were tested by PCR to confirm their status, using standard PCR primers [25]. As the related vector species *P. caladii* is sometimes found on banana [25], *P. nigronervosa* identity was verified by sequencing the mtCO1 barcoding gene and comparing it with GenBank accessions, using the primers LCO1490t1 and HCO2198t2 [26]. BBTV infection in both vectors and banana plantlets was verified by PCR (see Section 2.3, below).

2.2. BBTV Inoculation of Banana Plants

For each cultivar, three replicates of 10 plantlets each were inoculated by exposure to 10 viruliferous adult banana aphids placed in a clip cage, designed according to [27], and monitored for symptom appearance. The aphids were exposed to the first fully open leaf and allowed a 48 h inoculation access period. The aphids were then removed, and the exposed plantlets maintained in an insect-proof screen house for 20 weeks or till symptoms appeared. Every two weeks, starting a fortnight after exposure, the exposed, potentially infected, plantlets were tested for their ability to be a source of the virus for transmission to other healthy plants. For this, 10 healthy adult aphids were clipped onto the youngest fully open leaf of each plantlet and allowed a 72 h acquisition access period, before being transferred to the corresponding leaf of healthy test plantlets for a 48 h inoculation access (n = 4). All exposed plants (treatment group and test group) were monitored in a screen cage to document symptom development for 20 weeks. The control consisted of a similar setup, but with aphids accessing only the healthy plants of each cultivar.

2.3. Molecular Screening of Banana Plants and Aphids

PCR was used to assess whether the banana plantlets were infected and with subsequent sequencing to identify the aphid and virus involved. Total banana genomic DNA was isolated using the modified CTAB protocol [28]. The DNA pellet was re-suspended in 40 µL of 0.1 × TE buffer (10 mM Tris-HCL; 1 mM EDTA, pH 8). The resulting DNA sample was stored at −20 °C until use. DNA yield was assessed through electrophoresis and imaging on agarose gels in TBE.

PCR was used to detect BBTV and was performed in a duplex reaction to detect BBTV and the banana species-specific repetitive element (Brep 1) gene fragments [29]. The primers were specific for a 240 bp fragment of the BBTV master replication initiator protein (M-Rep) gene (DNA-R): BBTV-1F/5′-GCGTGAAACGCACAAAAGGCC-3′; BBTV-2R 5′-GCATACGTTGTCAAACCTTCTCCTC-3′ (240 bp; [30]). A 401 bp fragment of the banana species-specific repetitive element was amplified using the primers Brep-F 5′ GATTTTGTAGATTTTGGACACCG 3′; Brep-F-R: 5′-GAATAACAAATGCTCCAATACCC-3′, 401 bp [31]. The aphids were identified based on the amplification and sequencing of a 708 bp region of the mtCO1 gene using the DNA barcoding primers LCO1490t1 (5′-GCGTGAAACGCACAAAAG GCC-3′) and HCO2198t2 (5′-GCATACGTTGTCAAACCTTCTCCTC-3′) [25]. Two microliters of each the PCR products were loaded in their respective wells on a 2% agarose gel in Tris

Borate EDTA buffer (TBE) buffer and electrophoresed at 100 V (2 v/cm) with 1 × TBE running buffer.

Six aphid specimens and four infected leaf tissue specimens were submitted to INQABA Biotech, Ibadan, Nigeria, for PCR and DNA Sanger sequencing. The sequences were reviewed for base call accuracy, then joined using BioEdit [32]. These sequences were deposited in GenBank under the accession codes OM6861345-OM681347-OM249967 (BBTV) and OM630531-OM630536 (*Pentalonia nigronervosa*). The identity of the vector species and BBTV studied in this work was confirmed by BLAST analysis of the sequences obtained. Related sequences were selected from among the top 100 hits based on geographical location and extent of overlap. Aligned sequences were used to generate similarity matrices using MEGA X software [33], and consensus phylogenetic trees were generated to indicate the relationship of our sequences. Sequences of *Abaca* bunchy top virus (ABTV, *Babuvirus*, *Nanoviridae*) [34] and three *Aphis* spp. (*Homoptera*; *Aphididae*) were used as outgroups.

2.4. Identification of BBTD Symptoms

Each inoculated banana plant was examined every week for the appearance of typical BBTD symptoms on the leaf lamina, *Pseudostem* and leaf petioles (morse code streaks and dots; marginal chlorosis, bunchy aspect) [16,35]. The symptom classification was carried out according to a 5-scale classification [18]. The number of leaves with symptoms since exposure at the appearance of each symptom level was also recorded.

2.5. Statistical Analysis

Data on symptom development in individual plants were collected and summarized into proportions of plants showing BBTD per fortnight post exposure. The proportions of infected plants were compared per cultivar in R software version 4.0.3 (R Development Corporation). Successful infection rates were analyzed by ANOVA (analysis of variance) following the generalized linear model (GLM) procedure to compare cultivars by infection time, and finally a linear regression was performed on the data related to the average numbers of leaves showing the different stages of BBTD at different times of their occurrence.

3. Results

3.1. Symptom Development and Disease Evolution in Plants with Different Genotypes

The first symptoms appeared between 3 weeks post exposure in 'FHIA 25' and 16 weeks in '*Sotoumon*' (Table 1). The two plantain cultivars showed an intermediate rate of symptom development, with the first symptoms appearing four weeks after exposure. 'FHIA 25' showed the clearest symptoms of BBTD, i.e., dark green discontinuous streaks along the leaf petiole ('Morse code' streaks) and then on the leaf blade parallel to the secondary veins of the young leaves and marginal chlorosis of the leaf blade (Figure 1). The reduction in size of new leaf blades was apparent one week after the onset of these symptoms. At week 12, 'FHIA 25' plants developed dwarfing symptoms, with several new leaves emerging erect and stunted and forming a rosette-shaped cluster with a bunchy top appearance. In contrast, in '*Sotoumon*', no symptoms were observed until the fifteenth week, and consisted of stunting, narrow and shortened leaves, reaching level 5 (bunchy appearance) by the nineteenth week. Notably, this cultivar did not clearly show the typical early symptoms of BBTD (leaf streaks and marginal chlorosis). In '*Ebenga*' and '*Aloga*', the first symptoms (BBTD level 1) appeared during the 4th week, when dark green streaks were observed on the petiole and leaf blade with dark streaks extending to the *Pseudostem* (level 2). Marginal chlorosis (level 3) followed at about week 12 and 14 post inoculation, respectively. Similarly, dark green streaks parallel to the main veins were observed on the upper surface of the leaf blade. Beginning at week 15, a reduction in the size of the newly emerged blades was observed, and by the 17th week '*Aloga*' plants were no longer growing (level 4). All newly emerged leaves were stunted erect and formed rosettes (level 5) (Figure 1c,d). This final stage was observed in '*Ebenga*' at the 18th week. Of the four cultivars tested, 'FHIA 25' was the first to present BBTD symptoms, during the third week post exposure.

Table 1. Disease expression and progression in plants with different genotypes according to the five-stage rating scale used to assess the severity of BBTD [20].

Symptoms	Cultivars				Remarks
	FHIA 25	***Ebenga***	***Aloga***	***Sotoumon***	
Dark streaks along the petiole and young leaf	3 weeks	4 weeks	4 weeks	-	Presence of streaks only on the petiole of FHIA 25 plants
Dark streaks along the pseudostem	7 weeks	9 weeks	12 weeks	-	Presence of streaks on the petiole and on the plant blades
Marginal chlorosis of the leaf blade margin with normal size	8 weeks	12 weeks	14 weeks	-	Absence of marginal chlorosis of the leaf blade margin with normal size in *Sotoumon*
Reduction in the size of newly emerged blades	10 weeks	15 weeks	15 weeks	15 weeks	Presence of striae plus reduction in leaf size in all cultivars
Leaves form a rosette-like cluster-Bunchy top	12 weeks	17 weeks	17 weeks	19 weeks	Presence of bunchy top in all cultivars

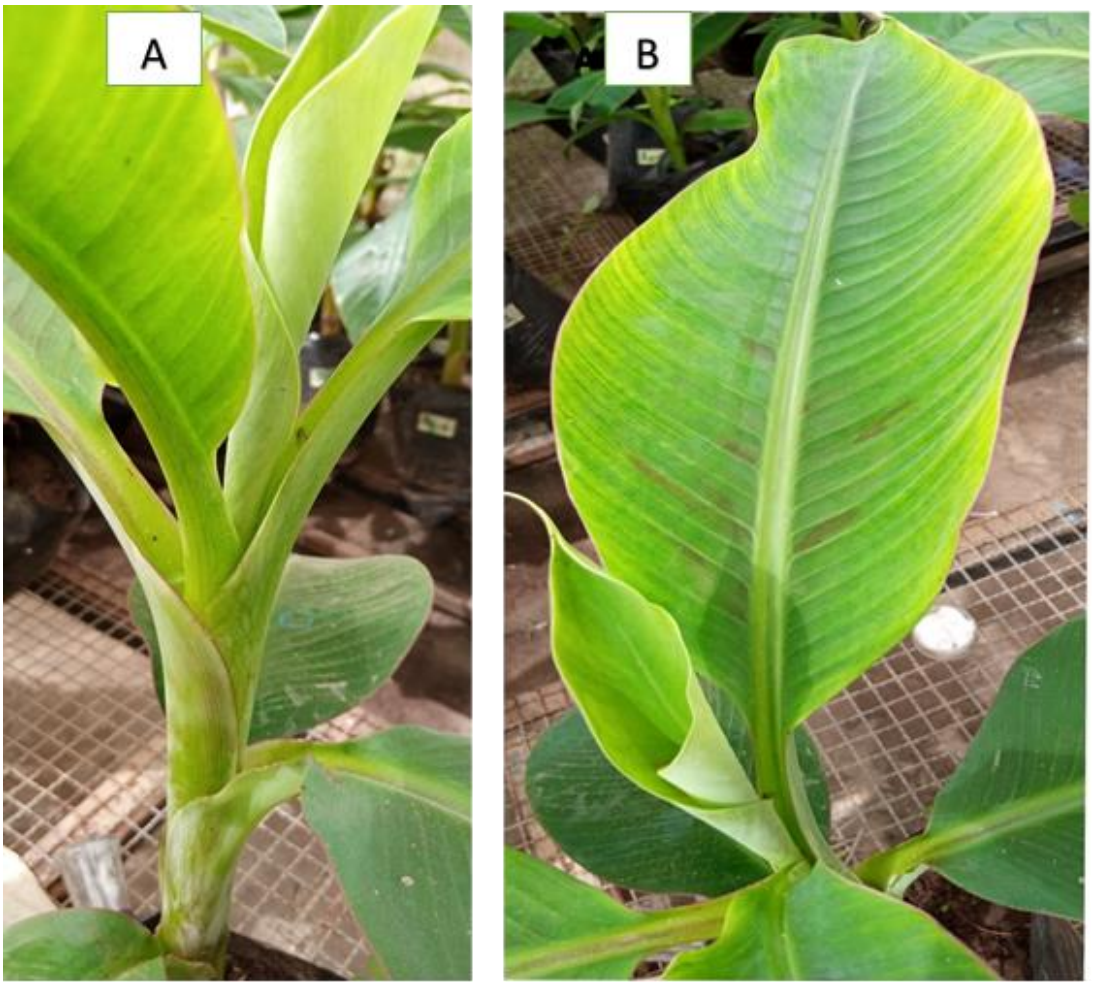

Figure 1. *Cont.*

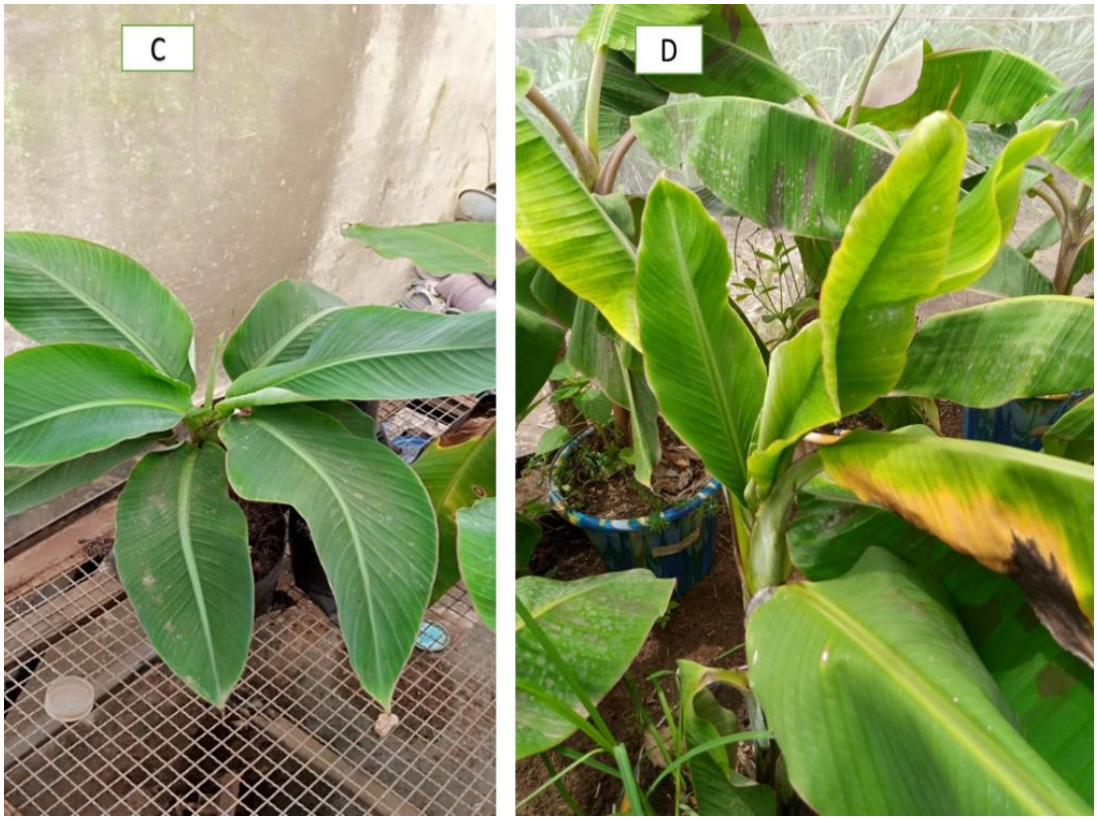

Figure 1. Early symptoms of BBTD. Dark green discontinuous 'Morse code' streaks (**A**) and marginal chlorosis (**B**) were the first typical symptoms of BBTD observed on the leaf lamina of all cultivars except '*Sotoumon*'. Late BBTD symptoms: incipient leaf size reduction (**C**) and advanced leaf dwarfing creating the bunchy top symptom (**D**) were observed in all cultivars. Where present, the earlier symptoms continued to show when the plants expressed the final stages of BBTD.

3.2. Leaf Positions at Symptom Expression

The time to onset of the Morse code streaks and the number of leaves emerging before the onset of this symptom were significantly different for 'FHIA 25', '*Sotoumon*' and '*Aloga*' (F-statistic: 575.8 on 3 and 82 DF, p-value: $<2.2 \times 10^{-16}$). The time to onset of marginal chlorosis and the number of leaves emerging before the onset of this symptom were significantly different between 'FHIA 25', '*Sotoumon*' and '*Aloga*' (F-statistic: 992 on 3 and 82 DF, p-value: $<2.2 \times 10^{-16}$). The time to onset of stunting and the number of leaves emerging before the onset of stunting were significantly different between 'FHIA 25', '*Sotoumon*' and '*Aloga*' (F-statistic: 291.2 on 3 and 82 DF, p-value: $<2.2 \times 10^{-16}$) (Table 2).

Table 2. Leaf positions at symptom expression.

	Leaf Number at Onset of Typical Symptom [1]		
Cultivar	**Morse Code**	**Marginal Chlorosis**	**Stunting Onset**
FHIA 25	3.59 ± 0.48^{c}	4.59 ± 0.48^{c}	5.59 ± 0.48^{a}
Ebenga	5.23 ± 0.37^{b}	6.18 ± 0.30^{b}	6.77 ± 0.49^{b}
Aloga	5.09 ± 0.17^{b}	6.09 ± 0.17^{b}	7.09 ± 0.17^{c}
Sotoumon	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	10.47 ± 0.56^{d}

[1] Means within a column followed by the same letter are not significantly different ($p < 0.05$).

3.3. BBTV Transmission at the Cultivar Level

The rate of infection of the cultivars varied. Of the 30 plants exposed for each cultivar, 'FHIA 25' presented the highest proportion developing BBTD symptoms, corresponding

to 90%, while only 50% of '*Sotoumon*' plants were infected ($F_{3,8}$: 6.952; DF, $p \leq 0.0128$). (Figure 2). BBTV was not detectable in any of the plants at two weeks after inoculation. The rates of transmission of BBTV from the infected plantlets of the four cultivars to the 'FHIA 21' test plantlets generally reflected the cultivars original infection rates. The rates of infection of the treatment plants ('FHIA 25'; '*Aloga*'; '*Ebenga*' and '*Sotoumon*') from which BBTV was transmitted at week 4 were 30%, 20%, 27% and 0%, respectively. These rates gradually increased to 92%, 83%, 86% and 50% at week 20, respectively (Table 3). No infection was observed from plants previously exposed to viruliferous plants before they developed symptoms. At four weeks post exposure, the rates of successful transmission from symptomatic plants were as follows: 'FHIA 25' (20.82%), '*Aloga*' (19.15%), '*Ebenga*' (16.65%) and '*Sotoumon*' (0%). These rates gradually increased and reached 98.7%, 72.5%, 55.75% and 10% for each cultivar, respectively, at week 20 (Figure 3). These results were confirmed by PCR.

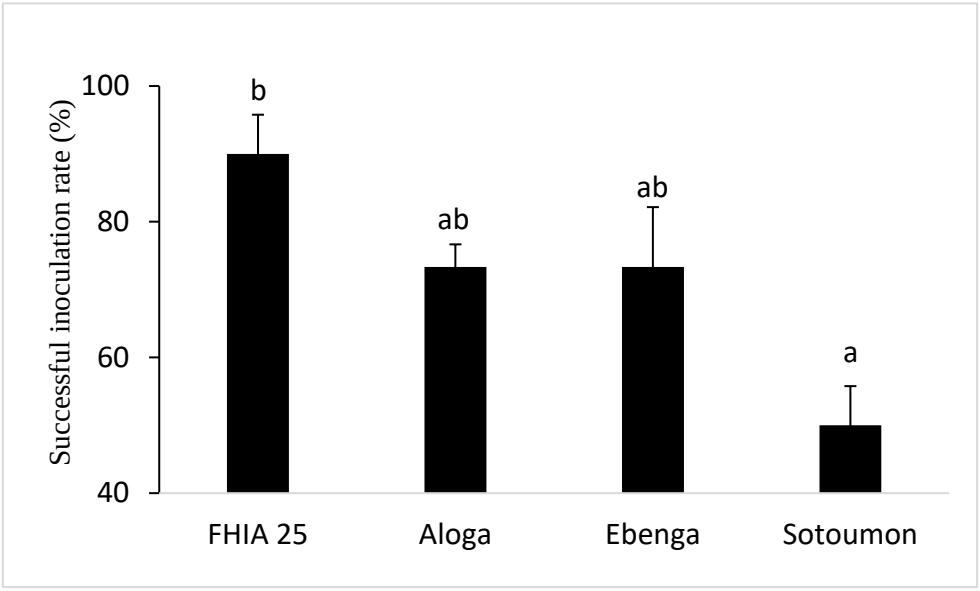

Figure 2. Infection rate of the treatment plants of each cultivar exposed to BBTV. Bars followed by the same letter are not significantly different, ($p < 0.05$).

Table 3. Percentage of plants of each infective cultivar at each date.

Treatment (Cultivars)	Percentage Transmission Per Fortnight (Weeks)									
	2	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
FHIA 25	0	30	30	37	44	56	69	81	87	92
Ebenga	0	27	27	36	45	64	73	73	82	86
Aloga	0	20	20	30	40	60	70	70	80	83
Sotoumon	0	0	0	0	0	0	0	0	25	50

3.4. Phylogenetic Analyses

Phylogenetic analyses based on the 580 bp barcoding region of the COX1 gene of *Pentalonia* spp. revealed a clear division into two clusters, representing the sibling species *P. nigronervosa* and *P. caladii*. Our specimens all clustered together with a nucleotide similarity of 100% among themselves and with other previously deposited *P. nigronervosa* sequences from the region (Figure 4). The *P. nigronervosa* clade further separated into two subclades with an identity difference of 1.7%., one including the samples from India and Nigeria, and the other including the rest of the samples. The two clades of *P. caladii* and *P. nigronervosa* showed a difference of 4.1% in nucleotide similarity. BBTV sequences for the OM6861345 BeninY01, OM6861347 BeninY03 and OM6861367 BeninY04 isolates were most

similar to other BBTV isolates from Benin (Figure 5). The African and Asian isolates of BBTV separated into different clades, consistent with previously reported sub-groupings [15].

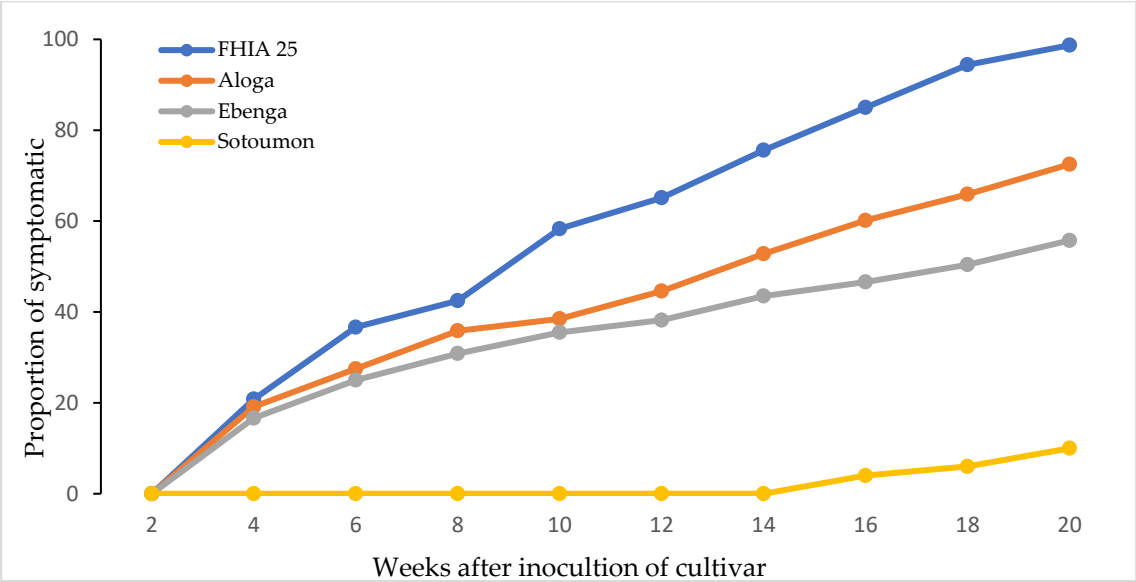

Figure 3. Rate of BBTV transmission from the treatment plants to healthy FHIA 21 plantlets.

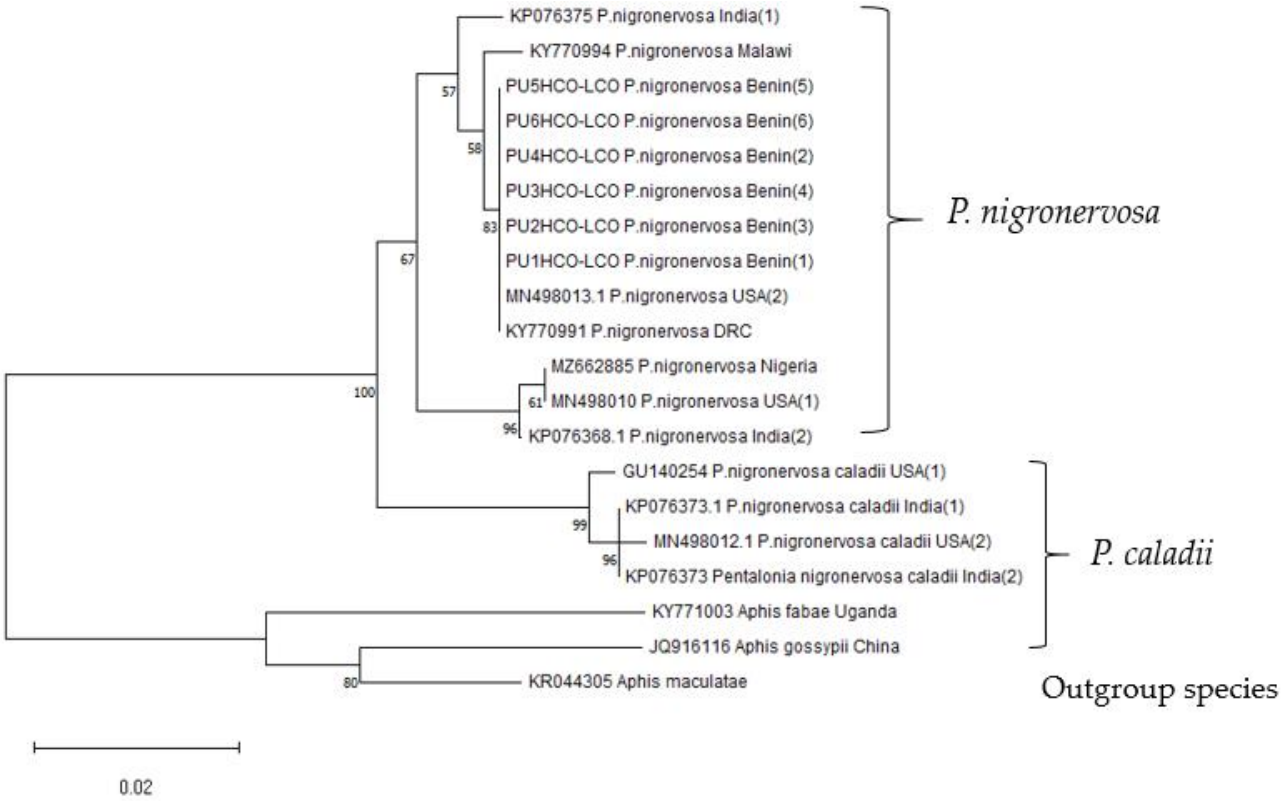

Figure 4. Phylogenetic analysis of the mitochondrial gene of cytochrome c oxidase subunit I (COI) of selected populations of the banana aphid *P. nigronervosa* and the sister species *P. caladii*. Values on tree branches represent the branch length between nodes.

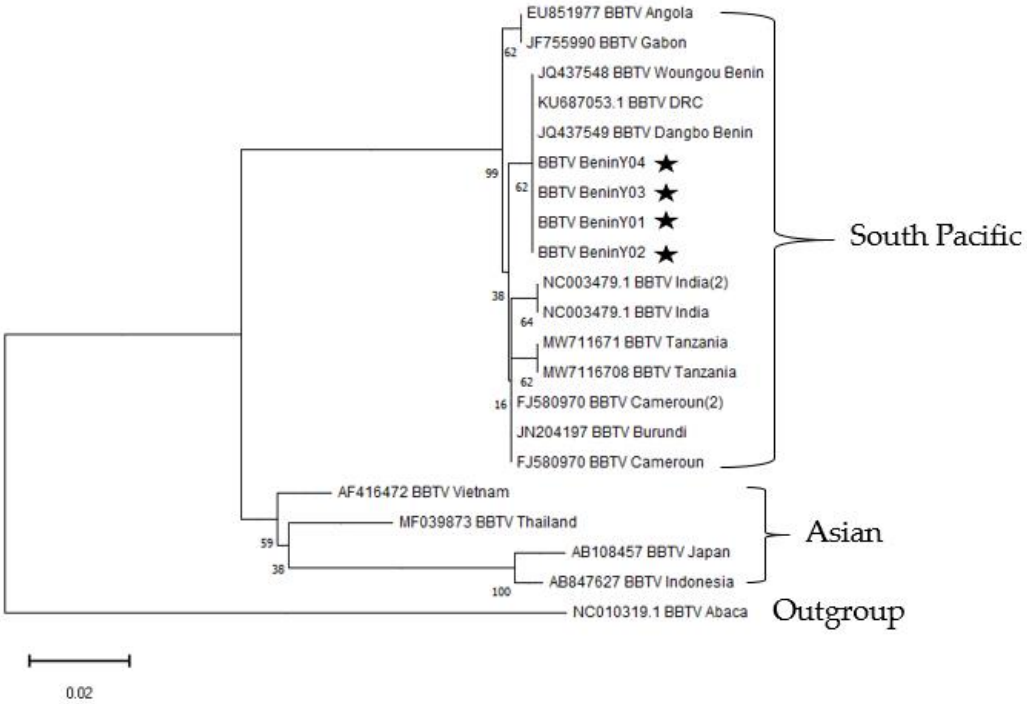

Figure 5. Phylogenetic analysis of the BBTV virus revealed two major clades: the Asian and the South Pacific, which cluster with the specimens from Benin (BBTV BeninY01, BBTV BeninY02, BBTV BeninY03, BBTV BeninY04). Values on tree branches represent the branch length between nodes. The accessions marked with * were the specimens from this study.

4. Discussion

In this study, we investigated the variation of BBTD symptom expression in banana plantlets of three AAB types: two plantain cultivars, one dessert banana and a hybrid cultivar (AAAB). The cultivars varied in terms of the proportion of plants showing symptoms, the date of the first symptom appearance and the progression of the symptoms to the late typical terminal bunchy expression. In addition, we noted a difference in the clarity with which these symptoms were expressed, and some symptoms were not observed. '*Sotoumon*' (Silk/Apple banana group) showed delayed symptom expression, poor or no expression of some of the early typical symptoms and reduced infectiousness. The infectiousness of the diseased plants was closely associated with the appearance of the first symptoms. Allen [20] established the link between the field risk of secondary disease spread and the disease stage in the cultivar 'Cavendish' (AAA). Differences in cultivar presentation of the disease has been shown before. To our knowledge, this is the first report linking symptom expression and infectiousness and comparing this relationship in different cultivars. Knowledge of differential symptom expression and its relationship with infectiousness of the plants can help inform the rouging threshold and frequency for the management of BBTD.

The elimination of the infected individuals is an intuitive approach to disease management, designed to minimize secondary infections. The relationship between the detection of infectious plants and secondary disease spread is a key factor in disease control. The early detection of symptoms before plants become infective makes the method of removing diseased plants more effective in controlling the spread of BBTD. The study of symptom expression and transmission of BBTD in different cultivars provides useful information to make the control and management of the disease more effective. Any biological or environmental factor influencing symptom expression is important in the control of BBTD, especially at the producer level. These variables may be plant-based (genetic factors influencing symptom expression), environmental or even human-based (accuracy of early symptom detection and speed of the removal of the infected plants).

This study showed that the expression and development of BBTD varies from one cultivar to another. In fact, three categories of cultivars with different reactions to BBTD were noted, namely, the highly susceptible cultivar 'FHIA 25′ (AAAB), the moderately susceptible cultivars '*Aloga*' and '*Ebenga*' (AAB) and the less susceptible cultivar '*Sotoumon*', which is probably in the AAB group. BBTV was infectious for 'FHIA 25′, '*Aloga*' and '*Ebenga*' 4 weeks after its inoculation with *P. nigronervosa*. In contrast, BBTV was first infectious 16 weeks after inoculation in '*Sotoumon*' plants, one week after the onset of BBTD symptoms. The differences in symptom expression and their relationship with the virus transmission rate could be a result of differences in viral load in the different cultivars at the different time points. Between the four cultivars, the relative BBTD severity expression level (FHIA 25 > *Aloga*/*Ebenga* > *Sotoumon*) was similar to the relative infection rate (susceptibility) and also to the suitability as a source of BBTV to infect the test plants. This could suggest a difference in the amount of virus in the plant, transferred by the feeding vectors and the infection and available for the subsequent transmission steps. We cannot rule out the effect of a different inoculation load of BBTV at the start and its potential influence in the subsequent virus titer in the plant. Studies of the vector feeding behavior in different cultivars and a comparison of the virus multiplication rates would be essential to clarify these differences.

These results have several implications for BBTD control, the recovery of production in BBTD endemic areas and the risk of banana seed degeneration in such areas [14]. Our data showed a significant variation in the symptom presentation and transmission of BBTV from exposed plants and hence a varied epidemiological risk at the landscape scale. Early symptom expression favors disease detection and elimination. Our data also showed a limited risk of BBTD transmission from asymptomatic plants which would reduce the field-level transmission. However, this poses a much greater risk, on seed systems, of seed degeneration through the inadvertent spread of infected, but symptomless, propagules. Furthermore, these results showed that the expression of symptoms and the transmission of BBTD are linked, since BBTD became infective in all four cultivars after the first symptoms of the disease appeared.

5. Conclusions

The early detection of diseased banana plants is the best method of controlling BBTD spread for effective disease management. To improve this control method, the present study focused on finding the relationship between symptom development and BBTD transmission from four banana cultivars. The expression of BBTD varied between cultivars in terms of time to onset of symptom expression and the typical symptoms showed. Overall, the earlier the symptom development, the earlier the possibility to transmit BBTV from plants of each cultivar. Transmission of BBTV from infected plants was achieved when the first symptoms were also detected; therefore, in our hands, only the symptomatic leaves were successful sources of inoculum. The more strongly symptomatic cultivars were also more susceptible to BBTV. Therefore, the expression of symptoms and the transmission of BBTD were linked. This information is crucial for the early detection of diseased plants in banana-producing areas. The variation in typical symptoms observed remains a challenge for optimizing rouging protocols especially among smallholders with genetically diverse holdings.

Author Contributions: Conceptualization, A.B.O., A.G.D., H.A.-S. and M.C.; methodology, M.C. and A.B.O.; validation, A.B.O., A.G.D., H.A.-S. and M.C.; formal analysis, M.C.; investigation, M.C. and A.B.O.; resources, A.B.O. and J.T.; data curation, M.C.; writing—original draft preparation, M.C.; writing—review and editing, M.C., A.B.O., J.T., A.G.D. and H.A.-S.; supervision, A.B.O., A.G.D. and H.A.-S.; project administration, A.B.O. funding acquisition, A.B.O. and J.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by grants from the CGIAR Programme on Root, Tubers and Bananas: BA 3.4—Resilient Cropping Systems, Banana Viral diseases; with staff time partly supported by the CGIAR's Plant Health Initiative. We thank all funders who have supported this research through their contributions to the CGIAR Trust Fund: (www.cgiar.org/funders/). This work was supported,

in part, by the Bill & Melinda Gates Foundation [INV010652]. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission.

Data Availability Statement: The raw data from this study are available from the corresponding author upon request.

Acknowledgments: We thank Y. Blandine Fatondji and the Melaine Gbeto of LaREGAME (FAST/UAC) for technical assistance in the molecular analyses of banana plants. We also thank Christian Allikponton for help with banana macropropagation and the rearing of aphid colonies.

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

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