Banana bunchy top virus in South Africa: The distribution, molecular relationship and transmission studies

by

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Submitted in fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Science

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

December 2021

THESIS SUMMARY

The first report of Banana bunchy top virus (BBTV) in KwaZulu-Natal (KZN), South Africa in 2016 has raised the need to study the virus in South Africa. The aim of this research project was to conduct surveys across banana-producing provinces, including KwaZulu-Natal, Mpumalanga and Limpopo in South Africa, to determine the spread of BBTV in banana production regions across the country. To date, the virus has been localized within the province of KZN in the South Coast region. Once positive samples were obtained, the genetic relationships between the South African isolates and those collected globally was investigated. This was done by studying five (DNA-C, -S; -N; -M; -U3) of the six components of the BBTV genome. No major differences between the isolates were observed. As the virus is only transmitted through infected planting material and through the vector, *Pentalonia nigronervosa*, BBTV transmission studies were conducted. Such studies have been conducted in different countries on this topic with conflicting results and BBTV transmission studies were included here as well. Plant species namely *Colocasia esculenta*, *Alocasia macrorrhizos*, *Alpinia zerumbet* and *Strelitzia reginae*, that are usually found growing around banana plantations, were investigated to determine if these plants act as reservoirs of the virus vector and also to determine if these potential alternative host plant can be hosts of the virus. Banana plants were included as controls in the experiment. A qPCR was optimised to test for BBTV in the plants and aphids at low concentrations. BBTV was detected in all of the plants except *A. macrorrhizos*. It was concluded that *A. zerumbet* was an alternative host of the banana aphid while *C.esculenta* and *S. reginae* are assumed to be intermediary hosts of the virus vector while *A. macrorrhizos* is neither a host of the vector nor of the virus.

DECLARATION

I, **Sinethemba Patience Fanelwe Ximba**, declare that:

i. The research reported in this thesis except otherwise indicated is my original work;

ii. This dissertation has not been submitted for any degree or examination at any other university

iii. This dissertation does not contain other person's data, pictures or graphs or other information, unless specifically acknowledged as being sourced from other persons

iv. This dissertation does not contain other person's writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written but the general information attributed to them has been referenced.

v. This dissertation does not contain text, graphics or tables copied and pasted from the Internet unless specifically acknowledged and the source being detailed in the thesis and in the References section.

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Dr A. Gubba (co-Supervisor)

ACKNOWLEDGEMENTS

- Praise be to the Almighty for making this happen. Indeed ‘when the time is right, I the Lord will make it happen’- Isaiah 60vs22
- A special thank you to my Supervisors Prof Gus Gubba and Dr Elize Jooste for the continued support throughout this journey.
- Dr Jacques Ibaba for your willingness to help me in all aspects of my academic career, for that I will always be grateful.
- To my parents who have always supported my vision and special mention to my Mom who has carried me in prayer at all times.
- I am grateful to my siblings Simtho, Thandaza and Mzomuhle for being a great support structure.
- To my wonderful and supportive husband, Thandinkosi Majikijela
- To my amazing children, Zethembiso and Alunamida Majikijela
- To Tumisho, thank you for being a great cheerleader
- To the ladies at ARC-Plant Pathology: Reshika, Thabile, Ayanda, Lebo, Zama, Saneh, Zanele and Ngcebo.
- To Lungile Linda, you have been extremely supportive and I am grateful for that.
- To Ronel Roberts, your supportive nature and willingness to share knowledge contributed significantly to this work.
- To the ever-supportive UKZN Plant Path 340 Family: Nonduduzo Ncwane, Phumzile Mkhize, Ncediwe Pute, Nompumelelo Dammie, Zandile Mngadi and Gilmore Pambuka.

DEDICATION

To my late maternal Grandmother, Lydia Busisiwe “maZakaliya” Khoza

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µl	Microliter
ARC	Agricultural Research Council
ARC-TSC	Agricultural Research Council Tropical and Subtropical Institute
BBTD	Banana bunchy top disease
BBTV	Banana Bunchy top Virus
BLAST	Basic local alignment search tool
Bp	Base pair
CP	Coat Protein
Cq	Quantification cycles
CR-M	Major common region
CR-SL	Stem-loop common region
CTAB	Cetyltrimethylammonium bromide
DAFF	Department of Agriculture, Forestry and Fisheries
DALRRD	Department of Agriculture, Land reform and rural development
DARD	Department of Agriculture and Rural development
DNA	Deoxyribonucleic acid
Dot-Iba	Dot immunobinding assay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
FAO	Food and Agriculture Organization
H	hour
IEM	Immune electron microscopy
KZN	KwaZulu-Natal
LAMP	Loop-mediated isothermal amplification
MARS	Multiple circular sequence Alignment using Refined Sequences
Min	minutes
mM	Millimolar
NaCl	Sodium Chloride
NCBI	Biotechnology Information
ORF	Open reading frame
PCR	Polymerase chain reaction
RCA	Rolling-circle amplification
RSA	Republic of South Africa
S	seconds
SEA	South East Asia
ssDNA	Single stranded DNA
TAE	Tris-acetate-EDTA
Tris	Trisaminomethane
U	Units of enzyme
USD	Currency of the United States of America

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THESIS INTRODUCTION

Bananas are the developing world's sixth most important food crop (after rice, wheat, maize, potatoes and cassava) in terms of gross value of production. The crop boasts an annual world production of around 115 million tonnes, of which around a third is produced in each of the African, Asia-Pacific, and Latin American and Caribbean regions (FAO, 2019). Around 87% of all the bananas grown worldwide are produced by small-scale farmers for home consumption or for sale in local and regional markets. They provide a staple food for millions of people, particularly in Africa, an area where the green revolution has had little influence (Frison *et al*., 2004). As well as providing a cheap and easily produced source of energy, bananas are also rich in certain minerals and in vitamins A, C and B6 (Frison *et al*., 2004). The crop is becoming more and more important as a source of revenue, sometimes providing the main source of income for rural communities. Bananas thus play an important role in poverty alleviation (Frison *et al*., 2004).

Problem Identification

Banana bunchy top disease (BBTD) was first reported from Africa in 1901. This disease is caused by the banana bunchy top virus (BBTV) and is considered the most devastating disease of banana. BBTV is a quarantine virus that is included in the South African Phytosanitary Services list of pathogens which must be absent in imported Musaceae propagation material (Article 3(1) of the Agricultural Pests Act, Act 36 of 1983 of South Africa). The disease is spread by the banana aphid (*Pentalonia nigronervosa*) and through infected propagation material. Over the last two decades it has spread to 16 Sub-Saharan Countries and in 2007 it was reported in neighbouring Mozambique (Gondwe, 2007; IPPC; 2016).

The presence of BBTV in neighbouring countries poses a huge threat to banana production in South Africa as Limpopo and Mpumalanga (both major producers) share a border with Mozambique.Recently, the virus was detected in an isolated area in the KwaZulu-Natal South Coast region (Jooste *et al*., 2016). The source of infection in this region is currently unknown. Yield losses of up to 100% can be experienced as infected plants generally fail to produce bunches.

The lack of survey data confirming the presence or absence of the virus in other banana growing regions of South Africa is a major concern. The last survey was conducted in 1996 in the Kiepersol area in Mpumalanga Province, which is only one of the six banana producing areas in South Africa (Pietersen *et al*., 1996). Since then, no other survey was done to investigate the presence of the virus locally.

As BBTV can be introduced through infected planting material (or planting material infested with infective aphids), or by wind-borne aphids, the threat BBTV might cause severe crop losses in the region where the virus was initially detected was a major concern. Infected plants don't always show symptoms immediately and if the virus can be detected in the aphid vector, prior to symptom development in plants, it will ensure a proactive management strategy towards the spread of the virus.

As the virus is transmitted by the aphid vector, *Pentalonia nigronervosa*, there is a concern that plants that are found growing close to banana plantations may act as overwintering plants for this vector. As a result, even with stringent monitoring strategies, potential alternative hosts may need to be examined as part of scouting for the virus.

Significance of Research

In the last 20 years, no detection surveys have been conducted locally to determine the spread of BBTV in South African banana production areas. The detection of the virus in the KwaZulu-Natal South Coast region warranted further action from a research perspective. This knowledge will support management strategies for the region, aimed to contain the virus. This is the first comprehensive study in South Africa to determine the distribution of the virus in banana plantations.

The objectives of this study were:

- To monitor disease presence by conducting surveys across the main-producing regions of South Africa and introduce a rural development and education program on identification of BBTV symptoms and management practices in the KZN South Coast region, the initial outbreak site.
- To analyse the different genetic components of BBTV in order to determine the relationship of the South African isolates to global BBTV isolates.

- To determine if plants growing in close proximity to infected plants serve as potential hosts of the banana aphid and for banana bunchy top virus.
- To evaluate results and publish research findings as well as attending local and international conferences to share results.

This dissertation comprises of four discrete chapters: Chapter 1 is a review of current literature on BBTV. Chapter 2 focuses on the surveys that were conducted in the three major banana-producing provinces. Chapter 3 reports on sequence analysis and phylogeny of five components of BBTV. Chapter 4 reports on the transmission study to determine potential alternative hosts of the aphid vector as well as the virus. This thesis format is adopted by the University of KwaZulu-Natal because it facilitates the publication of research output more readily than the older monograph form of thesis. There is, therefore, some unavoidable repetition of references and introductory information between chapters.

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CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Banana (*Musa* spp.) is among the important food security crops cultivated in over 120 countries worldwide (Rustagi *et al*., 2015). It is considered an important staple crop for millions of people in several developing regions of the world (Watanabe *et al*., 2013). It is the sixth-ranked food crop produced worldwide following maize, rice, wheat, potatoes and cassava. Exact figures on global banana production are difficult to obtain as smallholder farmers conduct the bulk of banana cultivation informally. Available data indicate that average global banana production is currently 115 million tonnes as of the 2017-2019 seasons, at an approximate value of 40 billion USD (FAO, 2019). Based on 2019 figures, the global banana export industry generates around 13.5 billion USD per year (FAO, 2019). It is, however, important to note that only about 18 percent of the total global banana production is traded in the international market. The rest is consumed locally, most importantly in large producing countries such as India, China, and Brazil, and in some African countries (including but not limited to Uganda, Angola, Rwanda, Nigeria and Cameroon) where bananas contribute significantly to people's diets (FAO, 2019).

Banana is mostly grown for local consumption in South Africa. This is mainly due to the country's location and its subtropical climate, which makes it difficult to compete with equatorial banana-producing countries on world markets. This limits the export potential for South African bananas (DAFF, 2017). Approximately 415 000 tons were harvested during the 2018/19 marketing season valuing the industry at approximately 137 million USD. This makes banana one of the most important subtropical fruits grown in South Africa (DAFF, 2019).

1.2. Threats to banana production

Even though it has a rich genetic diversity, the existence of bananas is threatened by a range of important pests and diseases (Mware, 2016; Jebakumar *et al*., 2018). All known commercial banana varieties are highly susceptible to fungal, viral and bacterial pathogens as well as nematodes and insect pests (Jones, 2000).

Black Sigatoka was first noticed in Fiji in 1963 and is regarded as the most economically important leaf disease of banana (Jones, 2000). Its widespread distribution in and around the Pacific suggests that it had been in the region for a while before being discovered in Fiji (Meredith, 1970). Its first authenticated report in Africa was in Gabon in 1978 (Blomme *et al*., 2013). Even though Black Sigatoka does not kill plants immediately, the crop losses increase gradually with the age of plantings. It destroys banana leaves, which leads to a reduction in yield and premature ripening of the fruit. (Jones, 2000).

Another very important disease of banana is Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense*; also known as Panama disease. By 1960, the disease had almost crippled the banana industry in Central America (Pegg and Langdon, 1987). The export industry had to change to cultivars in the Cavendish group (AAA) after an estimated 40 000 ha of Gros Michel (AAA) was destroyed. Fusarium wilt is believed to be one of the most catastrophic of all plant diseases (Simmonds, 1966; Ploetz and Pegg, 2000).

Similarly, banana bunchy top virus (BBTV) inflicted devastating effects in the Australian banana industry in the early 20th century. Strict quarantine regulations and enforcement ensured that the disease is contained (Magee, 1927; Dale, 1987).

1.3. Distribution of BBTV worldwide

BBTV was first reported in the Fiji Islands in the late 1880s (Magee, 1927) and has been reported in banana-producing regions of the world including Africa, Australia, Asia and the South Pacific Islands but is still absent in banana producing regions of the Americas (Figure 1.1). In Africa, BBTV was first reported in 1901 in Egypt. Currently, it has been reported in the Democratic Republic of Congo (Wardlaw,1961), Eritrea (Saverio,1964), Gabon (Manser,1982), Republic of Congo, Burundi and Rwanda (Sebasigari and Stover,1988), Central African Republic (Diekmann and Putter, 1996), Malawi (Kenyon *et al*.,1997), Angola (Pillay *et al*., 2005), Zambia and Mozambique (Gondwe *et al*., 2007) as well as Cameroon (Oben *et al*., 2009). It has also been reported in Benin (Lokossou, 2012), Nigeria (Adegbola *et al*., 2013) and South Africa (Jooste *et al*., 2016). In 2018, BBTV was reported in Togo but swift implementation of control measures were put in place to control its spread (IITA news,

2019; Kolombia, 2021). The virus has also been reported in Tanzania (Shimwela *et al*., 2022) and Uganda (Ocimati *et al*., 2021).

The continued spread of BBTV poses a major threat to any country's banana industry as production decreases drastically; for example; in Malawi 80% of Cavendish commercial production was adversely affected by the virus outbreak. Furthermore, an 88% reduction in Hill banana cultivation was reported in Tamil Nadu, India from 1970-2015 (Elayabalan *et al*., 2015; Jekayinoluwa *et al*., 2020). Losses are not restricted to reduced yields but also to abandonment of susceptible but high-yielding plantations by farmers (Jekayinoluwa *et al*., 2020).

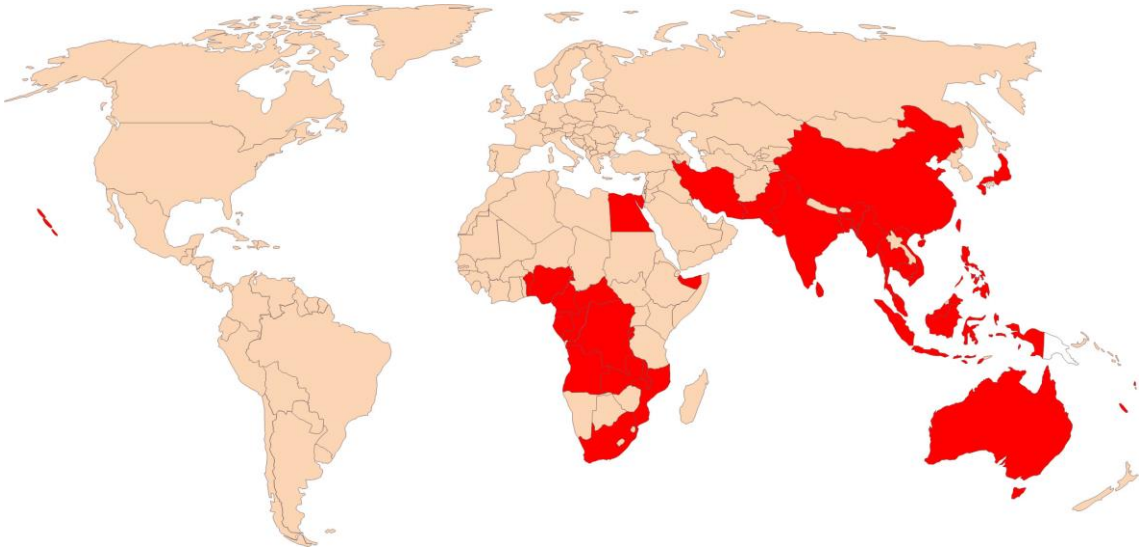

Figure 1.1. Map showing the reports of BBTD globally by 2020 (Jekayinoluwa *et al*., 2020)

1.4. Banana bunchy top virus

1.4.1. Genome Organization

BBTV has a circular single stranded genome and 18nm-20nm icosahedral virus particles, belongs to the genus *Babuvirus* in the family *Nanoviridae*. It is the causal agent of banana bunchy top disease (BBTD). The virus has a complex genome comprising six encapsidated circular, single stranded DNA components which are approximately 1kb each in length and are named DNA-R, -U3, -S, -M, -C, and -N, respectively, according to their putative functions (Gronenborn, 2004) (Figure 1.2). A few isolates have additional Rep-coding components (satellite Rep) (Qazi, 2016). DNA-R encodes a replication initiation protein (Rep) which supports the replication of other non Rep-encoding components (Fu *et al*., 2009; Horser *et al*., 2001). The open

reading frame (ORF) of DNA-U3 is not always present among characterized isolates, and the function of DNA-U3 in BBTV infection is currently unknown (Fu *et al*., 2009) while DNA-S encodes the viral capsid protein (Wanitchakorn *et al*., 1997; Tsao, 2008). DNA-M encodes a putative movement function protein with a hydrophobic N-terminus while DNA-N encodes a nuclear shuttle protein (Wanitchakorn *et al*., 1997; 2000). DNA-C encodes a protein that can presumably facilitate viral replication by binding retinoblastoma and may be involved in host-cell-cycle manipulation (Wanitchakorn *et al*., 2000). The genome components are characterized by the presence of a stem-loop common region (CR-SL) and the major common region (CR-M). The CR-SL structure has a conserved sequence, TA(G/T)TATTAC, in the loop region. The CR-SL can be identified in all single-stranded plant DNA viruses, including geminiviruses and nanoviruses. It contains the origin of replication for single-stranded DNA viruses (Hafner, 1997a). Notably, among BBTV integral components, the conserved loop sequence is TATTATTAC; however, in additional Reps, the sequence is TAGTATTAC (Heyraud-Nitschke *et al*., 1995; Hafner *et al*., 1997b, Fu *et al*., 2009). The CR-M is the binding site for DNA primers associated with complementary strand synthesis (Wanitchakorn, 1997; Su *et al*., 2003).

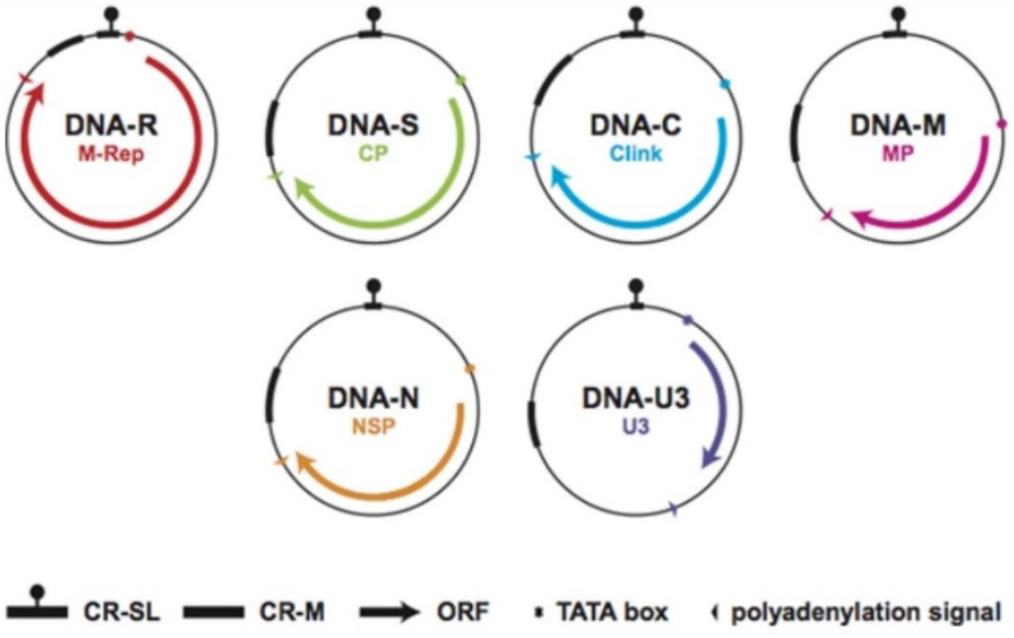

Figure 1.2. Genomic organization of the components of the BBTV genome (ICTV, 2021)

1.4.2. Hosts

BBTV has been detected in many cultivars of edible bananas, with varying susceptibility. BBTV infects *M. balbisiana*, *M. acuminata*, *M. acuminata* x *balbisiana*, *M.ornata* and *M.velutina* (Magee, 1927, 1948; Thomas and Dietzgen, 1991, Espino *et al*., 1993). BBTV also infects *Ensete ventricosum*, a closely related species in the *Musaceae* (Wardlaw, 1961). Reports outside of the *Musaceae* have not been substantiated due to conflicting results (Hu *et al*. 1996, Geering and Thomas, 1997; Ploetz *et al*. 2003; Watanabe *et al*. 2013). Ram and Summanwar (1984) reported *Coloscasia esculentum* as a host of the virus; however, Hu *et al*., (1996) were unable to demonstrate this. *Alpinia purpurata* (red ginger), *Caana indica* and *Hedychium coronarium* have also been reported as alternative hosts (Su *et al*., 1993). BBTV is also reported to infect *Calladium* sp, *Diefenbachia* sp and *Xanthosmoma* sp in the Araceae family as well as *Costus* sp and *Hedychium* sp in the Zingeberaceae family (Thomas, 2019).

1.4.3. Phylogenetic classification

Two broad phylogenetic groups for BBTV isolates have been identified based on nucleotide sequence analysis of the DNA-R, - N and –S regions (Karan *et al*., 1994; Bell *et al*., 2002; Hu *et al*., 2007; Amin *et* al., 2008). These include the ‘South Pacific’ subgroup comprising isolates from Australia, the Pacific Islands, India, Iran, Myanmar, Pakistan and Africa and the ‘Asian’ subgroup comprising isolates from Philippines, Vietnam, China (including Taiwan) and Indonesia (Bananej *et al*., 2007; Karan *et al*., 1994; Kumar *et al*., 2011; Wanitchakorn *et al*., 2000). The later grouping has been modified recently to the ‘Pacific-Indian Oceans’ group and ‘South East Asian’ group (Qazi, 2016). Based on analysis of full-length sequences of DNA-R, variability between isolates of the two subgroups was as high as 10%, while within isolates of each subgroup, the variability ranged from 1.9% in the South Pacific isolates to 3% in the Asian isolates. The nucleotide sequence of the CR-M is 96.6% conserved within South Pacific subgroup but only 68% between subgroups (Karan *et al*., 1994). When the nucleotide sequences of DNA-N were used in the analyses, the nucleotide similarity between the two subgroups was 85.5% (Mware, 2016).

1.4.4. Virus Transmission

In order to ensure survival in nature, plant-infecting viruses must be efficiently transmitted among plants and gain new hosts. In order to achieve this, a majority of plant viruses use specific vectors to move from plant to plant (Whitfield *et al*., 2015). BBTV is persistently transmitted by *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae) (Figure 1.3 A & B) in a circulative, non-propagative manner. This type of transmission occurs whereby the virus enters the insect body and spreads to different tissue systems. The virions move through the gut via a transcytotic pathway which is similar to luteoviruses. The virions are released from the gut cells, make their way into the insect hemocoel and enter the primary salivary glands before being transmitted into the respective host. The virus does not replicate in the body of the insect (Whitfield *et al*., 2015). Successful vector transmission depends on various factors such as temperature, vector life stage and a minimum access period from the plant (Anhalt and Almeida, 2008). Longer acquisition access periods increase the viral load in the hemolymph and salivary tissues of the aphid (Watanabe *et al*., 2013; Whitfield *et al*., 2015). For the aphids to be infective, they need to feed for at least 4 hours on BBTV-infected plants (Suparman *et al*., 2017). To transmit the virus, viruliferous aphids need at least 1.5 hours of feeding on susceptible plants. Anhalt and Almeida (2008) recorded that transmission of the virus is efficient at temperatures of 25°C to 30°C.

P. nigronervosa was previously reported to contain two forma specialis: “typica” and “caladii” (Eastop, 1996). Based on morphology and molecular data, Foottit *et al* (2010) re-classified *P. nigronervosa* f. *caladii* as a new species, *Pentalonia caladii* (*Hemiptera*: *Aphididae*), as originally proposed by van der Goot (1917). *P. nigronervosa* mainly colonizes banana (*Musa* spp.) plants, whereas *P. caladii* chiefly colonizes ginger (*Zingiber officinale*, *Alpinia purpurata*, *Hedychium coronarium*), heliconia (*Heliconia* spp.) and taro (*Colocasia esculenta*) plants (Foottit *et al*., 2010). Ginger, heliconia and taro plants often grow in close proximity to banana fields in Hawaii (Watanabe *et al*., 2013). Some transmission experiments have failed to demonstrate that taro and ginger plants serve as hosts for BBTV (Hu *et al*.,1996), and the *P. caladii* aphids colonising those plants may not play an active role in the transmission of BBTV, even when the aphids disperse from their primary hosts to banana plants (Watanabe *et al.*, 2013).

The virus can also be spread through the movement of infected plant material from one geographical location to another. BBTV is not transmitted mechanically or through tools used to carry out agricultural practices (Qazi, 2016).

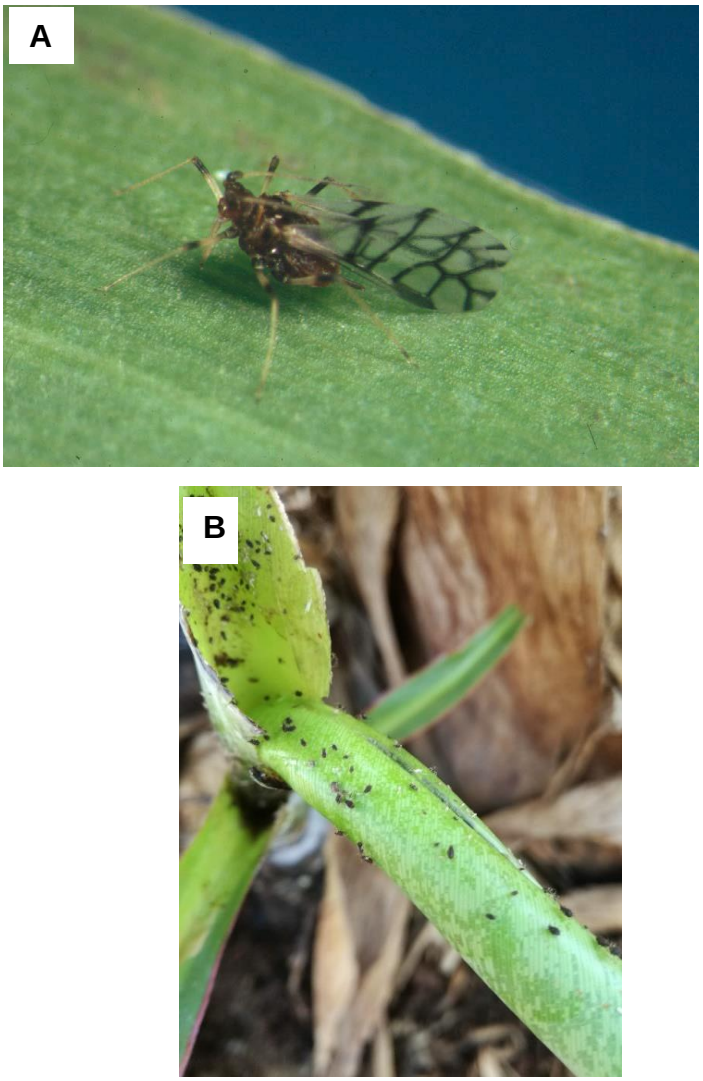

Figure 1.3 The (A) alate and (B) apterous forms of the aphid vector, *Pentalonia nigronervosa* (banana aphid) which transmits BBTV (Nelson, 2004)

1.5. Symptoms of BBTV

Banana plants infected early in their growth fail to produce fruits resulting in total loss of yield, while plants infected at later stages may produce deformed fruits therefore rendering them unmarketable (Dale, 1987; Thomas *et al*., 2003; Hooks *et al*, 2008; Kumar *et al*., 2011). BBTV colonizes in the phloem tissues and damages host cells (Tanuja *et al*., 2019). The virus spreads to suckers through the rhizome and the entire banana mat eventually becomes infected (Dale and Harding, 1998; Suparman *et al*.,

2017). According to Magee (1927) the first symptom of the disease is the appearance of dark-green streaks and flecking best viewed from the under surface (abaxial surface) of the leaf commonly referred to as “morse code”. As the infection progresses, the streak symptoms become more evident on the leaf blade (Fig. 1.4a & b). A very characteristic symptom of the disease is upright and crowded leaves at the apex of the plant. Each new leaf is narrower and shorter than the previous one giving the plant a “bunchy” appearance (Suparman *et al*., 2017) (Fig. 1.5). Another noticeable symptom of the disease is severe stunting of suckers (Kumar *et al*., 2015).

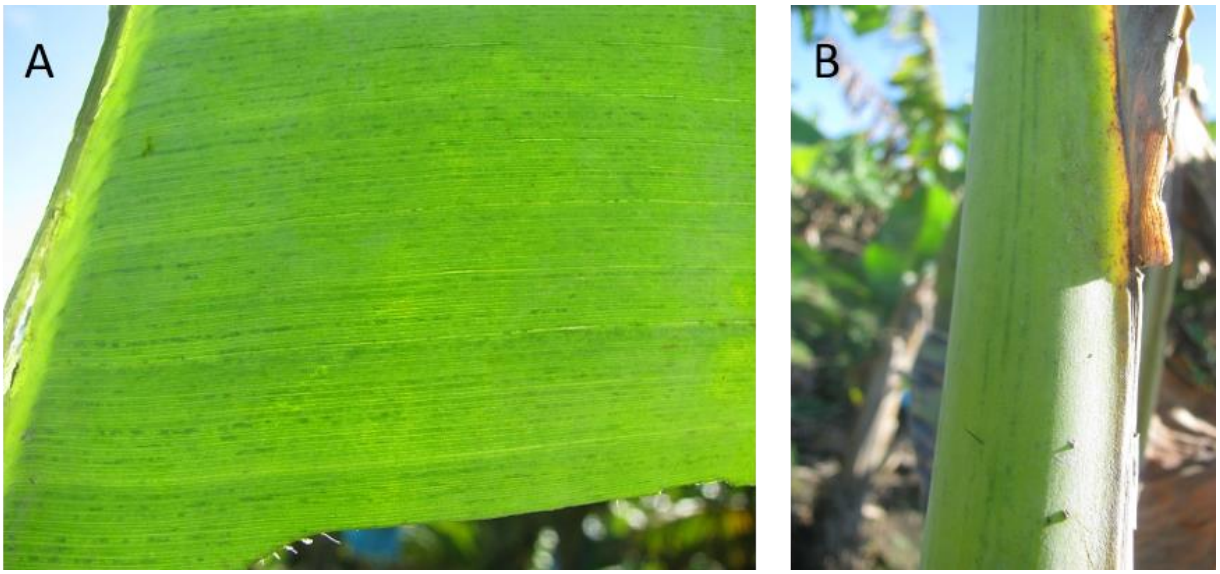

Figure 1.4. Typical symptoms of (a) BBTV-infected banana leaf showing distinct ‘Morse code’ symptom on the lamina and (b) dark-green streaks on the pseudostem

Figure 1.5. Banana bunchy top virus causes new leaves to be stunted and "bunchy", while leaf edges are deformed and yellow

1.6. Methods used in the detection of BBTV

A number of methods are used for BBTV detection. Among them are nucleic acid-based, serological and electron microscopy techniques. Polymerase chain reaction (PCR) which involves the enzymatic amplification of a DNA fragment defined by two oligonucleotide primers has been used to diagnose many plant viruses (Robertson *et al,* 1991; Rybicki and Hughes, 1990). Bashir *et al*., (2012) found that rolling-circle amplification (RCA) technology could also be used for characterization of Nanoviruses. Chen and Hu (2013) devised a high throughput Taqman® real-time PCR system for BBTV detection. They found that the system was highly sensitive, detecting as few as 2.73 copies of BBTV genomic DNA. The method is so efficient it can quantify BBTV in aphids and plants even before the appearance of symptoms of BBTD. Loop-mediated isothermal amplification (LAMP), a more rapid and simpler assay that is performed under isothermal conditions and uses only a waterbath or heating block is also used for BBTV diagnosis. Peng (2012) found that this assay is approximately 100-fold more sensitive than PCR in BBTV detection. Galvez *et al* (2020) improved the LAMP assay by developing loop primers and lowering reaction time from 90 minutes to 45 minutes.

Serology has been indispensable in the detection and identification of plant viruses. Serological techniques used to detect viruses are based on the reaction between viral nucleoprotein or viral protein and its specific antibody (Hsu, 1996). Two methods are commonly used namely enzyme-linked immunosorbent assay (ELISA) and Dot immunobinding assay. ELISA tests with monoclonal antibodies are commonly used for the accurate detection of BBTV (Wu and Su, 1990; Dietzgen and Thomas, 1991; Geering and Thomas, 1996). ELISA is normally used as it is rapid, cheap and easy to automate (Wangai and Lelgut, 2004) and although able to detect BBTV in field-infected plants and single aphids is less sensitive than PCR (Thomas and Dietzgen, 1991).

Virus particles are not always easily viewed under the electron microscope because of their minute size. A major constraint in EM is the expense involved in purchasing and maintaining the facility. Icosahedral particles found in highly purified BBTV sample can be seen under the electron microscope (Figure 1.6).

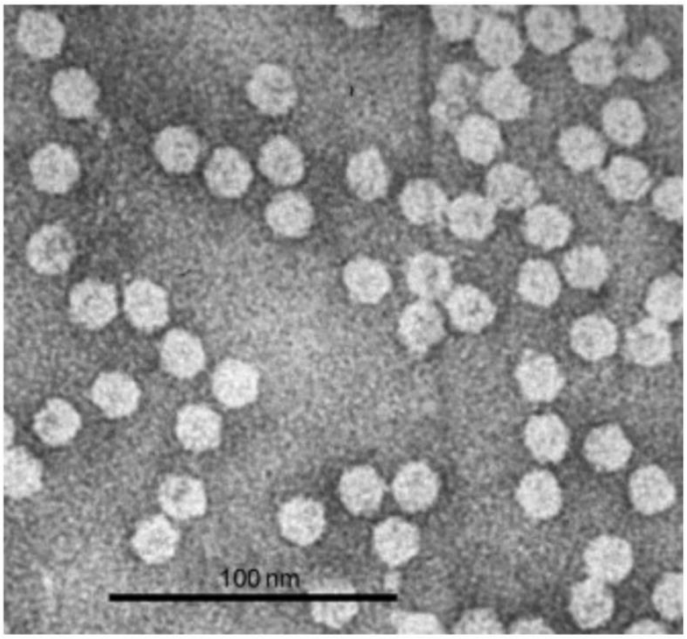

Figure 1.6. Negative contrast electron micrograph of icosahedral particles of BBTV under an electronic microscope. The bar represents 100 nm. (Harding *et al*., 1991)

Recombinase polymerase amplification (RPA) is a rapid, isothermal amplification method with high specificity and sensitivity**.** In a study by Kapoor *et al*., (2017) an assay was developed and evaluated for the detection of BBTV in infected banana

plants. Three oligonucleotide primer pairs were designed from the replication initiation protein gene sequences of BBTV to function both in RPA as well as in PCR. BBTV was efficiently detected using crude leaf sap in RPA and the results obtained were consistent with PCR-based detection using purified DNA as template. (Kapoor *et al*., 2017). The results showed the efficacy of RPA as a detection tool which is less labour-intensive and less time-consuming as crude leaf sap is used as a template (Kapoor *et al*., 2017).

1.7. Control Strategies

Virus infections lead to substantial yield losses on many crops. The adverse effects of virus infections can be limited if proper control measures such as minimising virus infection or suppression of virus are put into place. Individual measures used alone are not very beneficial and eventually become ineffective over long terms (Jones, 2004). A better approach is when integrated pest control strategies are put in place thus resulting in more effective control. According to Jones (2001), selecting such a combination requires comprehensive knowledge of the epidemiology of the causal virus and the mode of action of each control measure. Plant disease control strategies are usually developed on the concept that limiting pathogen spread will result in fewer diseased plants thus increasing plant yield (Hooks *et al*., 2009).

1.7.1. Regulatory Control

Once established, BBTV has not been completely eradicated from any country, it can only be localised (Qazi, 2016). It is however believed to have been eliminated from certain banana-growing districts in Australia (Thomas *et al*., 1994; Thomas and Iska-Caruana, 2000). BBTV is a quarantine virus that is included in the South African Phytosanitary Services list of pathogens which must be absent in imported Musaceae propagation material in the Agricultural Pests, (Act no. 36 of 1983 of South Africa). Spread of the disease is kept in check by strict legislation, which controls the source and movement of planting material, controls the issue of planting permits and requires the destruction of all plants with symptoms.

1.7.2. Cultural Control

Banana bunchy top disease can be effectively controlled by the eradication of diseased plants and the use of virus-tested planting material (Qazi, 2016). Rouging or

removal of infected plants has been found to be beneficial in smallholder farms as it may assist in recovering banana productivity if carried out accurately (Omondi *et al*., 2020). However, it needs to be carried out with utmost care to avoid disturbing vector colonies, which may result in increased pathogen spread (Hooks *et al*., 2009). The whole stool, including the corm and all associated suckers, must then be destroyed by uprooting and chopping into small pieces or by herbicide treatment to prevent regrowth. However, BBTD may persist indefinitely within plantations because inspectors are unable to identify all infected plants during a single inspection and non-symptomatic infected plants that remain between inspections may serve as a source of inoculum for virus spread (Allen, 1978). As such, an effective management strategy is dependent on early detection of infected plants so that potential source plants of BBTV can be destroyed promptly (Hooks *et al*., 2008). Cultural control must be implemented across the whole production area to avoid the rapid re-infection of virus-tested planting material (Thomas *et al*., 1994; CABI, 2017).

1.7.3. Chemical Control

Application of chemical control such as organophosphate insecticides (diazinon and paraffinic oil) has been employed to control the spread of banana aphids (Robson *et al*., 2007; Jekayinoluwa *et al*., 2020). However, the limiting factor to this approach is that applied insecticides may not reach aphids in the inner sections of the plant, such as the inner part of the cigar leaf and within leaf sheaths of the pseudostem where aphids tend to hide. Pesticides may also have negative effects on the applicator as well as killing other off-target species. Factors such as treatment concentration and the age of the leaves need to be taken into account as imidacloprid is not potent on young leaves (Robson *et al*., 2007).

1.7.4. Germplasm Management

To date, there is no known banana germplasm that is resistant to BBTV (Jekayinoluwa *et al*., 2020). However, the Germplasm Health Unit of CGIAR (Consortium of International Agricultural Research Centers (formerly known as the Consultative Group of International Agricultural Research) does some work in this field (Van den Houwe *et al*., 2020). Banana is amongst the major vegetatively propagated crops researched by this consortium. To mitigate risks of virus spread through movement of germplasm, MusaNet (an international network for Musa genetic resources) has

established a set of guidelines. The Bioversity International-CIAT Alliance (1617 accessions), and the IITA (393 accessions) germplasm collections are managed as in vitro cultures. A minimum of four plants for each accession are grown for a period of six months in a greenhouse. Leaf sampling is carried out from the petiole and midrib of the three youngest leaves after 3 and 6 months to test for the five most important viruses by PCR/RT-PCR: BBrMV, BBTV, banana streak virus (BSV), banana mild mosaic virus (BanMMV), and cucumber mosaic virus (CMV). Thorough indexing using electron microscopy is also carried out to search for any viral particle. Sanitation of the virus-infected banana accession is an intense process requiring a combination of meristem culturing, thermotherapy, and chemotherapy (Kumar *et al*., 2021). Despite stringent measures and the continuous optimization of the protocols, the success rate of banana sanitation is currently about 70%. An accession-indexed negative is added to an *in vitro* banana collection for further safe propagation and distribution. Necessary precautionary measures are taken to avoid any further infection to *in vitro* plants that could arise if the plant is transferred to the field or greenhouse before distribution (Kumar *et al*., 2021).

RNA interference (RNAi) is an immune response used by plants to silence the expression of viruses upon attack. This mechanism acts by suppressing transcriptional process (TGS- transcriptional gene silencing) or activating degradation of sequence-specific RNA (PTGS- post-transcriptional gene silencing; Agrawal *et al*., 2004; Jekayinoluwa *et al*., 2020). To counter-act this phenomenon, almost all plant viruses encode viral suppressors of RNA silencing (VSRs), obstructing the key steps of RNAi system to nullify the RNAi-based antiviral defense of the host plant. In BBTV, CP and MP encoded by DNA-S and DNA-R have been identified as suppressors of RNAi (Jekayinoluwa *et al*., 2020). Several studies have provided impressive findings in suppressing the expression of BBTV. Krishna *et al*. (2013) targeted four viral BBTV components (DNA-R, DNA-S, DNA-M, and DNA-C) through RNAi and attained partial resistance to BBTV in transgenic Grand Nain banana under controlled conditions. Elayabalan *et al*., (2017) targeted DNA-R in *Hill* banana and the result was symptomless plants with suppressed symptoms for BBTD.

1.8. Conclusion

BBTV is one of the most important viral diseases affecting banana. Banana-producing areas, where the virus has not been reported, are at risk if the vector *P. nigronervosa* is present. Because there is currently no known germplasm resistant to the disease, the only control measures with guaranteed success are planting disease-free material and killing the aphid vector. A lot of work has been done in trying to understand the BBTV genome and BBTV-host interactions however there is more work that can still be explored. An understanding of BBTV pathogenesis can improve studies related to pathogenesis-derived resistance.

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CHAPTER 2: MONITORING THE DISTRIBUTION OF BANANA BUNCHY TOP VIRUS IN SOUTH AFRICA: A COUNTRY-WIDE SURVEY[1]

Abstract

Banana bunchy top disease (BBTD) is the most devastating viral disease of bananas worldwide and is caused by banana bunchy top virus (BBTV). The disease is spread by the banana aphid *Pentalonia nigronervosa* Coquerel (Hemiptera: *Aphididae*) and through infected propagation material. In 2016, the virus was detected for the first time in an isolated area in the South Coast region of the KwaZulu-Natal Province (KZN), South Africa. The aim of this study was to conduct surveys across all banana-producing regions in South Africa, *viz.* KwaZulu-Natal, Mpumalanga and Limpopo provinces. Over 1700 plant and aphid samples were collected from commercial farms and rural households in the three provinces and more intense sampling was done in the affected KZN region. A BBTV-specific PCR, targeting DNA-R (encoding the Master replication initiation protein; M-Rep), was used to detect virus-infected samples and amplicons of the expected band size were sequenced. Comparative phylogenetic analyses showed that the South African BBTV isolates clustered within the Pacific-Indian Oceans genomic group that included isolates from India and other regions in Africa with a bootstrap value of 94%. To date, the virus has been identified only in the South Coast region of the KwaZulu-Natal province. Intense management strategies, including scouting, removal of infected plants and control of aphids, have been implemented in areas where positive samples were identified to minimize the spread of the virus.

Keywords: Banana, Banana bunchy top virus, Survey, Control, Phylogenetic analysis

[1]**Chapter 2 has been accepted for publication in *Archives of Virology* Journal**

2.1. Introduction

Banana (genus *Musa,* family *Musaceae,* order Zingiberales) is one of the most important economic crops for developing countries in tropical and sub-tropical areas and is cultivated in about 120 countries (Ghag and Ganapathi, 2018; Rustagi *et al*., 2015). Available estimates indicate that average global banana production rose from

69 million tonnes in 2000-2002 to 116 million tonnes in 2017-2019, at an approximate value of 31 billion USD. These values are an estimate as the bulk of banana production is conducted informally thus making it difficult to obtain accurate figures (FAO, 2019). An important subgroup is 'Cavendish', which may have originated in South China (Jones, 2000). The 'Cavendish' subgroup yields the most common fruit and form the backbone of the domestic industries in countries like Australia, India, China and South Africa (Robinson, 1996).

Banana bunchy top virus (BBTV), a multi-component, circular, single-stranded DNA virus with 18-20 nm diameter virions, is the type member of the genus *Babuvirus* in the family *Nanoviridae* (Amin *et al*., 2008). The viral genome comprises six encapsidated components (DNA-R; DNA-S; DNA-M; DNA-N; DNA-C and DNA-U3) each approximately 1100 nucleotides in length (Wickramaarachchi *et al*., 2015) and sometimes additional alphasatellite molecules of a similar size (Varsani *et al*.,2021). Phylogenetic relationships, largely based on the DNA-R, -N and –S segments, grouped BBTV isolates worldwide as 'South Pacific' or 'Asian' origin (Kumar *et al*., 2011) later proposed as the Pacific-Indian Oceans (PIO) and the Southeast Asian (SEA) groups based on their geographical delineation (Yu *et al*.,2012).

Banana bunchy top disease (BBTD), caused by BBTV, is spread in a circulative manner predominantly by *Pentalonia nigronervosa* Coquerel (Hemiptera: *Aphididae*) commonly known as the banana aphid (Magee, 1927). The first symptom of the disease is the appearance of dark green streaks on the minor leaf veins when viewed from the underside of the leaf with transmitted light or on the midrib. (Magee, 1927). As the disease progresses, infected leaves become progressively stunted and malformed and have an upright bearing eventually resulting in a 'bunchy' display. Yield losses of up to 100% can be experienced when plants are infected with BBTV and fail to produce bunches (Stainton *et al*., 2015).

BBTV was reported for the first time from the Fiji Islands in 1889 (Magee, 1927) and thereafter, it has been identified in 44 countries in Africa, Australia, Asia and the South Pacific Islands (CABI, 2020; Magee, 1927). In Africa, BBTD was first reported in 1901 in Egypt. Currently it has been reported in 17 African countries including Cameroon, Zambia, Mozambique, Malawi and Nigeria (Adegbola *et al*., 2013; Gondwe *et al*., 2007; Niyongere *et al*., 2012). The presence of BBTV in South Africa was

confirmed in 2016 from the banana production area located in the South Coast region of the KwaZulu-Natal Province (Jooste *et al*., 2016). The original source of infection in this region is currently unknown. Prior to the identification and confirmation of BBTV in South Africa in 2016, the last survey in 1996 provided no evidence for the presence of this virus in the country (Pietersen *et al*., 1996). BBTV is a quarantine virus included in the South African Phytosanitary Services list of pathogens that must be absent in imported *Musaceae* propagation material under the Agricultural Pests Act, 1983 (Act no. 36 of 1983).

Banana is amongst the most important commercial subtropical fruit grown in South Africa and is mostly grown for home consumption. Subsistence farming of banana also contributes as a staple food source for poorer communities and income is generated through informal trade at local markets. Only a small fraction of all the bananas produced is sold on the world market (DAFF, 2018). Approximately 415 000 tonnes were harvested during the 2018/19 marketing season valuing the industry at approximately 137 million USD (DALRRD, 2020). Based on the detection of BBTV, the extent of the spread of BBTV in South African banana production areas was investigated in order to establish effective management strategies in the affected regions.

2.2 Materials and Methods

2.2.1 Surveys

Follow up surveys and delimiting surveys were conducted during the period of March 2017 to February 2021 to determine the occurrence of BBTV in commercial farms and rural households in the main banana-producing regions in the KwaZulu-Natal, Mpumalanga and Limpopo provinces of South Africa. Farms and rural households were selected based on information provided by government extension services in each province. The co-ordinates were recorded for each site where sampling took place using a Global Positioning System (Garmin Etrex 20x Ltd) (Table 2. 1 and Appendix A).

In each field of the 31 commercial farms, or at individual households, plants were randomly selected and observed for typical BBTV symptoms. A section of emerging leaf tissue from the midrib and lamina of symptomatic and asymptomatic plants was collected and stored in a cooler box during transit from the field to the

laboratory. Aphids were collected from symptomatic and asymptomatic plants using a fine-tip brush followed by storage in a 2 ml microcentrifuge tube containing 99% ethanol. All samples were placed in labeled plastic bags and transferred to the laboratory for further analysis.

Yellow bucket traps, set up on metal stands, were filled halfway with water and a drop of Sunlight® liquid soap to trap aphids on commercial farms overnight (Figure 2.1). Twenty traps were placed at 10 commercial farms in the South Coast of KZN and the trapping was repeated four times per farm in the region. At the rest of the 21 commercial farms, located in the other production regions, trapping was done once. The traps had an opening on one side to allow for the drainage of excess water in the event of rain. This opening was covered with a very fine mesh cloth so that samples are not washed out. (Figure 2.1). The contents of the traps were sieved using muslin cloth and the cloth was then placed in a jar containing 99% ethanol and was later examined under a microscope to check for the presence of banana aphids.

Delimiting surveys were conducted at Marburg Farm (30°45'46.6"S 30°25'00.2"E), a commercial farm 42 km from the initial outbreak site. This was done according to a surveying protocol developed by the International Institute of Tropical Agriculture (IITA). In each field, observations for symptoms were randomly recorded on 100 plants by walking across a “W” shaped path inspecting 25 plants on each of the four transverses at an equal distance from each other. In blocks where symptomatic plants were observed, the midrib and lamina of five plants and aphids from these plants were collected for virus testing while in blocks with asymptomatic plants; the midrib and lamina from the last 25 plants were sampled (BBTV Survey Protocol, 2018).

Figure 2.1. Yellow bucket trap used for the trapping of P. nigronervosa aphids

2.2.2. Nucleic acid extraction from plant and aphid samples

Nucleic acid was extracted from aphids using a non-destructive extraction method (Robbertse *et al*., 2019). Four to six aphids from a sample stored in 99% ethanol were dried on tissue paper and placed in a 2 ml microcentrifuge tube containing 200 µl digestion buffer (10 mM of each of NaCl, Tris, EDTA) and Proteinase K (ThermoFisher Scientific, USA). Samples were incubated overnight in a shaking incubator at 55 °C and transferred to a water bath at 72 °C for 10 min. An aliquot of 180 µl of the lysate was transferred to a clean tube and the aphid specimens were stored. A one-tenth volume of sodium acetate (pH 5) was placed into each tube and samples were incubated for 30 min at -20 °C. Samples were centrifuged at 13 500 rpm for 20 min and 160 µl of supernatant was transferred to a new tube. A volume of 224 µl of 98% (v/v) ethanol was added to the samples and they were incubated at -20 °C for 3 h. The precipitated nucleic acid was collected by centrifugation at 13 500 rpm for 20 min and the supernatant was discarded. The pellet was washed twice with 70% (v/v) ethanol, once with 98% (v/v) ethanol, dried for 1 h and re-suspended in 20 µl of distilled water.

The modified cetyl trimethyl ammonium bromide (CTAB) protocol was used for nucleic acid extraction from symptomatic and asymptomatic banana tissue (Thomson and Dietzgen, 1995). Five hundred milligrams of banana leaves were cut into pieces

and crushed using a mortar and pestle in 2 ml of CTAB Buffer (pH 8). The homogenate was kept on a shaker at 60 °C for 30 min and then centrifuged at 3 000 rpm for 5 min. A volume of 900 µl of the supernatant was transferred to a new tube and an equal volume of chloroform:iso-amyl alcohol 24:1 (v/v) was added. The tubes were centrifuged at 13 500 rpm for 12 min. Cold isopropanol was added to the aqueous phase of the supernatant in a new tube which was then kept overnight at 10 °C for nucleic acid precipitation. Precipitated nucleic acid was collected by centrifugation at 13 500 rpm for 22 min and the supernatant was discarded without disturbing the pellet. The pellet was washed three times with 70% (v/v) ethanol, dried for 1 h and re-suspended in 100 µl of distilled water. The quality and quantity of each extraction was analyzed using a Nanodrop™ 1000 spectrophotometer (ThermoFisher Scientific, USA).

2.2.3. PCR detection of BBTV

Banana bunchy top virus detection was performed by polymerase chain reaction (PCR) using the primer pair, BBT-1: 5'-CTCGTCATGTGCAAGGTTATGTCG-3 and BBT-2: 5'-GAAGTTCTCCAGCTATTCATCGCC-3', designed to amplify a 349 bp product corresponding to a portion of the BBTV M-Rep gene (Thomson and Dietzgen, 1995). PCR was performed in a 25 µl reaction containing 1x reaction buffer, 0.5µM of each primer, 5 U MyTaq DNA Polymerase (Bioline, USA) and 1 µl of sample nucleic acid. The temperature profile was as follows: initial denaturation at 95 °C for 1 min, 40 amplification cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s, extension at 72 °C for 10 s and a final extension for 5 min at 72 °C. All PCR reactions were carried out using a Proflex PCR cycler (Applied Biosystems, USA). For electrophoretic analysis, 10 µl of the PCR product was run on a 1.5% (w/v) agarose gel in Tris-acetate EDTA (TAE) buffer, pre-stained with ethidium bromide. The amplified DNA bands were visualized using a UV transilluminator (Quantum CX 5, Vilber Lourmat, France).

2.2.4. Sequencing and Phylogenetic studies

Fourteen PCR-positive representative isolates from the KZN South Coast region were sequenced at Inqaba Biotechnical Industries (Pretoria, South Africa). The nucleotide sequences were aligned using MAFFT and BioEdit (Hall, 1999; Katoh *et al*., 2002) software. For genetic analyses, nucleotide sequences from DNA-R components were

aligned with closely related BBTV M-Rep gene sequences downloaded from the National Center for Biotechnology Information (NCBI) database including two South African isolates (GenBank accession numbers KY770984 and KY770985) from a previous study (Robbertse *et al*., 2019). Construction of the phylogenetic tree was performed using MEGA X (Tamura *et al*., 2021) and abaca bunchy top virus (ABTV, genus *Babuvirus*, GenBank accession no. EF546813) was used as an outgroup. The bestfit model was determined using MEGA X. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model with a Gamma distribution (Tamura, 1992). The bootstrap consensus tree was inferred from 1 000 replicates. Branches corresponding to partitions reproduced in less than 70% bootstrap values were collapsed.

2.3. Results

2.3.1. Surveys

Surveys were initially carried out in the KZN South Coast region where the BBTV outbreak was confirmed, including a 30km radius region from the initial outbreak site. This region consists of rural households that cultivate banana for home consumption and subsistence farming. Commercial farms 50km south of the initial outbreak site were also surveyed. The surveys were then extended to the North Coast of KwaZulu-Natal as well as the Mpumalanga and Limpopo provinces of South Africa covering over 5 000 ha in total (Table 2.1 and Appendix A). A total of 1 704 plant and aphid samples were collected in the three provinces (Figure 2.2). BBTV symptom expression was only observed in samples collected from the KZN South Coast region (Figure 2.3). Surveys were conducted in rural communities from the four local municipalities (Ray Nkonyeni, Umdoni, Umzumbe and uMuziwabantu) within the Ugu District Municipality. The number of banana plants per rural household ranged from three plants per household to more than 100 plants and the level of infection per household, ranged from just a few diseased plants to complete infection of all plants. The exact range of infection per household was not determined because the aim was to map infection sites. Therefore, the visual scoring of symptoms was done per site to determine the radius of BBTV spread from the initial outbreak site. Plant and aphid samples were collected from plants per site, even if no symptoms were observed. During the 2018 survey, BBTV was detected in banana plants at 5 households from a total of 15 visited sites (Appendix A) and a second trip that year, at a different location, resulted in 8

households with positive plants from the 10 sites visited. A further 7 sites surveyed in 2018, resulted in all 7 sites with one or more than one BBTV-infected plant (Appendix A). In 2019, 6 sites had BBTV positive plants and in 2020, 7 from 11 sites, at different locations, had BBTV-infected plants. During the 2021 survey, 9 from 14 sites had BBTV-infected plants. The impact of BBTV infection in commercial farms were restricted to the loss of 160 ha of banana at the initial outbreak site while infections in the second commercial farm was reported in three blocks throughout the farm. Here, the spread was contained with removal of infected plants and chemical control of the banana aphid. Symptom severity on the “Williams” cultivar, which is the most common cultivar in both rural households and commercial farms, ranged from mild to severe in the field and was determined by visual inspection of plant parts. Mild symptoms included the dot-dash symptoms visualized on banana leaves and the streak symptoms seen on the pseudostem (Figure 2.4A). Subsequently, the leaves become yellow and curl with a leathery feel (Figure 2.4B and 2.4C). The most distinctive symptom detected in severely affected plants is the upright growth of an infected plant with severe stunting on smaller plants (Figure 2.4D).

2.3.2. Nucleic acid extraction and PCR detection of BBTV

Regardless of symptom expression, all samples collected during the field surveys were screened with the PCR protocol and positive samples yielded amplicons of ~349 bp in size, which corresponded to a partial DNA-R sequence of the M-Rep gene. There was no amplification found from asymptomatic plant samples with the BBTV-specific primers. From 379 plants collected in the KZN South Coast region (Table 2.1), 76 tested positive for BBTV. A total of 236 aphids were collected (218 aphids directly from plants and 18 winged aphids from traps) and 50 of these tested positive for BBTV. In addition, there was no detection of BBTV from aphids collected from the traps.

Rural households accounted for a larger portion of positive aphid and plant samples (104 out of 126 positive samples) while BBTV infections were confirmed at only two commercial farms in the KZN South Coast region. Upon detection of the virus at the farm (30°45’46.6” S, 30°25’00.2” E), approximately 42 km from the initial outbreak site, delimiting surveys were conducted. Prior to the delimiting surveys at this commercial farm, two plants had tested positive for BBTV in a survey conducted in

October 2018. BBTV was detected on an additional four plants in two other blocks on the farm, using the delimiting survey strategy. This indicated that infections had spread to more sections of the farm by January 2019.

2.3.3. Sequencing and Phylogenetic studies

Fourteen randomly selected PCR positive amplicons were submitted to Inqaba Biotech (Pretoria, South Africa) for Sanger sequencing and the sequences were deposited into the NCBI GenBank. The accession numbers are MT023045-58. The phylogenetic results showed that all BBTV isolates from the South Coast region in KZN grouped within the Pacific-Indian Oceans (PIO) group along with isolates from India, Pakistan, Fiji and Australia with a 94% bootstrap value amongst the isolates (Figure 2.5). The tree topology and branch lengths indicate that the South African isolates are closely related to the other isolates within the PIO group.

Table 2.1. Number of samples collected from various banana-growing regions in South Africa

Location	**GPS-coordinates**[a]	**Number of samples**		**BBTV positives**	
		Aphids	Plants	Aphids	Plants
South Coast (KwaZulu-Natal)	-30.500933, 30.467350	236	379	50	76
North Coast (KwaZulu-Natal)	-29.639439, 31.067040	94	140	0	0
Mpumalanga	-25.452267, 31.968867	271	304	0	0
Limpopo	-23.811533, 30.181833	140	140	0	0

[a] Complete breakdown of all samples collected available in Appendix A

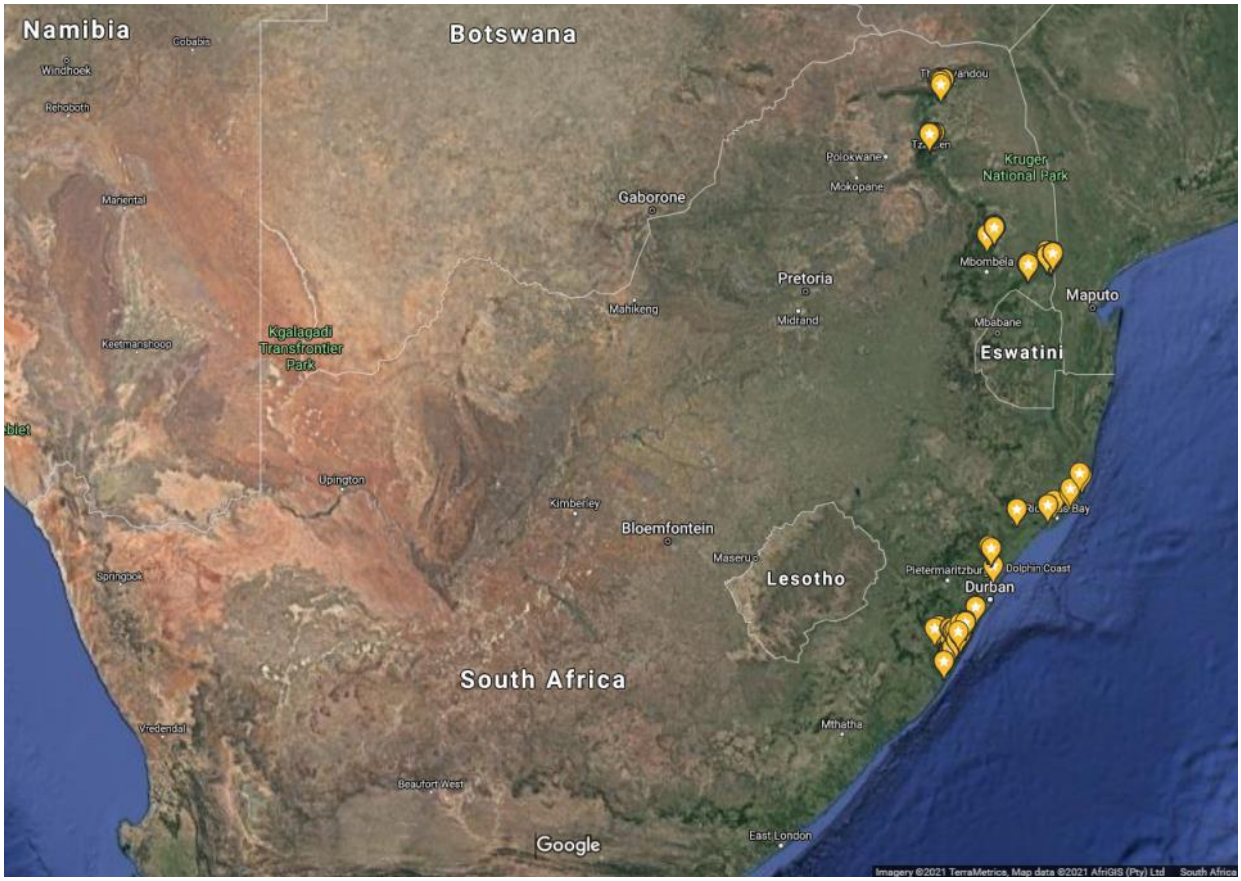

Figure 2.2. Map of surveyed locations indicated by yellow tear-drop points. Samples were collected from KwaZulu-Natal, Mpumalanga and Limpopo provinces.

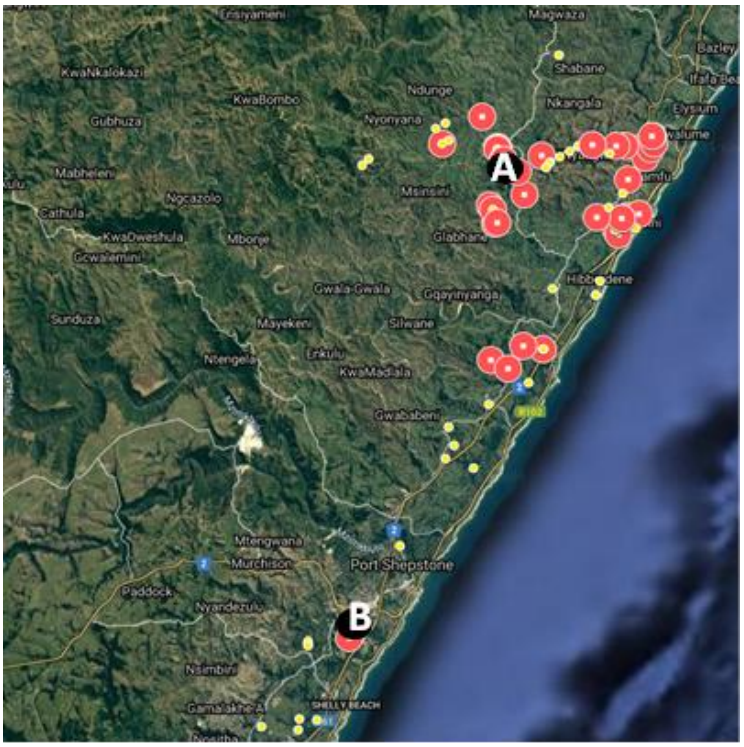

Figure 2.3. A close-up view of the KZN South Coast region where BBTV was discovered. The red circles represent areas where BBTV was detected. The black circle with letter A represents the initial outbreak site and the black circle with letter B is the other commercial farm with BBTV-positive samples. The yellow dots represent the sites sampled

Figure 2.4. Various symptoms visible on BBTV-infected plants. A: Arrow indicating dark green lines (streaky/dot dash symptoms) on the stem. B-C: Yellowing and curling of leaf margins on infected plants. D: A banana plant exhibiting the most characteristic BBTV symptom, a bunchy appearance

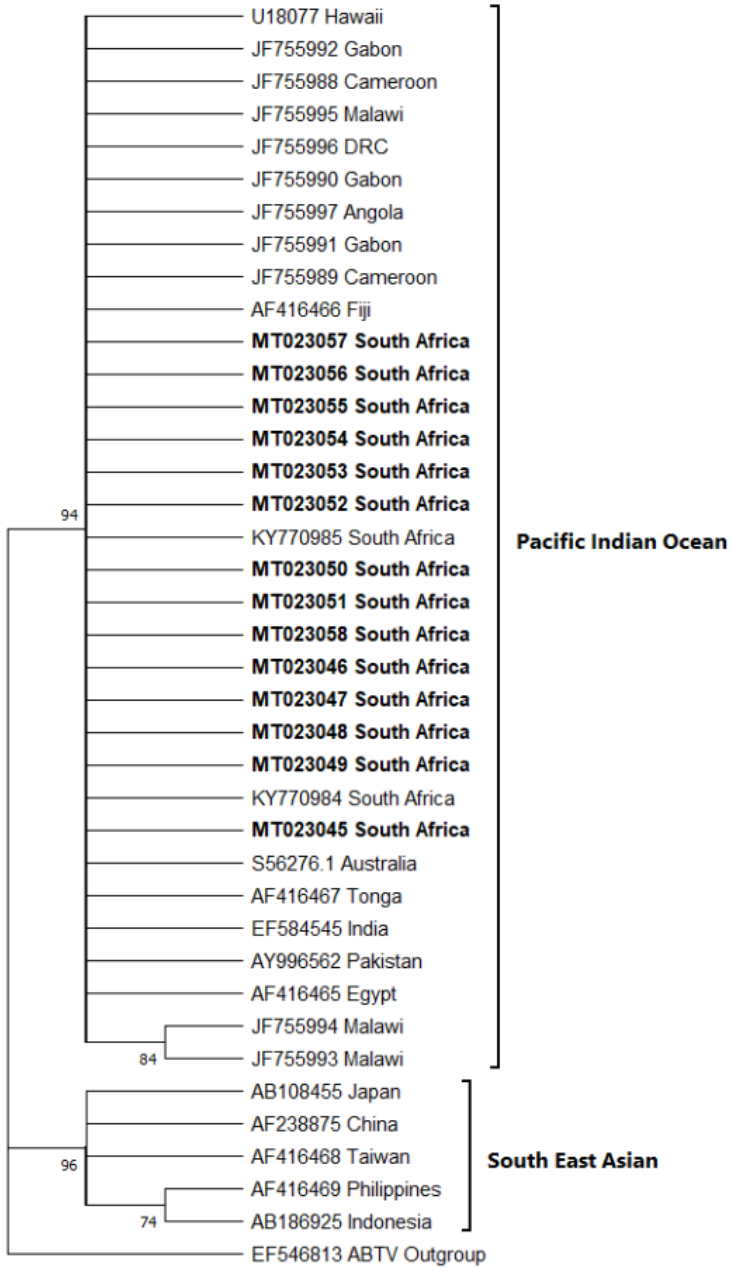

Figure 2.5. Phylogenetic tree showing the genetic relationships between sixteen randomly selected BBTV isolates from the South Coast region of KwaZulu-Natal, South Africa, other isolates of BBTV and an outgroup [abaca bunchy top virus (ABTV)]. The fourteen random samples from this current study are marked in bold

2.4. Discussion

Banana bunchy top virus poses a major threat to banana cultivation in South Africa, especially in the currently affected KZN region. In this study, plant and aphid samples were collected in the main banana producing provinces of South Africa for detection of BBTV (Table 2.1 and Figure 2.1). BBT-1 and BBT-2 primers were able to

detect the virus in the PCR analysis on positive plant and aphid samples. Multiple studies showed the reliability of using PCR as a detection tool for BBTV (Selvarajan *et al*., 2010; Su *et al*., 2003; Xie and Hu, 1995). Viruliferous aphids were detected on the majority of plants displaying clear BBTV symptoms and 78% of aphids collected off these plants tested positive for BBTV. *Pentalonia nigronervosa* Coquerel (Hemiptera: *Aphididae*), the known vector that transmits BBTV (Magee, 1927), was found in all surveyed banana fields across the country. In all surveyed fields, the occurrence of aphids was higher where no vector control was implemented. Interrupting the virus transmission chain is not always possible but removal of infected plant material will help contain the spread of BBTV. Poor maintenance of the banana crop and its dense canopy might also help in increasing the aphid vector population (Young and Wright, 2005). The dense canopy partially prevents rainfall from reaching the leaves and pseudostem and thereby favouring aphid multiplication (Young and Wright, 2005).

Different scenarios can be proposed for the introduction of BBTV into South Africa. Firstly, the virus may have spread by aphids from Mozambique into South Africa with which it shares a border. However, all samples collected from areas directly adjacent to the South African/Mozambican border tested negative for BBTV arguing against this proposition. Secondly, the virus may have been introduced unknowingly into the KZN South Coast region in South Africa through infected planting material and this resulted in its widespread dissemination. Since cultivated bananas are propagated vegetatively, the potential for human-meditated spread is high. Symptom expression can take between 25-85 days in a BBTV-infected plant so when an infected, but asymptomatic, banana propagule is introduced to a region where *P.nigronervosa* is present, there is potential for subsequent vector transmission (Stainton *et al*., 2015). Spread of BBTV from the first reported KZN outbreak site in a southerly direction to a farm approximately 42km away suggests that infective aphids were carried with wind currents to this farm.

Phylogenetic analysis of the DNA-R partial sequence was carried out to determine the relationship of the South African isolates with the other BBTV isolates detected worldwide (Figure 2.5). The use of the M-Rep gene and the coat protein gene sequences to construct phylogenic trees has been a common approach to display the evolutionary history of BBTV and other *Nanoviruses* (Wickramaarachchi *et al*., 2015).

Phylogenetic analysis showed two broad clades/groups of BBTV, namely, the South East Asian and Pacific-Indian Oceans groups, with high bootstrap support values of 96% and 94%, respectively. The KZN South Coast isolates from this study, clustered under the Pacific-Indian Oceans group along with isolates from other African countries, Australia, Hawaii, Fiji, Pakistan and India. The branch lengths indicated minimal genetic difference between the South African BBTV isolates from this study, the two South African reference isolates with GenBank accession numbers (KY770984 and KY770985) and the other isolates within this group. Even though the tree topology does not show the probable origin of the South Africa isolates, it does confirm its grouping within the Pacific-Indian Oceans group. The monophyletic origin of the isolates within the Pacific-Indian Oceans group is also confirmed by a 94% bootstrap value. Two Malawian isolates, GenBank accession numbers: JF55994 and JF55993, grouped within a separate clade, away from Malawian isolate with GenBank accession number JF755995.

Socio-economic factors such as lack of funds needed to purchase chemicals for pest control and resistance to proper/adequate removal of infected plant material in the rural community is a contributing factor responsible for the spread of the disease. Commercial farmers in the region follow management strategies such as stringent scouting, use of chemicals as part of aphid control for orchard management, and therefore are accustomed to chemical control methods while this is not the case for rural households. In this region, the use of chemicals for aphid control is not a sustainable option due to economic constraints and environmental impact. A study showing the effectiveness of consistent rouging in managing the disease concluded that it is possible for smallholder farms to recover banana productivity if such a practice is carried out diligently (Omondi *et al*., 2021).

In the affected region, some field-workers have lost their jobs in the commercial farms as infected plants in various plots have been uprooted. Some households that were visited mentioned that they had incurred loss of income generated from the sale of bananas (personal communication). This has a negative impact on food security and sustainability of banana production for the region. To reduce the impact of BBTV in the region, awareness campaigns, in conjunction with the Department of Agriculture, Land Reform and Rural Development (DALRRD) and Department of

Agriculture and Rural Development (DARD) have been launched in the KZN South Coast region and efforts are ongoing to contain the spread of BBTV.

2.5. Conclusion

Once established, BBTV has never been completely eradicated in any country. It is, however, possible to manage the disease (Jones, 2009). The spread of BBTV in the KZN South Coast region and monitoring thereof in the rest of the banana producing regions in South Africa was discussed here. Banana plants and aphids collected from other regions in the country tested negative for BBTV. Therefore, continuous scouting to monitor any outbreaks is critical, especially in the regions neighboring Mozambique which has positive BBTV sites. Integrated control strategies are the key to contain the spread of BBTV in a region. Awareness needs to be raised amongst stakeholders at all levels (policymakers, extension services, commercial and small-holder farmers) by promoting regular scouting for symptoms as well as removal of infected mats to reduce inoculum pressure. Applying strict quarantine measures to avoid movement of propagation material and the use of certified tissue culture material free of the disease is another recommendation.

The effectiveness of consistent rouging in managing the disease is worthwhile to investigate. Omondi *et al*. (2021) found that it is possible for smallholder farms to recover banana productivity if such a practice is carried out diligently. Such an approach can be explored in the KZN South Coast region as it is a practical control strategy that needs little to no financial implementation costs. Our findings demonstrate a restricted distribution of BBTV in South Africa. Continued monitoring is required to limit the spread in the KZN South Coast region and to prevent any further spread beyond this region.

2.6. References

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CHAPTER 3: INVESTIGATING GENETIC DIVERSITY BETWEEN SOUTH AFRICAN ISOLATES OF BANANA BUNCHY TOP VIRUS

Abstract

Banana bunchy top virus (BBTV), the causal organism of banana bunchy top disease, is one of the most economically important viruses affecting banana production globally. In South Africa, BBTV has only been detected in the South Coast region of KwaZulu-Natal. The aim of the study was to determine the genetic diversity of BBTV isolates from South Africa. From 10 representative isolates, partial genome component sequences of the DNA -S, -M, -U, and -N genes were obtained using Sanger methods and the generated sequences were subjected to phylogenetic analyses. The sequence identity data and phylogenetic analysis of the components indicated that BBTV in South Africa belongs to the Pacific-Indian Oceans (PIO) group and that genetic diversity of South African isolates of the virus is very low. The tree topology of the South African isolates showed that there are no noticeable differences amongst these isolates. The virus displays the highest levels of sequence identity to isolates from the Democratic Republic of Congo, India, Malawi, Rwanda, Sri Lanka, Burundi and Egypt with identity ranging between 96.5 - 99.8%. Sequence identity levels between the South African isolates and the isolates in the South East Asian group ranged between 76.2% and 94. 4%. The findings emanating from the current study therefore highlight the genetic diversity that exists with different BBTV isolates.

Keywords: Banana, Genetic diversity, BBTV components, Phylogenetic analysis

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3.1. Introduction

Banana bunchy top disease, caused by banana bunchy top virus (BBTV), is the most important virus disease affecting banana in Africa, Asia and the South Pacific (Das and Banerjee, 2018; Leiwakabessy, 2016). The disease can result in up to 100% yield loss in infected fields (Dale, 1987). BBTV spread occurs through infected planting material that is used for banana propagation and in a circulative, persistent, non-propagative manner by its specific vector, *Pentalonia nigronervosa* Couqerel (Hemiptera: *Aphididae*) (Magee, 1927; Allen, 1978; Jekayinoluwa *et al*., 2020).

Infected plants exhibit symptoms such as foliar chlorosis, vein clearing and dark green streaks on minor veins of the lamina and petiole (Magee,1927). Late infection of the virus results in stunted and unmarketable fruits (Thomas, 2008).

BBTV belongs to the family *Nanoviridae* which contains at least eight recognized viral species within two genera, the *Nanovirus* and the *Babuvirus.* The genus *Babuvirus* of which BBTV is the type species, includes *Abaca bunchy top virus* (ABTV) and *Cardamom bushy dwarf virus* (CBDV) (Watanabe *et al*., 2013; Thomas *et al*., 2021). BBTV is a multi-partite, single stranded DNA virus with a non-enveloped, icosahedral geometry (Lal *et al*., 2020). It consists of six integral components originally named DNA 1-6 that were renamed DNA-R; -U3; -S; -M, -C and -N. The DNA -R segment encodes the replication initiation protein (M-Rep) responsible for initiating viral DNA replication and was later shown to support the replication of other non-Rep encoding components (Abdel-Salam *et al*., 2012). DNA -S encodes the coat protein (CP). DNA -C encodes the cell-cycle link (Clink) protein. DNA -M encodes the movement protein (MP) and DNA -N encodes the nuclear shuttle protein. The function of the protein encoded from DNA –U3 remains unknown (Baldodiya, 2019; Jekayinoluwa *et al*., 2020). These components are about 1.1 Kb each in length and have a common genome organization consisting of a major common region (CR-M) involved in the second strand synthesis of circular ssDNA genomic components and a stem loop acting as the site of the origin of viral replication (CR-SL) (Mukwa *et al*., 2016). They also possess a major potential TATA box 3' of the stem loop, at least one open reading frame (ORF) for a major gene in the virion sense and polyadenylation signals associated with each gene (Burns,1995; Jekayinoluwa *et al*., 2020). Some BBTV isolates may also carry 1-3 satellite DNA components that are not essential for BBTV infectivity (Stainton *et al*., 2017; Yu *et al.*, 2019).

BBTV isolates identified to date can belong to one of the two genetic groups *i.e*. the Pacific-Indian Oceans (PIO) group and the South East Asian (SEA) group. The grouping is based on nucleotide sequence differences between the genome components and geographical delineation (Karan *et al*., 1994; Kumar *et al*., 2011; Das and Banerjee, 2018). The PIO group comprises isolates from Africa, Australia, Hawaii, India, Myanmar, Pakistan, Sri Lanka, Fiji, Western Samoa, Bangladesh and Tonga while SEA group comprises isolates form China, Indonesia, Japan, Philippines, Taiwan, Thailand and Vietnam. The groups differ from each other with an average of

9.6% amongst DNA -R; 11.86% amongst the DNA -S and 14.5% amongst DNA -N over the entire nucleotide sequence (Jekayinoluwa *et al*., 2020).

BBTV was reported for the first time in South Africa in 2016 from a commercial farm in the South Coast region of KwaZulu-Natal (KZN) province (Jooste *et al*., 2016). Systematic virus surveys conducted lately in all banana-growing provinces showed that the virus only occurs in the South Coast region of KZN. However, very little is known about the molecular characteristics of the South African isolates of BBTV. Unraveling the molecular characteristics of these isolates will provide information that can be used in the development of resistant banana and optimize detection protocols to curb the negative effect of BBTV on production. Therefore, the aim of this study was to determine genetic diversity between South African BBTV isolates in relation to BBTV isolates from banana-growing regions around the world.

3.2. Materials and Methods

3.2.1. Sample Source

A total of ten infected leaf samples from the KZN South Coast region, South Africa, were randomly selected as the representative population of BBTV isolates to be used in this study. Samples were selected from different localities within the region. (Table 3.1) The modified cetyl trimethyl ammonium bromide (CTAB) protocol by Thomson and Dietzgen (1995) was used for nucleic acid extraction from the leaf samples infected with the selected isolates.

Table 3.1. Origin of BBTV isolates used in this study

Isolate name	Locality	GPS co-ordinates
613	Umzumbe	-30.500081, 30.597965
615	kwaNtobela	-30.500816, 30.605412
616	Mthwalume	-30.497886, 30.607407
617	KwaBangibizo	-30.493447, 30.606983
620	Sipofu	-30.498851, 30.588735
626	Nyangwini	-30.517602, 30.590025
627	kwaNyathikazi	-30.504150, 30.535660
629	Mathulini	-30.536533, 30.597596
631	Sipofu	-30.539907, 30.576785
633	Mthwalume	-30.448176 , 30.538626

3.2.2. Molecular Identification

Amplification of the five different genomic segments of the BBTV genome was performed by PCR using the primers described and listed in Table 3.2 The sixth genomic component was studied in Chapter 2 using the same isolates. The PCR with a 25µl reaction mixture consisted of 1x reaction buffer, 0.5 µM of each forward and reverse primer, 5U MyTaq DNA Polymerase (Bioline, USA) and 200ng of sample nucleic acid. PCRs were carried out using a Proflex PCR cycler (Applied Biosystems, USA) according to the setup listed for the different primer pairs with expected amplicon sizes ranging from ~500 bp to ~ 1 100 bp (Table 3.2). Amplification products were visualized on a 1.5% (w/v) agarose gel in Tris-acetate, EDTA (TAE) buffer, pre-stained with ethidium bromide.

3.2.3. Sequencing and Phylogenetic comparison

PCR amplicons with expected sizes, from representative isolates, were sequenced at Inqaba Biotechnical Industries (Pretoria, South Africa) using the same primers used for their amplification (Table 3.2). The sequences generated were used to produce the consensus sequence of each isolate's component. All consensus sequences were subjected to blastn on National Center for Biotechnology Information (NCBI) website to confirm the identity of each component. Closely related respective gene sequences were downloaded from the GenBank database of the NCBI as representatives of the two phylogenetic groups of BBTV. Sequences of the South African isolates and their homologous representatives of the two phylogenetic groups for each genome component were rotated using MARS (Ayad and Pissis, 2017) before performing multiple sequence alignment. Clustal plugin in the MEGA X version 10.2.6 (Kumar *et al*., 2018) was used to perform a multi sequence alignment. MEGA X was also used to determine the best model fit for the dataset and for construction of the maximum likelihood phylogenetic trees with 500 replicates. Abaca bunchy top virus was used as the outgroup. Branches corresponding to partitions reproduced in less than 70% bootstrap values were collapsed. The online tool, Sequence Identity and Similarity (SIAS) (http://imed.med.ucm.es/Tools/sias.html) was used to perform sequence comparison.

Table 3.2. List of primers and PCR conditions for BBTV components

Primer name	Sequences	Target component	PCR conditions	Amplicon size	References
BBTVSF	5' ATCAAGAAGAGGCGGGTTGG3'	DNA-S full length	94°C-5 min: Initial denaturation Then 34 cycles	~1.1 Kb	Islam *et al*, 2010
BBTVSR	5'GGATTTCTTCGGATACCTA3'		94°C-45 s; 63°C-60 s; 72°C-1.3 min; 72°C-10 min: Final elongation		
BBT2.2F	5'CGGGCAGGGACATGGGCTTT3'	DNA- U3	94°C-4 min: Initial denaturation	~750bp	Anandhi *et al*, 2007
BBT2.2R	5'CGCCCTTGTATTTCATAGCGTGTTGTATT3'		Then 40 cycles 94°C-30 s; 60°C-45 s; 72°C-1 min; 72°C – 10 min: Final elongation		
BBTV CF	5'TGCCTGACGATGTCAAGAGAGAG3'	DNA-C full length	94°C-5 min: Initial denaturation	~1.1Kb	Islam *et al*, 2010
BBTV CR	5'TAGCAGACCATTCCCAGAACTCC3'		Then 34 cycles 94°C-45 s; 60°C-60 s; 72°C-1.3 min; 72°C-10 min: Final elongation		
BBTV MF	5'GTATATTAAGCAGCTCGTGAGG3'	DNA-M full length	95°C-5 min: Initial denaturation	~1.1 Kb	Islam *et al*, 2010
BBTV MR	5'TTCGGTACCTCAAAGAGCAAAACC3'		Then 27 cycles 94°C-30 s; 55°C-45 s; 72°C-1.1 min; 72°C-10 min: Final elongation		
BBTV NF	5'TGGAAGAAAGTCGCCTCGCAAGG3'	BBTV DNA-N full length	95°C-5 min: Initial denaturation Then 27 cycles	~1.1 Kb	Islam et al., 2010
BBTV NR	5'GCTCCAGAATCGACGCATGGTAC3'		94°C-30 s; 65°C-45 s; 72°C-1.1 min; 72°C-10 min: Final elongation		

3.3. Results

3.3.1. Molecular Identification

The five DNA components were amplified using specific primers and as expected, amplicons yielded the predicted sizes. The amplification was however not uniform amongst the isolates as can be seen in Table 3.3.

Table 3.3. Amplification rate in relation to the total number of the representative samples.

Target gene	Number of isolates	Number of isolates PCR positive
DNA -S	10	9
DNA -U3	10	7
DNA -M	10	8
DNA -C	10	6
DNA -N	10	1

3.3.2. Genome sequences of the components of the South African BBTV isolates

The consensus sequences of the genomic components of the South African isolates were deposited into GenBank and assigned accession numbers (Table 3.4). Only the open reading frame of the DNA U3 was complete while the other four components had partial ORFs (Table 3.5).

Table 3.4. Accession numbers of South African BBTV isolates sequenced in this study

Gene	Accession number
DNA S	OL791303
	OL791304
	OL791305
	OL791306
	OL791307
	OL791308
	OL791309
	OL791310
	OL791311
DNA-U3	OL791295
	OL791296
	OL791297
	OL791298
	OL791299
	OL791300
	OL791301
DNA-C	OL791289
	OL791290
	OL791291
	OL791292
	OL791293
	OL791294
DNA-M	OL791281
	OL791282
	OL791283
	OL791284
	OL791285
	OL791286

	OL791287
	OL791288
DNA-N	OL791280

The thirty one sequences of the different BBTV components were allocated accession numbers by NCBI.

The genome sequences of the different genes ranged from 689 to 1 104 bp. The open reading frames (ORF) were partial in DNA -S, -C, -M and -N while it was sequenced in full in DNA -U3 (Table 3.5)

Table 3.5. Sequence lengths of the South African BBTV target genes in this study

Target Gene	Amplified Size (bp)	Predicated ORF location
DNA -S	1014	1-474 (partial)
DNA -U3	689	84-319 (complete)
DNA -C	1003	1-405 (partial)
DNA -M	869	1-177 (partial)
DNA -N	918	1-352 (partial)

3.3.3. Phylogenetic analyses

Tamura 3 parameter model (Tamura, 1992) with a Gamma distribution and invariable sites was identified as the best fit model for datasets. For each genomic component, the phylogenetic results confirmed the grouping of the South African isolates within the Pacific-Indian Oceans (PIO) group (Fig. 3.1-3.5). The tree topology and branch lengths indicate that the South African isolates are closely related to each other. The following results were obtained:

Component S: The South African isolates shared high level of sequence identity (99.6-99.8%) with isolates from India, Malawi, Burundi and the Democratic Republic of Congo, which clustered in the PIO group. The South African isolates also shared 93-94.4% identity with isolates from Phillipines and Thailand in the SEA group. The South African isolates shared 75.4% identity with ABTV, the outgroup.

Component U3: The South African isolates shared high level of identity (98.1-98.8%) with isolates from Egypt, Malawi, DRC, India and Rwanda in the PIO group.

The South African isolates shared 82.4-83.1% identity with the isolates in the SEA group and 50.9% identity to the outgroup.

Component C: The South African isolates shared 96.5-97.4% identity with isolates from India, Australia, Tonga, Malawi and Rwanda in the PIO group. The South African isolates also shared 86.2-87.5% identity with isolates from Philippines, Taiwan, and Indonesia in the SEA group. The South African isolates shared 58.1% identity to the outgroup.

Component M: The South African isolates shared 97.6-98.8% identity with isolates from the Democratic Republic of Congo, Sri Lanka, India, Samoa and Hawaii that grouped under the PIO group. The South African isolates shared 76.2-78.3% identity with isolates from Taiwan and China in the SEA group and 46.6% to the outgroup.

Component N: The South African isolates displayed 97.9-99.1% identity with isolates from Sri Lanka, Burundi, Rwanda, Egypt, India, Malawi and the Democratic Republic of Congo, which all clustered under the PIO group. The South African isolates shared 86.2-86.6% identity to isolates from Taiwan, Philippines and Indonesia, which grouped under SEA.

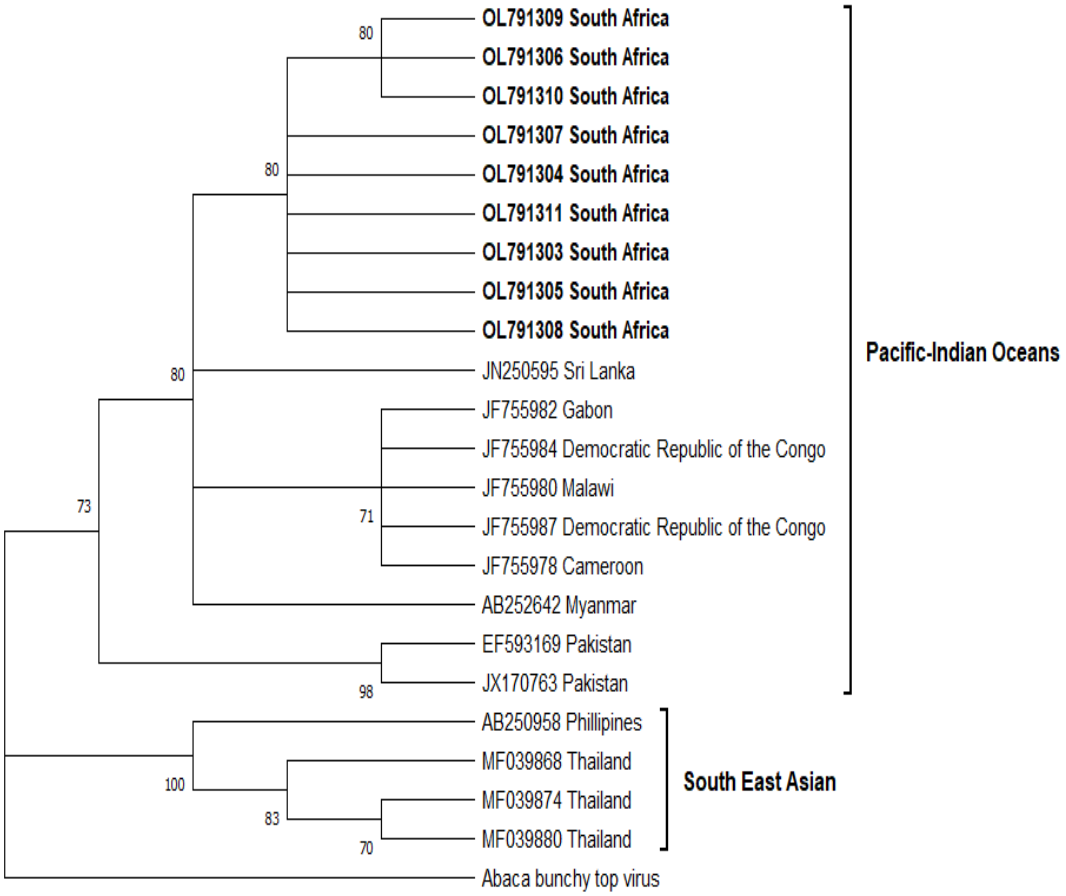

Figure 3.1. Phylogenetic analysis of the partial S gene of BBTV. The tree was rooted using abaca bunchy top virus, a closely related species of BBTV

The nine South African isolates sequences from the DNA S segment formed a subcluster with an 80% bootstrap value (Figure 3.1). Furthermore, these South African isolates clustered together with isolates from India, Burundi, Rwanda, the Democratic Republic of Congo and Malawi. They all grouped within the PIO and have a 73% support bootstrap value amongst them. In the SEA group, there was a 100% bootstrap value among the isolates (Figure 3.1).

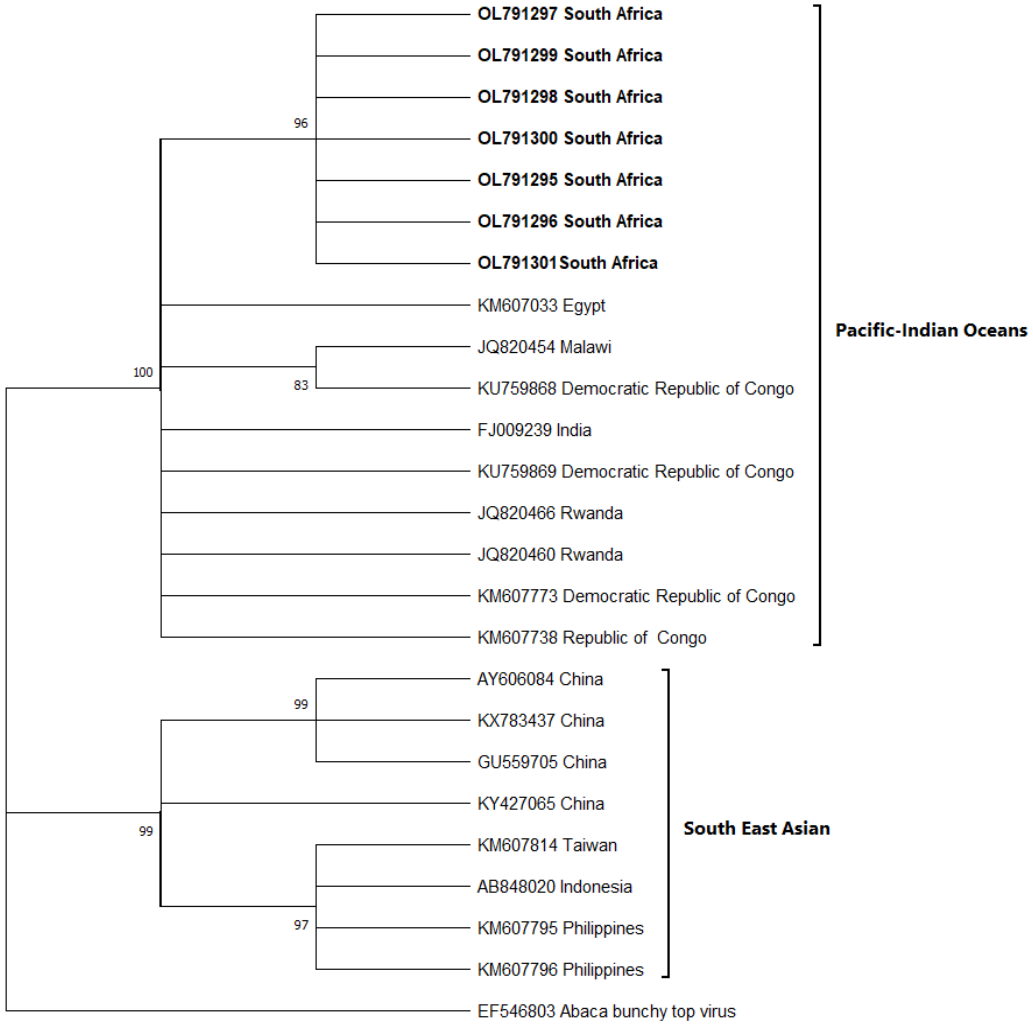

Figure 3.2. Phylogenetic analysis of the U3 gene of BBTV. The tree was rooted using abaca bunchy top virus a closely related species of BBTV.

The seven South African isolates sequenced in the DNA- U3 gene all grouped within the PIO within a 100% bootstrap value amongst these isolates. The South African isolates formed a separate group within the PIO group and there is a 96% bootstrap value. Out of the three DRC isolates with accession numbers, KU759868; KM607773 and KU759869, DRC isolate with accession number KU759868 has the closest relationship with the Malawian isolate with accession number JQ820454 with a bootstrap value of 83%. The isolates within the SEA group have a 99% bootstrap value amongst each other (Figure 3.2).

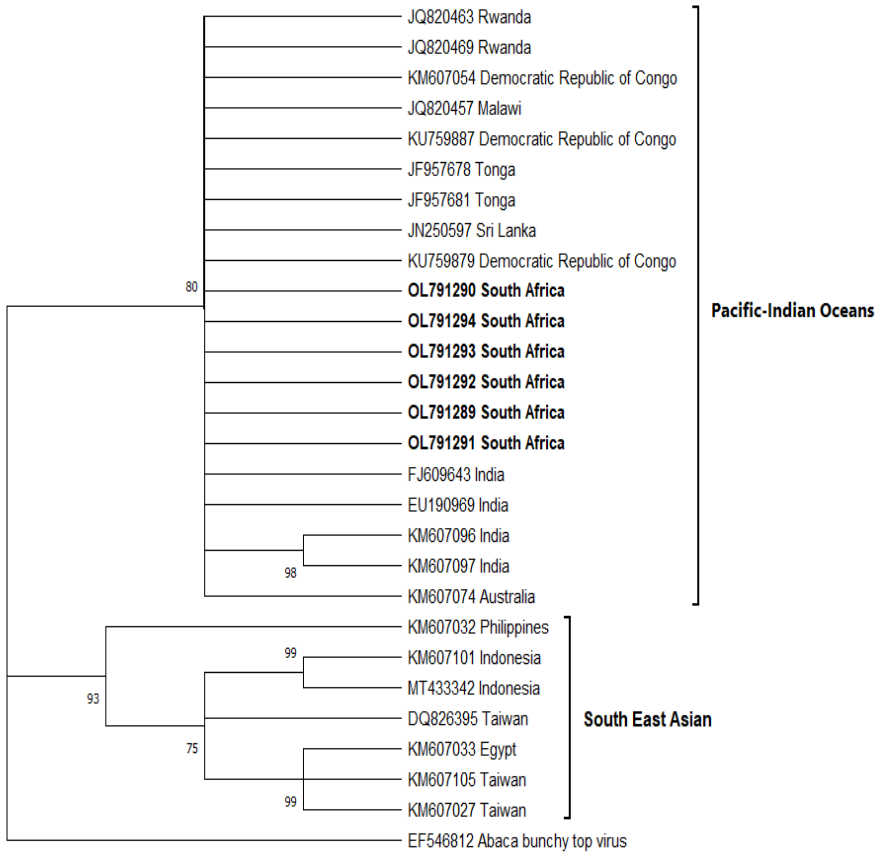

Figure 3.3. Phylogenetic analysis of the partial C gene of BBTV. The tree was rooted using abaca bunchy top virus a closely related species of BBTV.

The six South African isolates sequenced from the DNA-C gene grouped within the PIO group with an 80% bootstrap value. The bootstrap value for the isolates within the SEA group was 93%. An interesting observation was the presence of the Egyptian isolate with accession number KM607033. This isolate clustered in a subgroup with Taiwanese isolates with accessions KM607027 and KM607105 (Figure 3.3).

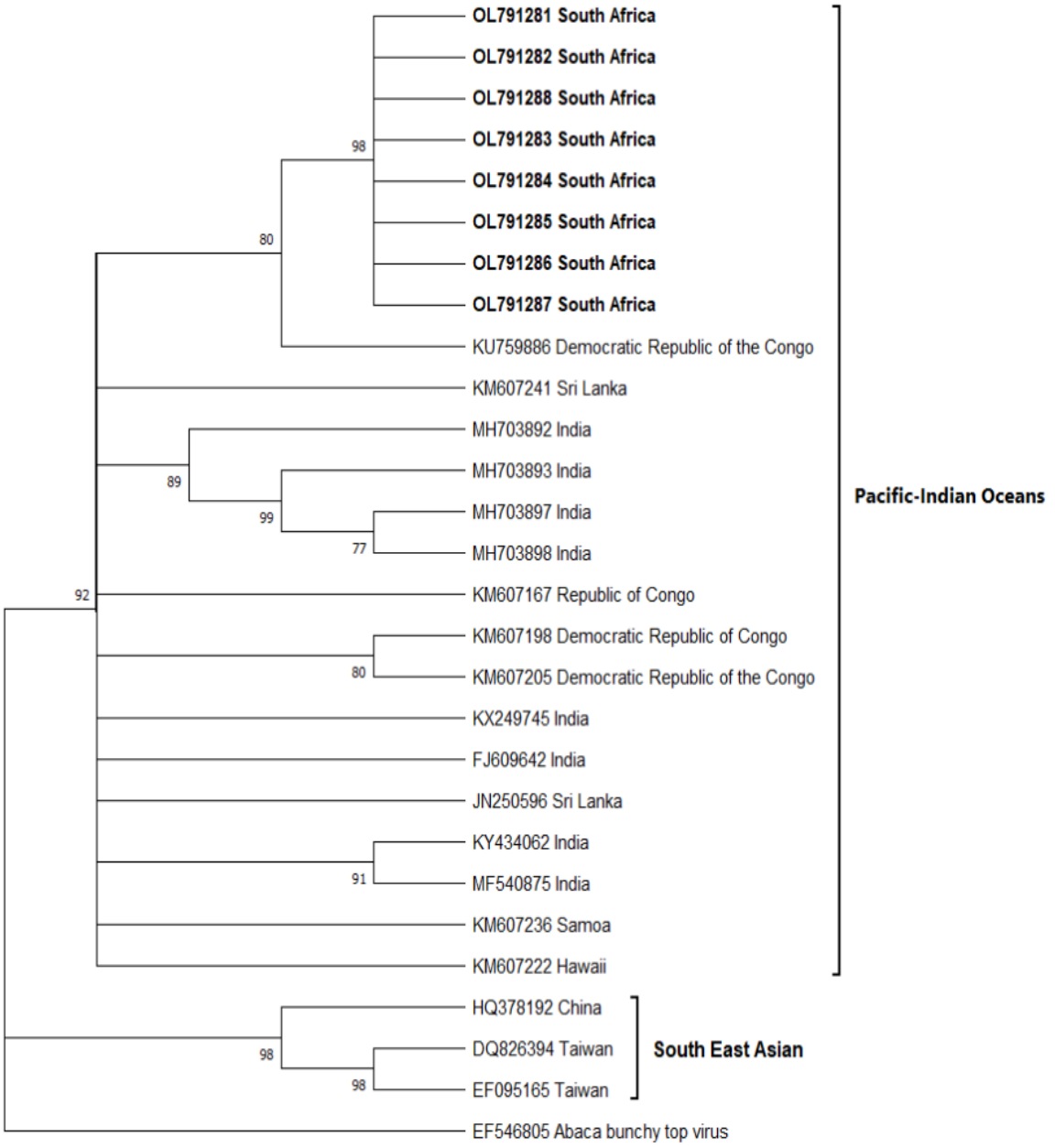

Figure 3.4. Phylogenetic analysis of the partial M gene of BBTV. The tree was rooted using abaca bunchy top virus a closely related species of BBTV.

The South African isolates sequenced for the DNA-M gene clustered together with a bootstrap value of 98% amongst each other and further grouped within the PIO group with a 92% bootstrap value. The South African isolates share a bootstrap value of 80% with DRC isolate with accession number KU759856. There is a 98% bootstrap value amongst isolates in the SEA group (Figure 3.4).

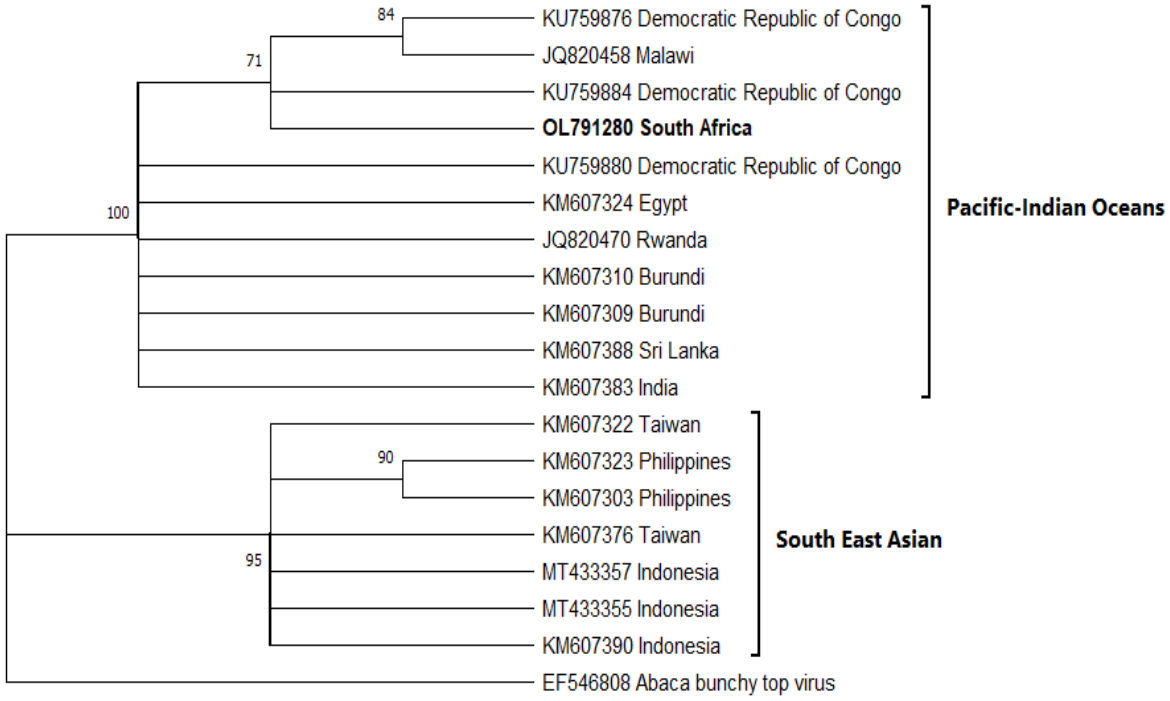

Figure 3.5. Phylogenetic analysis of the partial N gene of BBTV. The tree was rooted using abaca bunchy top virus a closely related species of BBTV.

The sequence result of the DNA-N gene of the South African isolate share close relations with accession numbers KU759876 (DRC); JQ820458 (Malawi) and KU759854 (DRC). The bootstrap value between these isolates is 71%. The South African isolate grouped within the PIO group with a 100% bootstrap value. There is a 95% bootstrap value among the isolates in the SEA group. The two isolates from the Philippines with accession numbers KM607323 and KM607303 share a 90% bootstrap value between each other (Figure 3.5).

3.4 Discussion

In the present study, molecular and phylogenetic characterization of the DNA -S; -N; -C; -U3 and -M components of BBTV isolates from South Africa were explored. Amplification of the different genes resulted in some genomic regions that were not detected in all ten samples (Table 3.3). DNA -S and DNA -M were amplified in almost all the samples; while DNA -N was only amplified in one sample. DNA-C was detected in six of the samples while DNA-U3 was amplified from seven of the samples. There is uncertainty whether nanoviruses invade individual vector cells with few or plenty viral particles allowing the segments to travel separately or together (Di Mattia *et al*.,

2020; Jekayinoluwa *et al*., 2020). The uneven distribution of DNA -N is fairly common in nanoviruses. In an experiment by Timchenko *et al*., (2006) where cloned DNA of faba bean necrotic yellows virus (FBNYV) was introduced into tobacco and tomato by agro-inoculation, systematic symptoms were induced on the plants. The interesting part is that even without including DNA -N, the plants showed symptoms similar to plants that had been infected with all eight cloned DNA components of FBNYV.

A total of 31 sequences from the different components were obtained from this study (Table 3.4). Prior to this study, only the BBTV DNA -R component from South African isolates had been deposited in the National Center for Biotechnology Information (NCBI) database (including those from Chapter 2), therefore the allocation of accession numbers for sequences from this study will contribute to this database as a result.

There was very little (0.4-2.3%) amino acid sequence differences detected between the South African BBTV components in comparison to the other isolates throughout the Pacific-Indian Oceans (PIO) group while the nucleotide variation was as high as 23.8% when compared to the South East Asian (SEA) group. According to Amin (2008), analyses of the genetic diversity of BBTV have shown isolates of the PIO group to be less variable than those of the SEA group. An estimate of ~10% intergroup and ~1.9-3.0% intragroup sequence variation has been reported on studies of PIO and SEA (Karan *et al*., 1994; Das and Banerjee, 2018). According to Stainton *et al*., (2015), India is currently considered a BBTV diversity hotspot and has been found to be the major donor location for BBTV dispersal events to other parts of the globe as well as the major recipient location of virus introductions. These include two dispersal events from the Indian subcontinent to Sub-Saharan Africa between 1825 and 1934, and one to Egypt (between 1929 and 1936), Australia (two events between 1843 and 1974), Tonga (one event between 1735 and 1882), and Samoa (one event between 1915 and 1934) (Stainton *et al*., 2015). It also includes introduction events from SEA (the oldest between 926 and 1619 and two more recent events between 1976 and 1991) and Africa (between 1972 and 1997) (Stainton *et al*., 2015).

The phylogenetic grouping based on the different sequences showed clear indication of two independent groups of BBTV; PIO and the SEA group, with the BBTV components and ORFs from South Africa clustering into the PIO group (Figure 3.1-

3.5). This grouping, which is largely based on the DNA-R, -N and –S segments, initially grouped BBTV isolates globally into the 'South Pacific' or 'Asian' origin before modification to the current grouping system (Kumar, 2011; Baldodiya, 2019). A bootstrap value of 100% was observed between the South African BBTV -S ORF and all the isolates that clustered under the PIO group. (Figure 3.1). The South African BBTV components of DNA -S, -U3, and –M, formed sub-clusters where they grouped alone within the PIO group with bootstrap values ranging from 80-98%. Interestingly in our analysis, four isolates from Egypt clustered under the PIO group while one clustered with the SEA group (Figure 3.3). The sub-clustering of the South African isolates of components DNA –S, -U3 and -M leads to a speculation that even though BBTV has not been in the country for a long time, it has already started mutating (Figure 3.1, 3.2 and 3.4). The South African component DNA -N formed a subgroup with isolates from the Democratic Republic of Congo and Malawi with a bootstrap value of 76% (Figure 3.5). In a study by Mendoza *et al* (2021), the formation of monophyletic clades by isolates from the Philippines was attributed to monophyletic origin of isolates from a common SEA ancestor. A similar conclusion can be reached about the origin of the South African isolates, that they all share a common SEA ancestor.

In conclusion, the findings from this study showed that there is not much diversity within the South African isolates alone and furthermore there is little diversity to the isolates clustered within the PIO group. On the other hand, there is noticeable diversity to the SEA group. Recommendations for future work can include cloning to avoid loss of critical information and hunting for satellites of BBTV as they may have contain useful information that may assist in understanding genetic evolution. It would also be important to characterize more complete genome segments of different BBTV isolates from South Africa and other countries with reported BBTV occurrence because currently the focus is on the DNA -R, -S and -N components (Banerjee *et al*., 2014; Mukwa *et al*., 2016). Nevertheless, the results from this work will contribute significantly in the NCBI database as work of this magnitude hasn't been carried out in South Africa before.

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CHAPTER 4: TRANSMISSION STUDIES TO DETERMINE THE SUSCEPTIBILITY OF ALTERNATIVE HOST PLANTS IN THE TRANSMISSION OF BANANA BUNCHY TOP VIRUS IN SOUTH AFRICA

Abstract

Banana bunchy top virus (BBTV) is transmitted through infected planting material and by its vector: *Pentalonia nigronervosa* (Hemiptera: *Aphididae*). In this study, Plant species, *Colocasia esculenta* (taro), *Alocasia macrorrhizos* (elephant's ear), *Alpinia zerumbet* (shell ginger*)* and *Strelitzia reginae* (bird of paradise*)*, that are usually found growing around banana plantations, were investigated to determine if these plants act as reservoirs of the banana aphid, in South Africa. Furthermore, the study sought to determine if these plants are potential alternative hosts of BBTV. A transmission study was done using a BBTV-infective and BBTV non-infective *Pentalonia nigronervosa* aphid colony. Twenty aphids sourced from banana plants were transferred to six plants of each of the plant species mentioned above using a fine brush; three replications from the infective colony and three from the non-infective colony. The trial was monitored for 88 days post-inoculation and data on the rate of aphid colonization and BBTV symptom expression were recorded at weeks 5, 9 and 12 of the trial. A qPCR protocol was optimised to test for BBTV in plant materials and aphids at each time interval. In addition, *Canna indica, Colocasia esculenta and Strelitzia reginae* growing in close proximity of BBTV symptomatic plants were sampled from the South Coast region of KwaZulu-Natal during surveys to determine if they are hosts of BBTV. In the transmission experiment, aphid numbers declined on *S. reginae*, *A. macrorrhizos* and *C. esculenta* to the point that there were no aphids on these plants by week 5 while aphids established on *A. zerumbet* and *Musa* spp, until the end of the trial. From BBTV transmission studies, all plant species tested positive for BBTV except *A. macrorrhizos*, at the end of the trial following qPCR assays. Furthermore, BBTV was not detected on *C. indica*, *C. esculenta* and *S. reginae* plants found growing in close proximity to BBTV-infected banana plantations. There were also no sign of aphid colonies on these plant species. Conclusions drawn from the transmission study suggest that *A. zerumbet* spp., is an alternative host plant of *P. nigronervosa* and BBTV in South Africa. Furthermore, *S. reginae* and *C. esculenta* are assumed to be intermediary hosts of the virus vector. The results from this

study contribute towards understanding the epidemiological implications of alternative host plants in banana plantations. Therefore, scouting for aphids on these alternative hosts can be implemented as a precautionary measure to mitigate the risk of potential BBTV transmission.

Keywords: Alternative Hosts, Transmission Study, Banana bunchy top virus, Banana aphid, virus-vector relationships, aphid colonization.

4.1. Introduction

Banana bunchy top virus (BBTV), the causal organism of banana bunchy top disease (BBTD), is an important virus affecting banana plantations worldwide. BBTD infects the fruit and foliage of banana plants. The virus colonizes in the phloem tissues and destroys host cells (Tanuja *et al*., 2019a). Early symptoms of BBTD include development of characteristic dark green streaks on the leaf veins with varying length to create a dot-dash appearance known as the Morse code pattern (Qazi, 2016). This symptom correlates internally with change of the phloem and surrounding tissue of the vascular bundles (Magee,1927; Suparman *et al*., 2017). As infection progresses, newly emerging leaves become narrower, develop chlorosis, are upright and dwarfed. This occurrence is the reason for the name of the disease “bunchy top” (Qazi, 2016). Symptom appearance ranges between 21-85 days post infection (Hooks *et al*., 2008; Suparman *et al*., 2017).

Insects play a major role in the rapid dissemination of numerous plant viruses between plants and from one geographic location to another. Their minute size, ability to fly and high reproduction rates make them efficient transmitters of several plant viruses infecting crops (Pinili, 2013). According to Hogenhout *et al*., (2008) aphid species can transmit over 197 (27%) plant viruses from different virus groups. To date, the only known vector of BBTV is *Pentalonia nigronervosa* Coquerel (Family *Aphididae;* Order *Hemiptera*) commonly known as the banana aphid (Pinili, 2013). Infected planting material is another important source for the transmission of BBTV. The virus is not spread mechanically nor is it spread through any other common mode of virus transmission (Suparman *et al*., 2017; Thomas and Dietzgen, 1991).

P. nigronervosa is widely distributed and is found in tropical and subtropical regions globally (Blackman and Eastop, 1984). The banana aphid transmits BBTV in a circulative, non-propagative manner (Magee, 1927, Anhalt and Almedia, 2008).

Circulative viruses enter the insect body with the sap of the infected plant. The virions cross the aphid's gut cell and reach the hemocoel where after they circulate and penetrate the salivary glands. When an infected aphid feeds on a plant, virus particles are transmitted into the plant along with saliva produced during feeding (Watanabe and Bressan, 2013; Suparman *et al*., 2017). Factors such as temperature, aphid life stage and plant acquisition period play a huge role in BBTV transmission (Anhalt and Almeida, 2008). The banana aphid cannot transmit BBTV below 16ºC (Wu and Su,1990). All life stages of the banana aphid can transmit the virus, but winged adult aphids can transmit the virus more efficiently as they move actively from one plant to another (Suparman *et al*., 2017). Jebakumar *et al*., (2018) reported that virus titre also determines the efficiency of *P. nigronervosa* to transmit the virus.

Studies of alternative host plants of BBTV have been done in different countries and the results have been contradictory (Ram and Summanwar, 1984; Geering and Thomas, 1997; Pinili *et al*., 2013; Suparman *et al*., 2017). In some cases, plants such as *Colocasia esculenta* and *Canna indica* acted as experimental hosts in one study and gave a negative result in another study. In this study, we tested *Alpinia zerumbet*, *C. esculenta*, *Strelitzia reginae* and *Alocasia macrorrhizos,* for their suitability as alternative hosts of *P. nigronervosa*. These plants were frequently found in and around banana plantations. Furthermore, the capability of BBTV isolates from South Africa to infect these potential alternative hosts was investigated. The use of real-time PCR, also known as qPCR, is an advanced technology from conventional PCR used to detect plant viruses and is commonly used in the detection of BBTV. It is highly sensitive and can detect viral loads at very low titres. This application uses SYBR Green or TaqMan chemistry (Tanuja *et al*., 2019b). A SYBR Green qPCR (Chen and Hu, 2013) for the detection of BBTV at high Cq (quantification cycles) values from plants and aphids was optimized in this study.

4.2. Materials and Methods

4.2.1. Source of virus inoculum: aphids and plants

Viruliferous aphids were sourced from BBTV-infected banana plants (Cavendish subgroup) growing in the South Coast region of KwaZulu-Natal (KZN). The aphids were maintained in Musa AAA Group cv. Williams plants which were sourced form Du Roi Nurseries (Tzaneen, Limpopo province). A BBTV-free aphid colony was

established on banana plants from aphids collected from the ARC-TSC, Burgershall Research Farm (Hazyview, Mpumalanga province) and maintained on banana plants for a period of 30 days when the desired size of the colony was reached. The plants on which the colony was established were tested by PCR before the colony was established and again before the transmission experiment was started to confirm BBTV-free status. Aphids were also tested. A mixture of adult and nymphs were used in the experiment. Twenty-four test plants, as outlined in Table 4.1, were obtained from local nurseries and were kept in an aphid-free glasshouse before being used in the experiment. Banana plants, *Musa* cv 'Williams' sourced form Du Roi served as transmission controls in the study.

4.2.2. Transmission assay

The experiment was carried out between August and November 2021 at the Virology Unit at ARC-PHP, Roodeplaat (Pretoria, Gauteng Province). It was carried out under very strict quarantine conditions in a growth room with 16:8 photoperiod at 25ºC according to the experimental design of Geering and Thomas (1997) with some modifications. Inoculation with aphids were done when plants were less than 30cm high (soil to stem apex). All plants used in this experiment were PCR negative for the presence of BBTV at the start of the study. The experimental design was completely randomized block design and is available as supplementary material (Appendix B). All test plants were equally replicated (three infected and three uninfected) including Musa cv 'Williams' which served as controls. To test aphid colonization and susceptibility to BBTV of the different plant species (Table 4.1), infective aphids that had completed their life cycle on BBTV-infected banana plants were transferred to 15 test plants consisting of 3 replications of each plant species with a fine tip brush in batches of 20 per plant and to the remaining 15 test plants, 3 of each plant species, non-infective aphids were placed in batches of 20 per plant**.** The aphids were left on each plant in a single plant cage for an inoculation access period of 88 days (Figure 4.1). Aphid colonization and disease prevalence was recorded at weeks 5; 9 and 12 post inoculation. At the end of the experiment, all plants were drenched with an insecticide (Kirchhoff's Ludwig's Insect Spray) before being destroyed.

Table 4.1. Description of plants used in transmission study

Scientific name	Family	Common name	Quantity
Musa cv Williams	Musaceae	Banana	6
Colocasia esculenta	Araceae	Taro	6
Alocasia macrorrhizos	Araceae	Elephant's ear	6
Strelitzia reginae	Strelitziaceae	Bird of paradise	6
Alpinia zerumbet	Zingiberaceae	Shell ginger	6

Figure 4.1. Layout of transmission experiment in the growth room.

The test plants were kept in individual cages throughout the experiment (Figure 4.1).

4.2.3. DNA extraction

Total DNA was extracted from aphids using a method described by Robbertse *et al*., (2019). The positive control consisted of DNA extracted from BBTV-infected aphids and for the negative control, total DNA from a BBTV-free aphid colony. For plant DNA

extraction, the modified cetyl trimethyl ammonium bromide (CTAB) protocol by Robbertse *et al*., (2019) was used on the midrib of symptomatic and asymptomatic plants. The positive control consisted of DNA from BBTV-infected banana plants from previous surveys described in Chapter 2. The negative control consisted of DNA extracted from a banana plant collected at the ARC-TSC, Burgershall farm.

4.2.4. Detection of BBTV using SYBR green quantitative PCR

A standard curve was optimized using a synthesized gBlock fragment (250 ng)(Whitehead Scientific,Cape-Town) from a portion of the coat protein gene (GenBank AB078023) as template. The gBlock was serially diluted up to 10^7 with each step differing by 10-fold from a 10 ng/µl stock solution. The concentration of DNA extracted from plants and aphid samples was determined using a Nanodrop™ 1000 spectrophotometer (ThermoFisher Scientific, USA) and the samples were diluted to 100ng/µl to achieve uniformity for qPCR. Detection of BBTV in aphid and leaf samples was carried out by qPCR using SYBR green chemistry as described by Chen and Hu (2013). Reactions were run in Rotor Gene-Q PCR system (Qiagen, Germany). The primers F1 (5'-ACCAGCCGACTACATGTCTG-3') and R1 (5'TCCTCAACACGGTTGTCTTC-3') were used to amplify 155 bp of the BBTV coat protein gene. A 15 µl reaction mixture containing 100 ng/µl template DNA was prepared using a SensiFAST™ SYBR® No-ROX Kit (Bioline) according to manufacturer's instructions. The standard amplification profile was initial denaturation at 95°C for 3 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s. Samples were amplified in duplicate and average Cq (quantification cycle) values were calculated for analyses. Threshold values for interpreting Cq values were determined with the Rotor gene software based on the established standard curve. The presence of the virus was determined by presence of Cq values where non-amplification was identified by the lack of a Cq value upon analysis at the end of the qPCR run.

4.2.5. Field Surveys of alternative hosts

C. esculenta, *S. reginae* and *C. indica* plants that were found growing close to BBTV-infected plants, were inspected for aphid colonization and typical BBTV symptoms or irregular appearance during field surveys that were conducted between 2018 and 2021 at the South Coast of KZN where BBTV is currently reported. Twelve samples

were collected and tested for BBTV by conventional PCR using primer pair: BBT-1: 5'-CTCGTCATGTGCAAGGTTATGTCG-3 and BBT-2: 5'-GAAGTTCTCCAGCTATTCATCGCC-3', designed to amplify a 349 bp product corresponding to a portion of the BBTV replication initiation protein (Thomson and Dietzgen, 1995).

4.3. Results

4.3.1. Aphid colonization and symptom expression

Upon observation, aphids colonized *A. zerumbet* and *Musa* cv 'Williams' plants only (Figure 4.2-4.3, Appendix B). Initially, 20 aphids were placed per plant. The aphid numbers increased by week 5 and 9 but at week 12 they decreased in both species. There was no aphid colonization on *S. reginae*, *A. macrorrhizos* and *C. esculenta* (Figure 4.2). The "morse code" symptom was observed at week 9 post inoculation on one infected *Musa* cv 'Williams' plant and by week 12 the infected plant displayed a typical bunchy top symptom (Figure 4.4). No symptoms were observed on any of the other test plants.

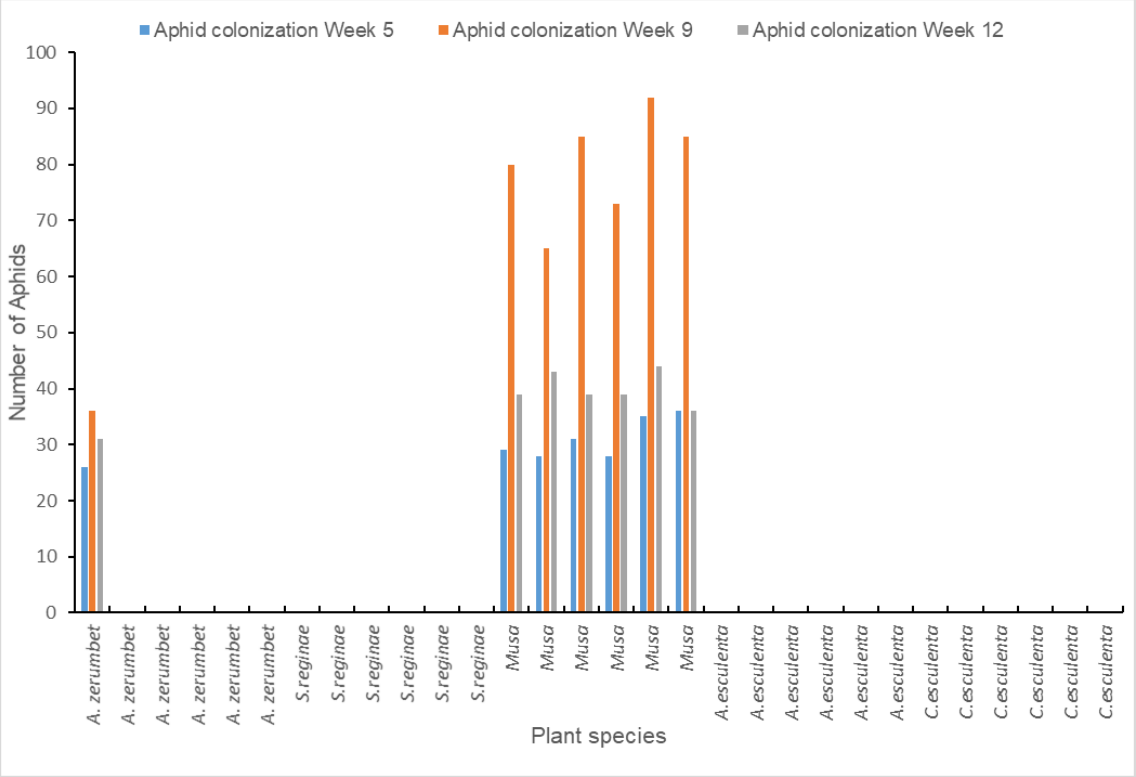

Figure 4.2. Aphid colonization on the different potential alternative host plants

Figure 4.3. High aphid infestation on *A. zerumbet* plant week 9 inoculation. The aphids were found on the top and underside of the leaf.

Figure 4.4. Bunchy symptom on a banana plant at week 12 post inoculation.

4.3.2. Detection of BBTV using SYBR green based qPCR assay

The qPCR was validated using the 10 ng/µl gBlock template with known concentration to determine the efficiency and sensitivity of the assay. An amplification efficiency of 0.91 with a linearity correlation coefficient (R^2) of 0.9995 using a seven-point 10× dilution standard curve was shown (Figure 4.5).

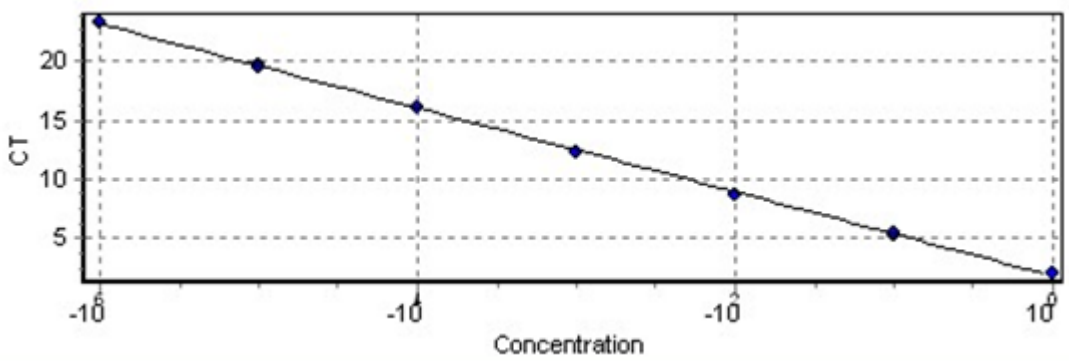

Figure 4.5. The standard curve obtained for BBTV DNA -S coat protein quantification

Results from the qPCR are summarized in Tables 4.2 - 4.4 where high Cq values represent low virus titres. One plant of *C. esculenta* tested positive in week 5 with a slight increase in concentration detected in weeks 9 and 12. Another plant of *C. esculenta* tested positive in week 9 and also showed an increase in concentration in week 12. One plant of the *Musa* cv 'Williams' tested positive in week 9 and an increase in concentration was detected in this plant over time. At week 12, very high Cq values were detected for two *S. reginae* plants while one plant showed no amplification; two of three *A. zerumbet* plants showed BBTV amplification as well. There was no amplification on any of the *A. macrorrhizos* plants throughout the experiment which was interpreted as a negative result (Table 4.2). Furthermore, there was no amplification on any of the uninfected plants (Table 4.3). In addition, none of the aphids which had colonized the plants tested positive for BBTV (Table 4.4).

Table 4.2. Cq values of the BBTV-infected test plants from week 5 to week 12

Plant	Sample Code	Week 5 (Cq value)	Week 9(Cq value)	Week 12 (Cq value)
Strelitzia reginae	**2**	**-**	**-**	**34.86**
	14	**-**	**-**	**38.0**
	28	-	-	-
Colocasia esculenta	**5**	**-**	**36.17**	**32.80**
	15	-	-	-
	26	**32.73**	**30.82**	**30.63**
Alpinia zerumbet	9	-	-	**41.62**
	16	-	-	**36.51**
	23	-	-	-
Musa cv 'Williams'	10	-	-	-
	12	-	-	-
	21	**-**	**36.11**	**33.50**
Alocasia macrorrhizos	6	-	-	-
	20	-	-	-
	22	-	-	-

- No amplification

The qPCR could only detect BBTV amplicons at week 12 on the *S. reginae* and A*. zerumbet* plants. BBTV amplification was observed on *C*. *esculenta* from as early as week 5, from week 9 on *Musa* cv 'Williams' and no amplification was observed on any of the *A. macrorrhizos* plants. In addition, BBTV amplification was not observed on any of the aphids that colonized the plants in the experiment (Table 4.2). The low Cq values were confirmed using the conventional PCR described in Chapter 2. There was no BBTV amplification on any of the control plants. This was an expected result as BBTV-free aphids had been placed on these plants.

Table 4.3. Non-amplification of BBTV on aphids that colonized the test plants

Plant	Sample code	Week 5 (Cq value)	Week 9 (Cq value)	Week 12 (Cq value)
Musa cv 'Williams'	4 (Control)	-	-	-
Musa cv 'Williams'	10 (Infected)	-	-	-
Musa cv 'Williams'	12 (Infected)	-	-	-
Musa cv 'Williams'	19 (Control)	-	-	-
Musa cv 'Williams'	21 (Infected)	-	-	-
Musa cv 'Williams'	24 (Control)	-	-	-
A. zerumbet	30 (Control)	-	-	-

- No amplification

BBTV was not amplified from aphids that colonized on plants in the experiment (Table 4.3)

4.3.3. Field Surveys of alternative hosts

Upon visual inspection of field plants, no aphids were found on any of the potential alternative host plants in banana plantations and there was no BBTV amplification reported on any of the plants either (Table 4.4)

Table 4.4. Absence of banana bunchy top virus in naturally occurring plant populations

Alternative Host	GPS co-ordinates	PCR result
C.esculenta	-30.8022, 30.3982	-
C.esculenta	-30.8022, 30.3982	-
C.esculenta	-30.8022, 30.3982	-
C. esculenta	-30.5317, 30.5028	-
C.esculenta	-30.5069, 30.5111	-
S. reginae	-30.5451, 30.5829	-
S. reginae	-30.7719, 30.3868	-
C. indica	-30.7719, 30.3868	-
C. indica	-30.5383, 30.5868	-
C. indica	-30.4995, 30.5593	-
C. indica	-30.6118, 30.5171	-
C. indica	-30.6118, 30.5171	-

- No amplification

4.4. Discussion

In this study, there was no evidence that *C. esculenta*, *S. reginae* and *A. macrorrhizos* act as reservoirs of the banana aphid (Figure 4.2). The aphids established on *Musa* cv 'Williams' as expected, and interestingly on one *A. zerumbet* plant which had non-viruliferous aphids. Aphid colonization was optimal on *Musa* cv 'Williams' and *A. zerumbet* from week 5 to week 9 but by week 12 there was a decline in aphid populations on both plant species. Aphids were found on the underside of the leaf blades as well as on the pseudostem, more noticeably closer to just above ground level (Figure 4.3). This observation supports a statement by Geering and Thomas (1997) and Pinili *et al* (2013) that *P. nigronervosa* has been found on *Alpinia* species. In a transmission study by Pinili *et al*. (2013) aphids were reported to multiply but at low levels compared to *C. esculenta* (Pinili *et al*., 2013). Colonization of *A. zerumbet* by the banana aphid also validates findings by Blackman and Eastop (1984) and Suparman *et al*., (2017) that plants from the Zingiberaceae family tend to act as reservoirs for the banana aphid. Suparman *et al.* (2017) listed three Zingiberaceous plants; *Curcuma zerumbet* Roxb. (bitter ginger), *Zingiber officinale* (ginger) and *Curcuma zanthorrhiza* Roxb. (Javanese ginger) as suitable alternative hosts for the banana aphid. In this study, no aphids were observed on *S. reginae* and *A.*

macrorrhizos plants from week 5 (Fig 4.2). It has been reported that aphids use chemical and morphological characteristics to evaluate the suitability of plants. Plant suitability may include trichomes and epicuticular waxes (Suparman *et al*., 2017). According to Bhadra and Agarwala (2010), different plant species provide different food environments for colonization despite belonging to the same family. Factors such as plant age, the number of aphids used for transmission, the feeding time and the inoculation access period all play a role on virus transmission (Boukari *et al*., 2020).

No aphids were observed on *C. esculenta* in field surveys as well as in the glasshouse trials (Figure 4.2 and Table 4.4). This contradicts findings by Ram and Summanwar (1984); Pinili *et al.* (2013) and Suparman *et al.* (2017) who reported *C. esculenta* to be a host of the banana aphid. In fact, Suparman *et al.* (2017) further added that *C. esculenta* (taro) is amongst the most suitable alternative hosts along with *A.galangal* (greater galangal) and *Xanthosoma sagittifolium* (blue taro). In a host transfer experiment by Bhadra and Agarwala (2010), when *P.nigronervosa* aphids from banana plants were transferred to *C. esculenta*; a 60% decline in the second and third generation was observed. This observation supports our study that *C. esculenta* is not a very suitable host for *P. nigronervosa*.

Despite not observing the presence of aphids on *C. esculenta* nor the plants exhibiting any symptoms, BBTV infection was detected with an optimized qPCR as early as week 5 (Table 4.2) from (2/3) *C. esculenta* plants used in the transmission study which allows us to conclude that taro is a good experimental host. It should be noted that BBTV was not detected on any *C. esculenta* plants collected from field surveys. According to Shange (2004), in South Africa, taro is mainly produced in the subtropical coastal belt, stretching from Bizana in the Eastern Cape to the KwaZulu-Natal north coast in South Africa. A number of households grow taro plants along with banana plants as an additional source of income in this region (personal communication). The detection of BBTV on the *C. esculenta* plants is therefore seen as a threat to these subsistence farmers. The economic impact of BBTV on *C.esculenta* is not known and its implication to host BBTV might be detrimental.

One banana plant displayed typical BBTV symptoms and tested positive for BBTV (Figure 4.4 and Table 4.2). Quantification cycles (Cq) values decreased which was an indicator of increased viral titre in the plant. As much as banana is the preferred host

for BBTV, it is possible that a low efficiency of transmission was occurring resulting in non-expression of symptoms. This was observed in this study as some *Musa* cv 'Williams' plants (2/3) that were treated as positive controls tested negative for BBTV (Table 4.2). Results from a study by Chen and Hu (2013) found that 14% of 'Williams' plants showed symptoms at 30-40 days post inoculation and that another 14% did not show any symptoms. As expected, BBTV was not amplified from any of the negative control plants in this study. The non-amplification of BBTV from aphids in this study could be attributed to the fact that no transovarial transmission of the virus occurred to aphid offspring as aphids were only tested from week 5 post inoculation (Table 4.3). The life span of an aphid ranges from 19 to 26 days (Niyongere *et al*., 2013). In an experiment by Hu *et al*. (1996), aphids used in their study retained the virus for a lifespan of 15-20 days but none of the 131 offspring from adult reared on infected banana plants tested for BBTV.

Banana bunchy top virus is considered an economically important disease in South Africa and as such, it is included as a pest that is governed under the Agricultural Pests, (Act no. 36 of 1983 of South Africa). Movement of infected plants is therefore prohibited. The common trend in controlling banana bunchy top virus has been mainly ensuring restricted movement of infected planting material, the planting of certified virus-free material as well as controlling the aphid vector. In this study we have highlighted the importance of scouting for the vector on neighboring plants as some may act as reservoirs thus hampering control strategies. The amplification of BBTV on *C. esculenta* and *S. reginae* plants could be explained based on the hypothesis that these plant species serve as intermediary hosts for the banana aphid. An optimized qPCR assay for the detection of BBTV in plants and aphids has been developed and this can help improve in the early detection of the virus. Future research should include more economically important plants such as *Zingiber officinale* (ginger) and *Curcuma longa* (turmeric). It is important to note that inclusion of such plants would need to happen during summer as these two plants lose their leaves in winter and it is for that reason that they were not included in the current study. Another plant that can be included is *C. indica* which couldn't be included in the current study due to non-availability at nurseries. The findings from this study will contribute towards understanding the epidemiological implications of alternative host plants in banana plantations.

4.5. References

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THESIS OVERVIEW

Major Findings

The different experiments carried out in this research were motivated by the discovery of banana bunchy top virus (BBTV) in KwaZulu-Natal (KZN), South Africa in 2016. The experimental chapters have been written as discrete research chapters; Chapter 2 has been submitted for peer-review in one journal while Chapter 3 and Chapter 4 will be submitted for peer-review after thesis submission.

The first research Chapter, *i.e.* Chapter 2, describes the surveyed regions and findings that have been covered in the various provinces of South Africa to determine if BBTV is present in any other banana-growing region besides the South Coast of KZN. Surveys were conducted from July 2017 to February 2021 in KwaZulu-Natal, Mpumalanga and Limpopo provinces with the assistance of extension officers from Department of Agriculture Land Reform and Rural Development. A partial gene characterization of the Replicase gene was performed to confirm the identity of BBTV. Furthermore, phylogenetic analysis was performed to determine the relationship of the isolates collected in relation to global BBTV isolates.

Results reported in Chapter 3 on the comparative molecular analysis of BBTV components to global sequences showed insignificant differences between South African isolates to those in the Pacific-Indian Oceans group. This could be due to few introductions within this group. It was also noticed that not all BBTV components could be detected in each sample.

The findings in Chapter 4 on the study of alternative hosts of banana bunchy top virus in South Africa showed that all the plants used in the transmission study except *Alocasia macrorrhizos* are hosts of the virus. *Alpinia zerumbet*, acts as a reservoir for *Pentalonia nigronervosa*, the vector of BBTV, while *Strelitzia reginae* and *Colocasia esculenta* are assumed to be intermediary hosts of the virus vector. These findings have proven to be contradictory to other researchers and in agreement to some researchers who have conducted similar transmission studies.

This thesis provides the most comprehensive survey of BBTV status in South Africa where three provinces were surveyed. The lack of sequences in the NCBI database especially for the five components of BBTV that are studied in Chapter 3 shows the originality of this work. Transmission studies of BBTV in different host plants hasn't been reported in South Africa according to our knowledge so the study is a first of its kind in the country.

Way forward

Efforts to contain the spread of BBTV in the South Coast region of KwaZulu-Natal needs to be continued through active involvement of all stakeholders. Continuous monitoring of BBTV presence across banana-producing regions also needs to be ongoing in order to curb further spread of the virus. Removal of infected banana plants will be crucial for the eradication of BBTV from the affected KZN region. This could be achieved by constant scouting and raising awareness about the virus. Extensive studies of the BBTV genome needs to be carried out in order to shed more light on how resistance can be developed against BBTV. A full Replicase gene needs to be amplified in order to add to the list of the ones that were sequenced. The study of alternative hosts can be improved by using more replications as well as more plant species such as *Canna indica* and *Zingiber officinale.*

CONFERENCE OUTPUTS TO DATE

Conference: ARC Postgraduate Day
Title: Monitoring strategies regarding the spread of Banana bunchy top virus in South Africa. - Best PhD Oral presentation award
Year and location: November 2021, Mpumalanga, South Africa

Conference: Postgraduate Research & Innovation Symposium (PRIS) - UKZN
Title: Surveying the distribution and monitoring of banana bunchy top virus outbreak in South Africa (Oral presentation)
Year and location: October 2019, Western Cape, South Africa

Conference: 51st Congress of the South African society for Plant Pathology
Title: Monitoring the spread of Banana bunchy top virus in the South Coast of KwaZulu-Natal. (Oral presentation)
Year and location: January 2019, Western Cape, South Africa

Conference: ARC Professional Development Programme Presentations
Title: Monitoring the spread of Banana bunchy top virus in the South Coast of KwaZulu-Natal (Oral presentation) - 2nd Best Oral presentation Award
Year and location: August 2018, Pretoria, RSA

Conference: XXX International Horticultural Congress: IHC 2018
Title: Monitoring the spread of Banana bunchy top virus in South Africa (Oral presentation)
Year and location: August 2018, Istanbul, Turkey

APPENDIX A *

APPENDIX B

Aphid colonization on the different test plants

Species (Cage number)	Experiment Type	Aphid colonization		
		WEEK 5	WEEK 9	WEEK 12
1.Alpinia	Control	26	36	31
2.Strelitzia	Infected	0	0	0
3.Alocasia	Control	0	0	0
4.Banana	Control	29	80	39
5.Colocasia	Infected	0	0	0
6.Alocasia	Infected	0	0	0
7.Colocasia	Control	0	0	0
8.Strelitzia	Control	0	0	0
9.Alpinia	Infected	0	0	0
10.Banana	Infected	28	65	43
11.Alocasia	Control	0	0	0
12.Banana	Infected	31	85	39
13.Strelitzia	Control	0	0	0
14.Strelitzia	Infected	0	0	0
15.Colocasia	Infected	0	0	0
16.Alpinia	Infected	0	0	0
17.Alpinia	Control	0	0	0
18.Colocasia	Control	0	0	0
19.Banana	Control	28	73	39
20.Alocasia	Infected	0	0	0
21.Banana	Infected	35	92	44
22.Alocasia	Infected	0	0	0
23.Alpinia	Infected	0	0	0
24.Banana	Control	36	85	36
25.Alocasia	Control	0	0	0
26.Colocasia	Infected	0	0	0
27.Colocasia	Control	0	0	0
28.Strelitzia	Infected	0	0	0
29.Strelitzia	Control	0	0	0
30.Alpinia	Control	0	0	0

*Separate Excel file