OCCURRENCE, CHARACTERIZATION AND SCREENING FOR RESISTANCE TO BANANA BUNCY TOP VIRUS IN BURUNDI, DEMOCRATIC REPUBLIC OF THE CONGO AND RWANDA

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Occurrence, Characterization and Screening for resistance to Banana Buncy Top Virus in Burundi, Democratic Republic of the Congo and Rwanda

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this thesis to my brothers' families Albert and Serges, my parents-in-law Célestin Sindatuma families, my lovely wife Adélaïde Niyonkuru and our son Jean Lucas Niyongere

Thank you all for your encouragement and Love

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

ABTV	Abaca bunchy top virus
ANOVA	Analysis of variance
BBTD	Banana bunchy top disease
BBTV	Banana bunchy top virus
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CIALCA	Consortium for Improving Agriculture-based Livelihoods in Central Africa
CP	Coat protein
DNA	Deoxyribonucleic acid
FACAGRO	Faculté des Sciences Agronomiques de l'Université du Burundi
FAO	Food and Agriculture Organization
FHIA	Honduras Foundation for Agricultural Research
GEB	General extract buffer
GLRA	Great Lakes region of Africa
GPS	Global positioning system
IGEBU	Institut Géographique du Burundi
ISABU	Institut des Sciences Agronomiques du Burundi
ISAR	Institut des Sciences Agronomiques du Rwanda
IRAZ	Institut de Recherche Agronomique et Zootechnique
IITA	International Institute of Tropical Agriculture
ITC	International Transit Center
OD	Optic density

ORF	Open reading frame
PCR	Polymerase chain reaction
SL-CR	Stem-loop common region
SsDNA	Single-stranded deoxyribonucleic acid
TAS ELISA	Triple Antibody Sanduich-Enzyme Linked Immuno sorbent Assay

ABSTRACT

Banana bunchy top disease (BBTD) is caused by *Banana bunchy top virus* (BBTV) which is transmitted by the aphid vector, *Pentalonia nigronervosa* Coquerel and through infected planting materials. BBTD is the most common and destructive viral disease that hinders banana production worldwide. It was reported for the first time in the Great Lakes Region of Africa (GLRA) in 1958 in the Democratic Republic of the Congo (DR Congo) and in 1987 in Burundi and Rwanda. Since then, no specific study has been undertaken on the disease in the GLRA. Therefore, in this study, the BBTD incidence, occurrence of the vector and farmers' awareness about the disease were assessed. Nucleotide sequences of GLRA isolates were characterized, whereas the varieties were screened for their resistance/tolerance to the disease. Additionally, seasonal and spatial distribution of *P. nigronervosa* was assessed.

A survey was conducted from September to October 2008 across five provinces in Burundi, Eastern DR Congo and Western province, Rwanda. In total, 7830 banana mats were assessed in 261 farms where 30 mats were considered per farm. Farmers were also interviewed on BBTD management. The regional averages for BBTD incidence and aphid occurrence were 25% and 46%, respectively. Among the interviewed farmers, 90% of them were able to recognize BBTD symptoms, while 95% were not informed on the disease management options and that none of the farmers' varieties was resistant to the disease.

A study on molecular characterization was performed using 22 and 19 isolates-based on BBTV DNA-R and Coat protein, respectively. These GLRA isolates in phylogenetic analysis were classified in the South Pacific group and followed by the Indian isolate. Molecular analyses revealed that the disease occurred in the region

from 1970s. Compared to other South Pacific group isolates, the GLRA isolates showed a relatively higher genetic variability.

Screening for resistance to BBTD was conducted using 40 different *Musa* genotypes at the ISABU Mparambo research station, from March 2007 to September 2010. There was no cultivar totally resistant to BBTD. However, five tolerant cultivars [‘Corne plantain’ (AAB-plantain), ‘FHIA-03’ (AABB), ‘Highgate’ (AAA, Gros Michel subgroup), ‘Igjindi’ (AAB-plantain) and ‘Saba’ (ABB)] out of 40 genotypes tested were selected based on their relative advantages for farmers’ adoption. The last activity was carried out to determine how aphid vectors influence disease spread in different locations. At nine months after trial establishment, BBTD incidence ranging from 21.8 to 56.4% was observed in plots established within BBTD-affected banana fields, while 0 to 12.3% incidence was reported in plots located outside BBTD-affected banana fields. Aphids were reported in the three study regions in Burundi with higher aphid numbers observed in the dry season (i.e, July and August). These aphid vectors were able to acquire and transmit the virus irrespective of altitude. A disease incubation period of 21 and 84 days was observed at low (780masl) and high (2090masl) altitudes, respectively.

From these results, the use of tolerant cultivars could be hampered by the genetic variability of BBTV and widespread nature of aphids. Hence, in context of small-scale agriculture, sustainable BBTD management consists of raising famers’ awareness on early detection and eraducation of BBTV-infected mats and adopting cultural practices aimed at the control of aphid populations. In addition, quarantine measures should help to prevent disease spread between BBTD affected and unaffected areas within the African Great Lakes region.

CHAPTER ONE

INTRODUCTION

1.1. Background

Banana (*Musa* spp.) is cultivated in more than 130 countries in the tropics and subtropics and is a staple food crop for millions of people, particularly in Africa (Frison and Sharrock, 1998). Banana is also the most important fruit crop used as dessert, and about 16 million tonnes are exported from these banana growing countries each year (Daniells, 2009). It is a source of carbohydrates to about 70 million people in Africa (INIBAP, 2000). In the Great Lakes Region of Africa (GLRA), which includes Rwanda, Burundi and the Democratic Republic of the Congo (DR Congo), the dependence on banana production is particularly evident by the high levels of per capita annual consumption of 382, 236 and 69 Kg/capita/year in these countries, respectively (Frison and Sharrock, 1998). Banana is also used to produce beer in Burundi and Rwanda, making the crop one of the main dependable cash crops in these two countries (Karamura *et al.*, 1998). Banana produces fruits all year-round, thus supplying food and income to the farmer on a continuous basis. In this respect, the crop is a major contributor to food security worldwide (Olorunda, 1998).

Banana cultivars are mostly diploid and polyploid hybrids of two wild banana species, *Musa acuminata* and *Musa balbisiana* (Stover and Simmonds, 1987). Five main genomic groups of cultivated banana designated as AA, AAA, AB, AAB, ABB

and four tetraploids hybrids namely, AAAA, AAAB, AABB and ABBB have been identified (Heslop-Harrison and Trude, 2007).

These banana genotypes potential is greatly hampered by several biotic and abiotic constraints prevailing in different banana producing regions. Among them, banana bunchy top disease (BBTD), which is caused by the *Banana bunchy top virus* (BBTV), is the most economically important disease in banana cultivating areas such as the GLRA. BBTV belongs to the genus *Babuvirus* in the family of *Nanoviridae*, with an approximately 18 - 20 nm isometric virions (Karan *et al.*, 1997; Su *et al.*, 2003). The virus has isometric virus particles containing six circular single stranded DNA (ssDNA) components, each measuring about 1kb in size (Wanitchakorn *et al.*, 2000). Genetic diversity within the BBTV species has revealed the existence of two distinct groups of BBTV isolates. These groups were designated as Asian group and South Pacific group (Karan *et al.*, 1994). The South Pacific group contains isolates from different regions of the South Pacific region (Australia and Fiji) as well as regions located outside this region such as Burundi, Egypt and India (Wanitchakorn *et al.*, 2000).

BBTV is a systemic virus whose asymptomatic suckers are responsible for the disease spread at long distances. Besides, across banana plantations, the virus is transmitted by the banana aphid, *Pentalonia nigronervosa*. However, the virus is not transmitted mechanically (Magee, 1927; Yasmin *et al.*, 2001).

The *P. nigronervosa* exists as either wingless or winged aphids. The winged adults which transmit BBTV often develop after 7 to 10 generations of wingless individuals

(Nelson, 2004). Dispersing winged adults establish new colonies on other new host plants. Although they are not strong fliers, they may be carried over considerable distances by light winds (Allen, 1987; Ferreira *et al.*, 1997).

Controlling these aphids with contact insecticide sprays is very difficult, because the spray must drench behind leaf sheaths and reach the protected areas to kill aphids (Ferreira *et al.*, 1997).

Various factors such as BBTD occurrence and spread, genetic variability of BBTV isolates, source of resistance among different genotypes, seasonal and spatial distribution of the banana aphids, need to be taken into consideration for an effective BBTD integrated management approach.

1.2. Problem statement and justification

Banana is a staple food and cash crop in the GLRA. However, yields have been declining to levels below 10 t/ha (Karamura *et al.*, 1998) due to various constraints such as soil fertility, pests and diseases. These have resulted to an aggravated food deficit situation in the region. Diseases constitute one of the most serious production constraints in the region (Tushemereirwe and Bagabe, 1999) and BBTD is considered as one of the most destructive diseases affecting banana production worldwide (Kavino *et al.*, 2007; IITA, 2010). In GLRA, BBTD was identified in 1987 and ever since, it has been confined within the Bujumbura valley (850-1200m) with a few cases reported in Cibitoke province “Cibitoke, Kaburantwa communes” in Burundi and the adjacent Bugarama valley (800-850m) in Rwanda (Sebasigari and Stover, 1988). The disease occurrence was concentrated in a small area where it

could have been eradicated. Since farmers did not foresee BBTD as a limiting factor in banana production (Sebasigari and Stover, 1988), the little effort was made to control its spread. It was considered as a mystery that the disease has not spread widely in the region which led to the thought that the vector was inactive or absent (Sebasigari and Stover, 1988). In 1987, Sebasigari and Stover (1988) recommended a program meant for the eradication of BBTD in Burundi and Rwanda. However, no action was taken in this regard at the time.

Tushemereirwe and Bagabe (1999) confirmed absence of information on the impact of BBTD although the virus was reportedly important in some areas of the region. According to the Consortium for Improving Agriculture-based Livelihoods in Central Africa (CIALCA), farmer baseline surveys carried out from June, 2006 to February, 2007 indicated that farmers complained of the destruction of their banana plantations by BBTD (CIALCA, 2008). Thus, famers classified BBTD as a serious constraint to banana production in major banana producing areas such as Cibitoke province in Burundi, an area that acquired its name from banana as a crop (CIALCA, 2008).

Although the continuous spread of BBTD is a fact in the GLRA, no specific in-depth investigation has been carried out to generate more significant data which could allow development of suitable strategies to permanently combat this disease which is likely to significantly hamper banana production in the region. This is the motivation behind which this study was carried out. The study therefore aimed at identifying approaches for integrated management of banana bunchy top disease in African Great Lakes region.

1.3. Objectives of the study

1.3.1. The overall objective

To determine the occurrence, source of resistance among *Musa* genotypes and molecular status of Banana bunchy top virus in the Great Lakes region of Africa.

1.3.2. The specific objectives

The specific objectives of this study were:

(i) To determine the occurrence and distribution of BBTD in major banana producing regions of Burundi, Eastern DR Congo and Rwanda;

(ii) To characterize BBTV isolates collected from Burundi, Eastern DR Congo and Rwanda;

(iii) To evaluate the response of *Musa* genotypes to *Banana bunchy top virus* and

(iv) To determine the influence of seasonal and spatial occurrence of *Pentalonia nigronervosa* Coquerel on banana bunchy top disease spread in Burundi.

This study focused on establishing the occurrence of the BBTD and its vector *Pentalonia nigronervosa* (aphididae). The molecular characterization of the virus associated with the disease was assessed and Musa genotypes screened for resisatnce. The study aimed at developing an integrated disease management for BBTD in the Great Lakes Region of Africa.

CHAPTER TWO

LITERATURE REVIEW

2.1. Banana plant (*Musa* spp.)

The banana is a perennial herb plant that grows from a bulb or rhizome and replaces itself regularly (Morton, 1987). The plant consists of a subterranean stem or corm that bears developing suckers, the root system, the pseudostem, the leaves and the inflorescence that bears the flowers and subsequently the fruit. A stool or mat of banana is composed by mother plant and shooting daughters or suckers (Karamura, 1995). The pseudo-stem of banana is formed by upright concentric layers of leaf sheaths that constitute the functional trunk. Approximately 44 leaves appear on the plant before inflorescence. The time between planting a banana plant and harvesting ranges from 9 to 12 months (Morton, 1987).

2.2. *Musa* genetics

Banana cultivars are mostly diploid and polyploid hybrids of two wild banana species, *Musa acuminata* and *Musa balbisiana* (Stover and Simmonds, 1987). Ploidy and genome composition of the different clones are designated as A and B in *M. acuminata* and *M. balbisiana,* respectively (Rowe and Rosales, 1996). Based on morphological observations of the characters that differentiate these two species and on the ploidy level of different clones, five main genomic groups of cultivated banana designated as AA, AAA, AB, AAB and ABB have been identified (Heslop-Harrison and Trude, 2007). Within each group, related clones are associated in a

subgroup (Sauer, 1993; Carreel *et al.,* 2002). For example, the dessert banana, which include AAA (i.e. triploid banana is composed of only *M. acuminata*), or AA (diploid banana is only composed of *M. acuminata*) and AAB (triploid banana is composed of 2/3 *M. acuminata* and 1/3 *M. balbisiana).*

The same genomic classification applies to banana for cooking and banana for beer making. On the other hand, plantain belongs to only one genomic group AAB (Margaret, 2007). Additionally, the four groups of tetraploids hybrids AAAA, AAAB, AABB and ABBB were genetically developed in order to improve their tolerance to diseases and increase banana yields (Bakry *et al.*, 2001; Heslop-Harrison and Trude, 2007; Geering, 2009).

2.3. Historic of banana and plantain cultivars

The centre of greatest diversity of wild *Musa* species is in Indochina and South East Asia where the early domestication occurred (Simmonds, 1962). In fact, hybridazation between sub-species of the polymorphic species *M. acuminata* led to a range of diploid cultivars (AAs). Diploid AAs gave rise to triploid AAA types. Hybridazations between AAs and *M.balbisiana* (BB) gave rise to the various AAB and ABB types. Simmonds postulates that AA and AAAs genotypes were dosmeticated in Malaysia and spread after through South East Asia where *M. balbisiana* occurred and led to hybridization and appearance of AB hybrids and the more vigourous AAB and ABB types (Simonds, 1962, Champion, 1976).

Introduction of banana and plantain in Africa was by Arabs who considered banana as a holy plant in the Koran (Reynolds, 1927, Kervégant, 1935). Distinct and

multiple introductions of banana and plantain in Africa were also reported, such from Indonesia through Madagascar to the cost of Africa from around the 5th century (Vérin, 1981). Many think that the Island was an important conduit for introductions either from the Indian sub-continent where AAB and ABB varieties predominate, or directly from South East Asia where AA and AAAs genotypes are more common. The varieties unique to Africa, in particular of AAA East African high Land banana and also of AAB plantains, have arisen as a result of somatic mutations (Simmonds, 1966).

The vast majority of cultivated types are currently triploid: AAAs providing sweeter cultivars, AAB plantain and ABBs often providing more starchy cooking types. Sterility, itself a result of triploidy, would have led to vegetative propagation by suckers (Gowen, 1995).

2.4. Utilization of *Musa* cultivars

The *Musa* cultivars grown are used for different purposes such as dessert bananas, AAB plantain and cooking bananas (Stover and Simmonds, 1987; Margaret, 2007). The plantain (AAB) is eaten cooked even when is ripe. It is a much starchier food than the banana: while in the ripe banana 80% of solids are sugar and less than 5% are starch, in the plantain sugars make up only 66% of solids and starch accounts for 17% (Ketiku, 1973).

Banana and plantain are not only used as staple food crop, but they also provide an essential source of income through different utilizations (Chandler, 1995). Banana fibre is used for making baskets, carpets and other handcrafts. Lower grade banana

and green leaves may be used as animal feed. The leaves are also used for covering food when cooking or packaging cooked food in many countries (Ogazi, 1996). Furthermore, banana in perennial production systems maintains soil cover throughout the year and if their biomass is used for mulch, soil fertility and organic matter remain stable with respect to soil and water conservation as well as nutrient storage and bio-recycling (Delvaux, 1998).

Apart from being cheap and easily produced source of energy, banana and plantain are also rich in vitamins A, C and B6 and potassium (Dickinson, 2000). Banana food contributes 25% of the source of carbohydrate requirements for over 70 million of people in Africa (IITA, 1998).

A high content of carbohydrates in banana and plantain makes them a very good source of energy for people practising sports whereas potassium helps to improve brain functioning (Raut and Ranade, 2004).

2.5. Banana production

Banana and plantain are cultivated in over 130 tropic and subtropic countries. The crop products constitute a major staple food crop and a source of income through local and international trade for millions of people (Frison and Sharrock, 1998). Furthermore, banana crop contributes to food security and generates income to smallholder producers all year-round (Olorunda, 1998). The crop is grown in over 10 million hectares, with an annual production of around 88 million metric tonnes. Thus, banana is the developing world's fourth most important food crop after rice, wheat and maize in terms of gross value of production (Frison and Sharrock, 1998).

Africa produces nearly 30 million tons of bananas yearly, which is equivalent to 34% of world's production; and mostly for local consumption (Karamura *et al.*, 1998). The Eastern region of Africa is also the world's leading consumer of banana with an annual per capita consumption rate of about 400-600 kg. In Rwanda and Burundi, the production of banana for beer making is a major commercial activity (Karamura *et al.,* 1998). In 2008, banana was ranked as number one crop in terms of domestic earning in Burundi and Rwanda, contributing estimated annual incomes of 263.6 million $ and 576.7 million $ in these two countries respectively. In DR Congo, banana was ranked as number two crop after cassava in terms of importance, contributing about 267.7 million $ in domestic earnings (FAOSTAT, 2009). However, banana and plantain production is limited by biotic and abiotic constraints.

2.6. Constraints of banana production

Banana and plantain are cultivated worldwide, especially in the tropic and subtropic regions of the world and in the Mediterranean, under various types of cropping systems with yields ranging from 5 to 70t/ha per year (Delvaux, 1998). The yields vary due to different constraints such as diseases and pests. In some areas, the pest and disease impact have been aggraved by the degradation of the natural resource base resulting from population pressure on land use (Karamura *et al.,* 1998). Consequently banana becomes very vulnerable to attacks from such pests as nematodes, fungi, bacteria, and weevils as well as viral diseases.

Different species of nematodes such as *Meloidogyne spp., Pratylenchus spp., Radopholus similis* and *Helicotylenchus multicinctus* have spread across Africa

attacking all banana cultivars, causing severe damage to the crop (Speijer and Fogain, 1998; Sarah, 2000). The nematodes are disseminated through planting material. The current management practices against the nematodes include disinfecting suckers by paring and using hot water treatment, planting clean tissue culture derived plantlets and using biological agents such as fungal endophytes (Viaene *et al.*, 2006; Viljoen, 2008).

Among fungi, black sigatoka affects most of banana cultivars including East African highland banana (EAHB) and plantains (Viljoen, 2008). The fungi are disseminated over long distances through planting material and by means of windborne ascospores, and within and between plants in the same plantation by conidia carried by the wind and water (Carlier *et al.*, 2000; Viljoen, 2008).

Effective control of black Sigatoka is through regular foliar fungicide application, which can be achieved in commercial plantations, but is not sustainable among smallholder producers in Africa. Therefore, among smallholder producers the disease is controlled through cultural practices, such as leaf pruning, and the use of black leaf streak-resistant hybrids such as FHIA-17 and FHIA-25 (Viljoen, 2008). Furthermore, other fungi such as *Fusarium* (*Fusarium oxyporum F.sp. cubense)*, a soil-borne fungus, infects the plant through the roots, and blocks the xylem vessels resulting in a lethal wilt. This soil-borne fungus was introduced in Africa from Asia through infected planting material across banana growing areas (Ploetz and Pegg, 2000; Viljoen, 2008). Over short distances, *Fusarium* is disseminated by rain, soil, and contaminated field implements. Once introduced, the fungus can survive as chlamydospores in the soil for decades, thus the only means to control the disease is

either through preventing it from being introduced or through using *Fusarium*-resistant cultivars in fields where the pathogen occurs (Ploetz and Pegg, 2000; Viljoen, 2008).

Bacterial diseases including Moko, caused by the bacteria *Ralstonia solanacearum* and blood disease have caused localised crop losses (Ploetz and Pegg, 2000). The most significant bacteria disease of banana in Africa for the past decade has been *Xanthomonas Wilt* (BXW), caused by *Xanthomonas campestis pv. Musacearum (Xcm),* which was reported for the first time in Ethiopia on *Ensete spp.* in 1968 and on banana in 1974. The disease has now spread to all East African countries of Burundi, DR Congo, Kenya, Rwanda, Uganda and Tanzania since its introduction and confirmation in the region in 2001 (Karamura and Tinzaara, 2009). The disease is systemic and is disseminated by infected plants, cutting tools, and insects visiting flowers of the infected plants (Tripathi *et al.*, 2009).

BXW control options consist of removal of male bud, cut and heap infected plants, regular sterilization of tools and use of clean planting materials (Karamura and Tinzaara, 2009).

Banana weevil (*Cosmopolites sordidius*) is among the pests that cause damage to banana by having their larvae feed on the central cylinder and lower pseudostem. This results in lower yields and snap-off of plants (Gold and Messian, 2000). The pest is managed by paring and using hot water treatment of planting material, sanitation practices such as splitting and drying of harvested pseudostems. The pest can also be controlled by insecticides, pheromone traps and fungal agent such as

Beauvaria bassiana or *F. oxysporum*. However, due to the cryptic nature of the weevil larvae, management of the disease still remains a challenge (Gold *et al.*, 2003).

Banana and other *Musa spp*. are also affected by five known, relatively well-characterized viruses. These are *Banana bunchy top virus* (BBTV) genus *Babuvirus*; *Banana streak virus* (BSV) genus *Badnavirus*, *Cucumber mosaic virus* (CMV) genus *Cucumovirus*, *Banana bract mosaic virus* (BBrMV) genus *Potyvirus* and *Abaca mosaic virus* (AbaMV) genus *Potyvirus.* Recently, there are other viruses that were identified namely *Banana mild mosaic virus* (BanMMV) and *Banana die-back virus* (Pietersen and Thomas, 2000). These viral diseases can be managed by using clean planting material of tolerant/resistant genotypes, eradication of infected plants, control of vectors, good sanitation practices and/or biocontrol (Lockhart and Jones, 2000; Thomas and Caruana, 2000; Viljoen, 2008; Muratori *et al.*, 2009). Among these viral diseases, *Banana Bunchy Top Virus,* is considered to be the most serious disease affecting banana worldwide (Dale, 1987; IITA, 2010; Kumar *et al.*, 2011).

2.7. Banana bunchy top disease

2.7.1. Importance of banana bunchy top disease

Banana bunchy top disease, which is caused by *Banana bunchy top virus* (BBTV), is the most common and destructive viral disease in the world (Kavino *et al.*, 2007). The first written reports of the disease were from Fiji in 1889; although the disease was probably known to have existed there as early as 1879 (Magee, 1927). The disease spread rapidly across Fiji where production declined from 778,000 bunches

in 1892 to 114,000 bunches in 1895. The economic impact of the disease was also reported in India where more than 18,000 ha were reduced to only 2000 ha under cultivation (Keshavamoorthy, 1980).

A survey carried out by Magee in 1927 revealed that the incidence of BBTD in banana plantations and gardens was between 5 and 30%. Currently, 13 African countries have reported incidence of BBTD namely, Egypt (1901); DR Congo (1958); Eritrea (1964); Gabon, Congo-Brazaville and Equatorial Guinea (1982); Burundi and Rwanda (1987); Malawi, Angola, Cameroon, Central African Republic and Zambia (1990s) (Fahmy, 1927; Wardlaw, 1961; Saverio, 1964; Fouré and Manser, 1982; Sebasigari and Stover, 1988; Kumar and Hanna, 2008; IITA, 2010).

2.7.2. Banana bunchy top disease spread

BBTV is primarily transmitted by planting materials and secondly by an aphid vector, *Pentalonia nigronervosa* Coquerel (Fig. 2.1), which is widely distributed throughout tropical and subtropical areas of the world (Magee, 1940; Allen, 1987; Hu *et al.*, 2007; Foottit *et al.*, 2010).

The *P. nigronervosa* was described on banana for the first time by Coquerel in 1859 in India, although occasionally found on other hosts. Its reproduction is almost totally asexual (Foottit *et al.*, 2010). The transmission of the virus by aphids is confined to short distance and the mean distance of new infections from their source of inoculum in an established plantation was estimated at 17.2 m (Allen, 1987). The movement of infected planting material is the major mechanism for disease spread

over long distances across areas and countries (Thomas and Caruana, 2000; Kumar and Hanna, 2008).

Fig. 2.1. Illustration of banana aphid (*P. nigronervosa*) at different stages, (a)-wingless form feeding on young banana leaf in colonies, (b) - typical wingless aphid and (c)-winged form (Photos from UH-CTAHR, Nelson, 2004).

2.7.3. Banana bunchy top disease symptoms

Disease symptoms usually appear about 25 days after inoculation in optimal conditions and this period is directly correlated with the age of the host plantlets (Dale *et al.*, 1987; Wu and Su, 1990). The phloem and its associated parenchyma of the infected plants become disorganized with excessive and irregular divisions. Symptoms develop more quickly at higher temperatures than at lower temperatures both in the field and in controlled environmental greenhouses (Sun, 1961; Dale *et al.*, 2000).

From the primary infections which occur when a ratoon arises from BBTV-infected "mother" plants, the plants are usually severely stunted, with leaves that do not expand normally and remain bunched at the top of the pseudostem. Suckers with these symptoms will not bear any fruits (Dale, 1987; Su *et al.*, 2003). On the other

hand, the secondary infection of plants occurs by aphid transmission after an initial period of BBTV-free plant growth. The symptoms of secondary infection are milder and only appear in tissues formed after the infection (Magee, 1927).

BBTD symptoms include development of dark green streaks of variable length in the leaf veins, midribs and petioles; dwarfing of leaves and development of marginal leaf chlorosis; upright and crowded leaves at the apex of the plant, hence the name bunchy top is attributed to the disease (Fig. 2.2).

Despite this clarity in symptom expression of the disease, symptoms on their own are not enough for a diagnosis. The limitation of visual diagnosis of BBTV is the incubation period of the disease which lasts for about one month. This complicates the disease management in banana fields, thus increasing the risk of pathogen spread (Allen, 1987; Drew *et al.*, 1989).

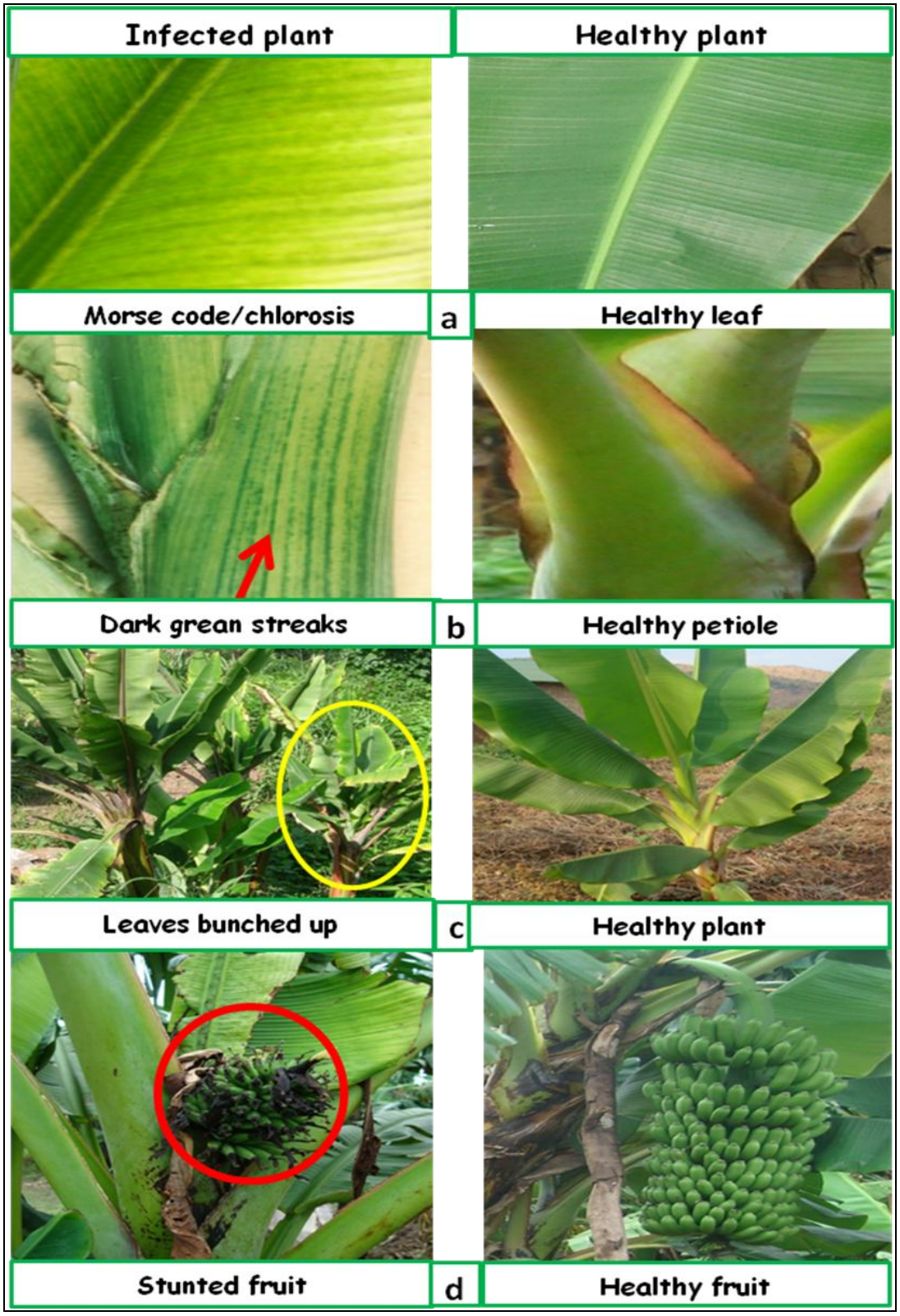

Fig. 2.2. BBTD symptoms on leaf (a), petiole (b), young plant (c), plant bears fruit (d) of infected plant compared to those of a healthy banana plant.

2.7.4. Diagnosis of Banana bunchy top virus

Based on the variability in BBTV symptom expression, detection methods that are more sensitive have been developed. The enzyme-linked immunosorbent assay (ELISA) is commonly used for BBTV detection, although this may be of limited sensitivity especially where there is very low virus concentration in banana tissues (Wanitchakorn *et al.*, 2000). The most sensitive methods currently available are based on polymerase chain reaction (PCR) undertaken to amplify specific DNA sequence of the virus (Su *et al.*, 2003).

There are studies which have been done using molecular methods to detect BBTV, but most of these have been carried out using purified total DNA extracts as the template material (Furuya *et al.*, 2005). The need for molecular detection with DNA extracts was a significant limitation to routine use of PCR detection method to diagnose BBTV infection. Some variants of PCR have also been developed by inclusion of an initial step of immunocapture (Sharman *et al.*, 2000) to allow immobilisation of virions on the PCR reaction tube wall using antibodies.

This preliminary step of immunocapture is time-consuming and necessitates having specific antibodies. The Gembloux Agricultural University developped a protocol of PCR detection of different banana viruses including BBTV by using banana crude extracts (Jacques *et al.*, 2005). This protocol, based on sampling using the PhytoPass (DNAlis Gembloux, Belgium) kit to harvest the material to be analysed, is a simplified process of preparing plant material to be used for pathogen detection (Fig. 2.3).

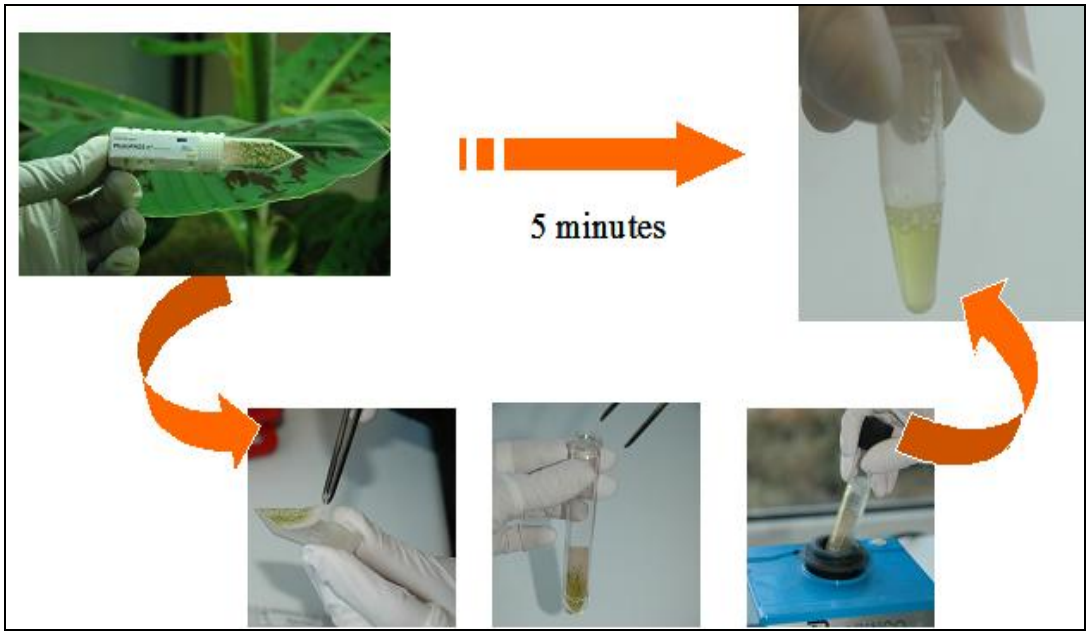

Fig. 2.3. Illustration of the banana sampling process using the PhytoPass kit for PCR amplification using crude extracts to detect BBTV infections (Busogoro *et al.*, 2009).

Additionally, silica gel has a structure with a high capacity desiccant making it an ideal material for field preservation of leaf samples for DNA studies. Therefore, fresh leaves placed in bags with the silica gel, can be transported and still arrive in a suitable condition for molecular analysis using banana crud extracts (Chase and Hills, 1991).

2.7.5. Banana bunchy top virus taxonomy

BBTV is a member of the genus *Babuvirus* in the family *Nanoviridae* and has a genome divided into six circular single stranded (css) DNA components of approximately 1kb in size (Wanitchakorn *et al.*, 2000; Vishnoi *et al.*, 2009) labelled as DNA-1, DNA-2, DNA-3, DNA-4, DNA-5 and DNA-6. These six DNA components have been consistently associated with BBTV worldwide in all geographical isolates (Dale *et al.*, 2000; Horser *et al.*, 2001a) (Fig. 2.4).

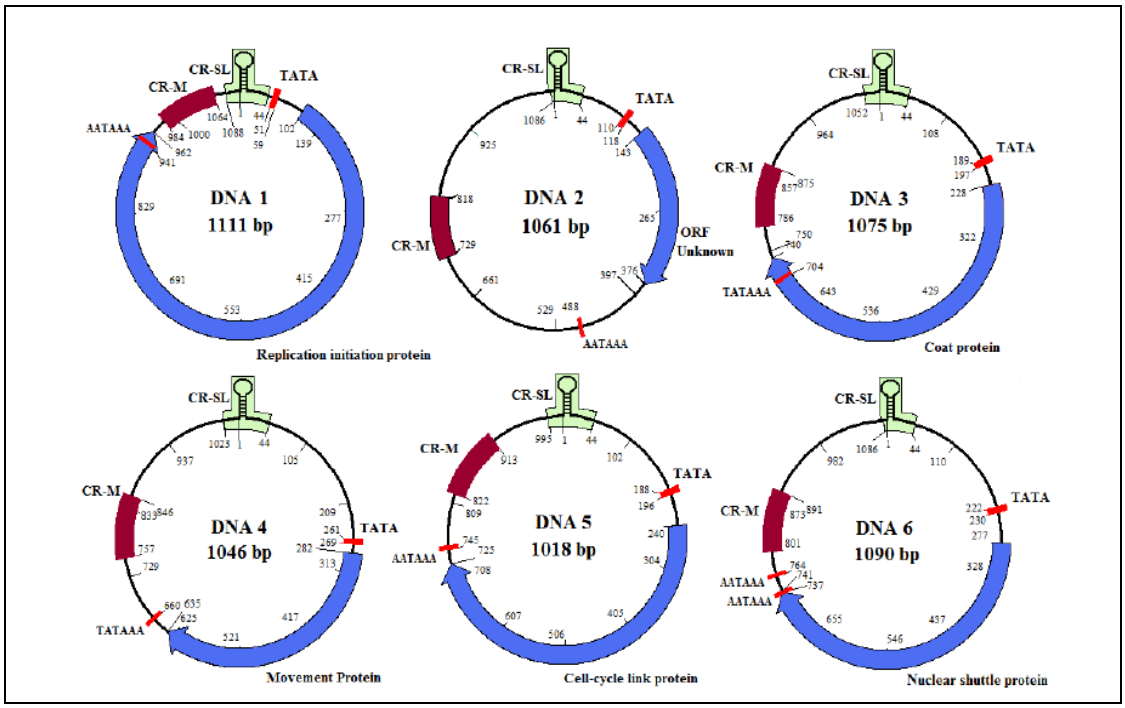

Fig. 2.4. Genomic organization of all six BBTV DNA components showing CR-SL, CR-M, TATA Box, Poly A signal and ORF, DNA-1(R) econdes Replication initiation protein and CP is encoded by DNA-3(S) (Islam *et al.*, 2010)

Genetic diversity among the BBTV species has been revealed using different analysis methods including pathological and molecular patterns. At the pathological level, there is variability in symptom expression (Su *et al.*, 2003).

The molecular characterization, using BBTV genomic components such as BBTV DNA-R and coat protein (CP) of different geographical isolates of the virus, revealed the existence of two distinct groups of BBTV isolates (Karan *et al.*, 1994).

These two groups were designated as Asian and South Pacific groups. The Asian group comprises of isolates from countries such as the China, Philippines, Taiwan and Vietnam. On the other hand, the South Pacific group contains isolates from different areas of the South Pacific region of Australia and Fiji as well as isolates from regions located outside this South Pacific region such as Burundi, Egypt, Pakistan and India (Wanitchakorn *et al.*, 2000; Horser *et al.*, 2001b).

CHAPTER THREE

OCCURENCE AND DISTRIBUTION OF BANANA BUNCHY TOP DISEASE IN BURUNDI, EASTERN DR CONGO AND RWANDA

Abstract

Banana bunchy top disease (BBTD) was first reported in 1958 in African Great Lakes region at the Yangambi research station in DR Congo. Cases were reported in 1987 in the Rusizi valley encompassing the borders of Burundi, DR Congo and Rwanda. Since then, no study about BBTD had been carried out in this region. A survey was conducted from September to October, 2008 in three provinces (Bujumbura rural, Cibitoke and Bururi) of Burundi, two districts (Kamanyola and Nyangezi) in South Kivu, Eastern DR Congo and the Rusizi district in the Western province, Rwanda. A total of 7,830 banana mats, 30 mats randomely selected per plot, were assessed on 261 farms. A structured questionnaire was used to assess, cultivar diversity, BBTD incidence and severity, presence and occurrence of the aphid vector and farmers' awareness about BBTD management. Leaf samples were randomly collected on symptomatic plants for further PCR analysis to confirm the disease. PCR results of samples collected in the three countries confirmed the presence of BBTV. The regional average of BBTD incidence and aphid occurrence was 25% and 46%, respectively. However, no significant relationship between aphid occurrence and BBTD incidence ($R=0.3$, $P= 0.623$) was observed. Similar banana varieties are grown across the three countries, indicating the cross-border movement of planting materials which may have influenced disease spread over the past decade.

Among the interviewed farmers, 90% were able to recognize advanced BBTD symptoms; while 95% of farmers were unaware of disease management options and stated that no local cultivar is resistant to the disease. This pinpoints the need for farmers' awareness raising and that tolerant cultivars should be part of control option packages.

3.1. Introduction

Banana (*Musa* spp.) is a staple food crop for about 70 million people in Africa including Burundi, DR Congo and Rwanda (Frison and Sharrock 1998; INIBAP 2000). Unfortunately, banana yields are low due to constraints such as the banana bunchy top disease (BBTD) which is one of the most devastating viral diseases in many banana producing regions of Africa, Asia, and the South Pacific (Dale 1987; Su *et al.*, 2003). The occurrence of BBTD represents a serious threat to food security in the regions where banana is one of the main staple crops for small-scale growers.

The disease was reported for the first time in Fiji Islands in 1889 (Magee, 1927) and so far has been recorded in 33 countries which include Africa, Asia, Australia and the South Pacific Islands but does not yet occur in Central and South America (Diekmann and Putter, 1996; Ferreira *et al.*, 1997; Amin *et al.*, 2008). In Africa, BBTD was first reported in Egypt in 1901 and subsequently in 1958 in sub-Saharan Africa at the *Institut National pour l'Etude Agronomique au Congo Belge* (INEAC) Yangambi agricultural research station in central DR Congo (Wardlaw, 1961; Fouré and Manser, 1982). It was also reported in 1964 in Eritrea (Saverio, 1964). Cases of

the disease were reported in 1987 in the Rusizi valley encompassing parts of Burundi and Rwanda (Sebasigari and Stover, 1988).

In 1982, BBTD was reported in Gabon, Congo-Brazaville and Equatorial Guinea (Fouré and Manser, 1982). In the early 1990s, it was described in Malawi and Angola (Kumar and Hanna, 2008). Currently, BBTD has been reported in 13 countries in Africa including Cameroon, Central African Republic and Zambia (IITA, 2010).

BBTD spreads with exchange of infected propagules from place to place and through banana aphid, *P. nigronervosa* Coquerel *(*Hemiptera: Aphididae) from plant to plant (Magee, 1927; Ferreira *et al.*, 1997; Robson *et al.*, 2006). Winged aphids which fly across the banana plantations and thus transmit the virus from a diseased to a healthy plant often develop after around 7 generations of wingless individuals (Nelson, 2004). The history of BBTD spread has mainly been attributed to the exchange of planting materials, and this has been the case with the spread of the disease in sub-Saharan Africa which includes the Great Lakes Region (Kumar and Hanna, 2008; Kumar *et al.*, 2011). For instance, the presence of a BBTD susceptible cultivar 'Yangambi Km5' (AAA) as its name suggests, originates from Yangambi in DR Congo and spread across the Great Lakes region of Africa (GLRA).

In the field, characteristic BBTD symptoms are easily observable and different from other known banana viruses. These symptoms include development of "morse code" streaking of variable length in the leaf veins, midribs and petioles as first symptoms; followed by progressive dwarfing of leaves and development of marginal leaf

chlorosis, upright and crowded leaves at the apex of the plant, hence the name bunchy top disease (Magee, 1927; Fry, 1982; Dale, 1987; Ferreira *et al.*, 1997).

Plants infected by BBTV at an early growth stage are unable to produce bunches whereas those infected at a later stage of growth produce small bunches often of poor quality (Dale, 1987; Ariyatne and Liyanage, 2002; Su *et al*., 2003).

In most regions, such as GLRA, where banana is produced for local consumption, research on BBTD has been limited. Indeed in most of African countries where there is an occurance of BBTD there are little or no resources for virus diagnosis (Dale, 1987), resulting in few works on BBTV-isolates prevailing in Africa, with the first publication on large number of BBTV isolates of sub-Saharan Africa, in 2011 by Kumar *et al.* (2011).

Despite viral disease presenting the major threat of banana production in GLRA, no specific in-depth investigations have been undertaken to generate sufficient information for the development of suitable disease management strategies. Therefore, the survey was conducted in six localities of the GLRA, known to be BBTD-affected. The study aimed at assessing the incidence of banana bunchy top disease, its severity and the importance of its vector *P. nigronervosa*. Farmers' awareness on the disease, management options and *Musa* cultivars grown were also recorded.

3.2. Materials and Methods

3.2.1. Study area

The study was conducted in three countries bordering the Rusizi valley namely Burundi, DR Congo and Rwanda (Fig.3-3). Altitudes at the surveyed localities in Burundi range from 1,297 to 2,096 metre above sea level (masl) in Bujumbura rural province, 906 to 1,330 masl in Cibitoke province, and from 769 to 934masl in Rumonge of Bururi province. The surveyed localities in the Eastern South-Kivu Province of DR Congo have an altitude ranging from 895 to 972 masl at Kamanyola district, and from 1,254 to 1,937 masl at Nyangezi district. The surveyed sites in Rwanda are located in the Rusizi district of the Western Province with altitudes of between 964 and 1,652 masl.

The geographical coordinates of each surveyed location were recorded using a Global Positioning System (GPS) receiver (Magellan Sport Trak Pro 2003). GPS coordinates of surveyed sites were plotted in ArcMap Graphical User Interface (GUI) of ArcGIS 9.3 software (Sreejit *et al.,* 2011).

3.2.2. Survey

The survey for the current study was carried out from September to October 2008. A structured questionnaire (Appendix 6.1) was used to obtain information on incidence and severity of BBTD and the *P. nigronervosa* occurrence. Other types of information obtained and analysed for this study include the source of planting material, *Musa* cultivars grown, possible existence of resistant varieties, seasonal

influences on the expression of BBTD symptoms, farmers' knowledge and perceptions on the disease and its management in the surveyed areas. Overall, 261 households' farms ranging from 10 to 75 per locality were visited. The distance between two farms within the same village was at least 100 m, and the distance between one and another surveyed village was 5 km. In each plantation, thirty mats were randomly selected for data collection. Mats containing one or more suckers with moderate to severe visible BBTD symptoms were considered as infected. Subsequently, the disease severity was assessed using an ordinary rating scale ranging from 0 to 5 with the higher ratings indicating increasing severity of the disease. The specific ratings used are shown on table 3.1 below. Photographic representations of characteristic BBTD symptoms are in appendix 3.2.

Table 3.1. Rating scale used to assess banana bunchy top disease severity

Rating	**Description**
0	Symptomless
1	Dark green streaks on the leaf veins
2	Dark green streaks on leaf midribs and petioles
3	Marginal leaf chlorosis
4	Dwarfing of leaves
5	"Bunchy Top" aspect: upright, crowded, and brittle leaves at the apex of the plant

The presence of *P. nigronervosa* was also assessed on all 30 mats and the occurrence of *P. nigronervosa* was assessed using an ordinary rating scale ranging from 0 to 5 with the higher ratings indicating increasing of aphid populations (Table 3.2). Photographic representations of aphid colonies are in appendix 3.3.

Table 3.2. Scale scored from 0 to 5 used to assess importance of the disease vector, *Pentalonia Nigronervosa*.

Rating	**Description**
0	No aphid
1	A simple colony (no winged individuals)
2	Several simple colonies (no winged individuals)
3	A large colony with one or more winged individuals
4	Several colonies with winged individuals
5	Generalized colonies on leaves and pseudostem with numerous winged individuals

The molecular detection using PCR (polymerase chain reaction) was conducted on 110 banana leaf samples collected during the survey. The sampling strategy consisted of taking at least three symptomatic leaf samples per village in the six localities across the three countries. Hence, 60 samples were collected in Burundi (21 in Bujumbura rural province, 21 in Cibitoke Province and 18 in Rumonge of Bururi province), 26 in DR Congo (6 in Kamanyola and 20 in Nyangezi districts) and 24 in the Rusizi district of Rwanda. Sampling was carried out using the PhytoPass system, with a single to two abrasive membranes used per BBTD symptomatic banana plant. The plant tissue fragments were recovered by putting the abrasive membrane into a Falcon tube containing 1ml of extraction buffer with subsequent vortexing (Busogoro *et al.*, 2009). For each sample, 500µl of crude extract was recovered and stored at -20°C pending PCR analysis. PCR was performed using primer pair BBT1

used as forward and BBT2 as reverse, designed to amplify a 349-bp fragment of the BBTV putative replicase gene (Thomson and Dietzgen, 1995).

3.2.3. Data analysis

The data were analyzed using the Statistical Package for Social Scientists (SPSS) version 16.0. The relationship between BBTD and *P. nigronervosa* populations was determined by a correlation analysis. The disease incidences as well as the aphid occurrence were determined as the proportion of plants showing BBTD symptoms or containing aphids of the total number of plants assessed (Saghir *et al*., 2002).

3.3. Results

3.3.1. BBTD distribution across Burundi, Eastern DR Congo and Rwanda

The cases of BBTD were reported in five provinces of Burundi (Bujumbura rural, Bururi, Cibitoke, Makamba and Muramvya provinces) including the Bujumbura town. Among these, three surveyed provinces (Bujumbura rural, Cibitoke and Rumonge in Bururi province) were highly affected by BBTD. In DR Congo, BBTD was found in the territory of Walungu, in Kamanyola and Nyangezi districts, while the western province, Rusizi district around Bugarama valley was the region highly affected by BBTD in Rwanda (Fig. 3.3).

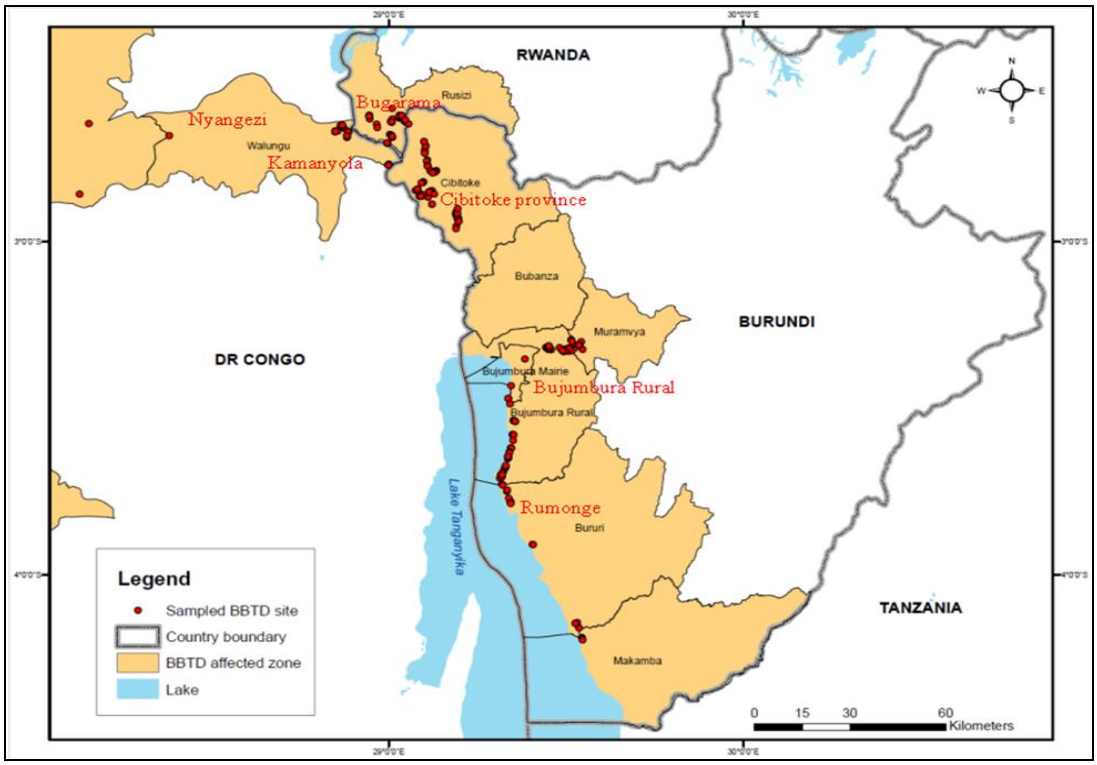

Fig. 3.1. Map showing BBTV distribution in Burundi (Bujumbura rural, Bururi, Cibitoke, Makamba and Muramvya provinces), in two districts of Eastern South-Kivu DR Congo (Kamanyola and Nyangezi districts) and in Rusizi district in the Western province of Rwanda.

3.3.1. *Musa* varieties grown in six localities surveyed

A total of 7830 banana mats were assessed in this study of which 73% were brewing varieties, 12% were dessert, 11% were cooking and 4% consisted of plantain varieties. Majority of farmers (72%) reported that the establishment of plantations dates back to more than a decade ago. Intercropping was practiced on 83% of surveryed farms.

The number of banana varieties per location varied from 6 to 10 with similar varieties being found in different localities surveyed across the three countries. The frequently reported varieties were differently named in each locality such as ‘Igisubi’/’Kayinja’/‘Pisang awak’ (ABB, brewing), ‘Yangambi Km5’/‘Ibota’ (AAA brewing), ‘Igisahira’/ ‘Barabesha’/ ‘Gisamunyu’ (AAA-EA, cooking), ‘Igitsiri’/‘Intuntu’ (AAA-EA, brewing), ‘Indarama’ (AAA-EA, brewing) and ‘Imizuzu’ (AAB, plantain). The ‘Yangambi Km5’ and ‘Kayinja’ varieties were the most reported amongst surveyed varieties across Burundi, DR Congo and Rwanda (Table 3.3).

Table 3.3. *Musa* cultivars commonly grown in the six surveyed localities in Burundi, Eastern DR Congo and Rwanda.

Location	Country	Use of different cultivars		
		Brewing	**Cooking**	**Dessert**
Bujumbura rural province	Burundi	1.'Indarama'(AAA,EA) 2.'Kayinja'(ABB) 3. 'Inkira'(AAA,EA) 4. 'Igitsiri' (AAA, EA)	5. 'Igisahira' (AAA,EA) 6. 'Ibiganda' (AAA, EA)	7. 'Ikimaraya'(AAA) 8. 'Gros Michel'(AAA) 9. 'Ibigurube'(AAA)
Cibitoke Province	Burundi	1. 'Yangambi Km5' (AAA) 2. 'Kayinja'(ABB) 3. 'Indarama'(AAA,EA)	4. 'Ibihanda'(AAA,EA) 5. 'Ibiganda'(AAA,EA)	6. 'Ikimaraya' (AAA, EA)
Rumonge in Bururi province	Burundi	1. 'Kayinja'(ABB) 2. 'Indarama' (AAA, EA) 3. 'Yangambi Km5'(AAA)	4. 'Igisahira' (AAA, EA)	5.'Ikimaramasenge'(AAB) 6.'Ibigurube'(AAA)
Kamanyola district	DR Congo	1. 'Yangambi Km5'(AAA) 2. 'Kayinja'(ABB)	3. 'Igisahira' (AAA, EA)	4. 'Kampala' (AAA, EA)
Nyangezi district	DR Congo	1. 'Yangambi Km5' (AAA) 2. 'Kayinja'(ABB) 3. 'Magizi' (AAA, EA)	4. 'Igisahira' (AAA, EA) 5.'Imizuzu' (AAB, plantain) 6. 'Rufufu' (AAA, EA) 7.'Naruvu' (AAA, EA)	8. 'Musheba'(AAB) 9.'Ikimaramasenge' (AAB) 10. 'Gros Michel'(AAA)
Rusizi district	Rwanda	1. 'Yangambi Km5' (AAA) 2. 'Kayinja'(ABB) 3.'Igitsiri' (AAA, EA)	4.'Igisahira' (AAA, EA) 5.'FHIA17'(AAAA) 6.'Barabesha' (AAA, EA)	7. 'Kampala' (AAA, EA) 8. 'Gros Michel' (AAA)

3.3.2. BBTD incidence and occurrence of *P. nigronervosa*

Banana bunchy top disease was reported in all the six surveyed localities in GLRA (Table 3.4). The BBTD incidences ranging from 23 to 30% were observed in five of the six localities. On the other hand, a relatively lower incidence of 14% was reported in Burundi at Rumonge. The occurrence of aphids in Bujumbura rural province, located at relatively higher altitude, was 26% whereas in the other surveyed localities it was more than 40% (Table 3.4).

Table 3.4. BBTD incidence and occurrence of *P. nigronervosa* in six surveyed localities in Burundi, Eastern DR Congo and Rwanda (across 261 farms with 30 mats per surveyed farm).

Location	Country	Number of mats assessed	BBTD incidence(%)	Occurrence of *P. nigronervosa* (%)
Bujumbura rural province	Burundi	1830	26	26
Cibitoke province	Burundi	2250	30	56
Rumonge in Bururi province	Burundi	1800	14	42
Kamanyola district	DR Congo	300	23	40
Nyangezi district	DR Congo	720	29	41
Rusizi district	Rwanda	930	28	64
Total/ Mean		7830	25	46

The BBTD severity was assessed on infected mats using a scale range of 0 to 5. The scores of 3 to 5, which are characterized by marginal leaf chlorosis to a bunchy top appearance, were more frequent than the scores of 1 or 2 which represent the initial symptoms manifested by dark green streaks. The average severity of BBTD which was 18% (i.e. 3-5) was higher than that of BBTV-infected plants at early stage (i.e. 1-2) which was 7.1% for the GLRA.

The percentage of surveyed mats per location with higher disease severity which could not bear fruits was 16, 22, 9, 21.5, 21 and 24% in Bujumbura rural, Cibitoke, Rumonge, Kamanyola, Nyangezi and Rusizi district, respectively (Table 3.5).

Table 3.5. BBTD severity ranging from 0 to 5 scores (%) for the six surveyed localities.

Locations	Country	Scores ranging from 0 to 5					
		0	**1**	**2**	**3**	**4**	**5**
Bujumbura rural province	Burundi	74	7	3	7	5	4
Cibitoke province	Burundi	70	5	3	9	7	6
Rumonge in Bururi province	Burundi	86	3	2	3	4	2
Kamanyola district	DR Congo	76.5	2	0	7	7	7.5
Nyangezi district	DR Congo	71	5	2	5	8	9
Rusizi district	Rwanda	72	3	1	9	8	7
Mean		74.8	4. 5	2.6	6. 8	6. 0	5. 2

The *P. nigronervosa* colonies were found on both symptomatic and asymptomatic mats. Apterous (i.e. wingless) *P. nigronervosa* in simple colonies (scoring 1 to 2) were most frequently observed (36%) while winged individuals (scoring 3 to 5) were observed on an average of 9% of the surveyed mats. The mats containing winged aphids (scoring 3 to 5), potential vectors transmitting BBTV from plant to plant, varied according to each location with 4, 8, 7, 16, 17 and 18% of mats surveyed in Bujumbura rural, Cibitoke, Rumonge, Kamanyola, Nyangezi, and in the Rusizi district, respectively (Table 3.6).

Table 3.6. Typology of *P. nigronervosa* colonies (scores ranging from 0 to 5) in the six surveyed localities.

Locations	**Country**	**Scores ranging from 0 to 5 (%)**					
		0	**1**	**2**	**3**	**4**	**5**
Bujumbura rural province	Burundi	74	18	5	3	0.5	0.5
Cibitoke province	Burundi	44	36	12	6	1.5	0.5
Rumonge in Bururi province	Burundi	58	28	7	5.5	1	0.5
Kamanyola district	DR Congo	60	15	9	15	0.5	0.5
Nyangezi districts	DR Congo	59	16	8	15	1	1
Rusizi district	Rwanda	36	29	17	16	1	1
Mean		55.1	23.6	9.6	10.1	0.9	0. 6

The generalised colonies were most frequent on the plants severely infected (scoring 3 to 5) rather than on plants infected with the scores of 1 or 2 (Table 3.7). For instance, the highest aphid colonies (i.e, scoring 5) were reported at 36.84% on BBTV-infected plants displaying severe symptom (i.e, score 5) with bunchy top aspect. However, the aphids' typologies (i.e. scores ranging from 0 to 5) across the six localities were not significantly correlated ($R = 0.2$, $P = 0.16$) with BBTD severity (i.e. scores 0-5). Moreover, the occurrence of *P. nigronervosa* colonies and BBTD incidence were not significantly correlated ($R = 0.3$, $P = 0.623$) across the 3 countries.

Table 3.7. Values (%) comparing surveyed mats according to aphid typologies (0 to5) and scores (0 to 5) of BBTD severity.

Aphids typology	BBTD severity scored from 0 to 5 (%)					
	0	**1**	**2**	**3**	**4**	**5**
Without aphids (0)	80.26	3.82	2.68	4.97	4.26	4.00
Simple colony (1)	72.32	5.48	2.35	9.03	6.20	4.61
Several simple colonies (2)	66.67	4.78	3.14	10.25	7.92	7.24
Large colony with winged individuals (3)	63.31	4.78	1.71	7.51	13.82	8.87
Several colonies with winged individuals (4)	54.93	7.04	4.23	8.45	11.27	14.08
Generalized colonies with winged individuals (5)	26.32	5.26	2.63	18.42	10.53	36.84

Aphids were observed on all assessed banana varieties in the six surveyed localities (Table 3.8). In addition, aphid colonies were sporadically observed on tomato (*Solanum lycopersicum*) and taro (*Colocasia esculenta)* which are frequently intercropped with banana in the region.

Ninty five percent of farmers indicated that no BBTD-resistant *Musa* varieties were present in their plantations. Based on the data collected during the survey, BBTD incidences, ranging from 25 to 48% were observed on dessert varieties [‘Gros Michel’ (AAA) and ‘Kamaramasenge’ (AAB)], ‘Yangambi Km5’ (AAA-brewing) and ‘Indarama’ (AAA, EA-brewing), while 13 to 17% were reported on ‘Igitsiri’ (AAA, EA-brewing), ‘Kayinja’ (ABB, brewing) and ‘Igisahira’ (AAA, EA-cooking). A relative low incidence of 9% was reported on ‘imizuzu’ (AAB-plantain) cultivars. BBTD incidence on banana varieties, which were established using tissue culture derived plantlets, was lowest (5%), although high aphid populations were reported on their mats (Table 3.8).

Table 3.8. Occurrence of *P. nigronervosa* and BBTD incidence of the common banana varieties grown in the Great Lakes region of Africa.

Cultivars (genome)	Number of mats assessed	Occurrence of *P. nigronervosa* (%)	BBTD Incidence (%)
'Yangambi Km5' (AAA-brewing)	2168	61	36
'Kayinja' (ABB-brewing)	2045	41	16
'Igisahira' (AAA-EA-cooking)	1061	36	17
'Igitsiri' (AAA-EA-brewing)	815	34	13
'Indarama' (AAA-EA-brewing)	695	43	48
Dessert varieties:'Gros Michel' (AAA) and 'Kamaramasenge'(AAB)	832	33	25
'Imizuzu' (AAB-plantain)	107	59	9
Tissue culture derived plantlets [FHIA01(AAAB), FHIA17 (AAAA) and FHIA25 (AAB)]	19	58	5
Total of surveyed mats/averages	7830	46	21

P. nigronervosa occurrence was higher to the regional average (46%) on 'Yangambi Km5' (AAA-brewing), 'Imizuzu' (AAB-plantain) and banana varieties grown from tissue culture plantlets. About 40% of aphids occurrence were reported on 'Indarama' (AAA, EA-brewing) and 'Kayinja' (ABB-brewing), while around 35% were reported on the highland varieties 'Igisahira'(AAA, EA-cooking),'Igitsiri' (AAA,EA-brewing) and the dessert varieties (Table 3.8).

3.3.3. Farmers' knowledge on BBTD management

Most of the 261 interviewed farmers (90%) were able to recognize advanced BBTD symptoms in their fields, while the remaining 10% did not follow the disease symptoms. Of these farmers, 67% used local names [e.g. 'Kagazi'= leaves appearance of BBTV-infected plant are compared to those of palm tree, 'Saturubwato'=means no necessary to keep brewing-material once banana plantations are BBTD-affected, 'Sindika'= means that a BBTV-infected plant become stunted] to identify the disease. All of the interviewed farmers reported that

the disease is systemic and, overtime, it spreads to all plants within a mat. Seventy two percent of farmers reported that banana production ceases within a year after infection. Nine percent indicated that production could continue for up to two years after a mat gets infected, while five percent reported three years of post-infection production. A small proportion (11%) of the farmers was not aware of the effect of BBTV infection on banana production.

Among the interviewed farmers, 83% reported to have used their own suckers in the establishment of new plantations, 65% obtained suckers from nearby (<10 Km) plantations, while 31% obtained suckers far away (>10 Km) from their home villages.

The use of *in vitro* derived plantlets was reported by 11% of the interviewed farmers. The tissue culture derived plants were mainly distributed by government institutions and non-governmental organizations.

Regarding the occurrence of the disease in their fields, 56% of the interviewees attributed the origin of the disease either to their own or to neighbouring fields, 39% attributed the source to remote (>10km) villages, while 10% suspected that the contamination was coming from bordering countries.

In the new plantations established with suckers either from own or from neighbours' fields, four percent noticed the disease after about three weeks, 36% of the interviewees noticed BBTD symptoms after about three months, 25% identified the symptoms after more than one year whereas, 31% did not pay attention to the disease development after planting. Among the 11% of farmers who had established banana

fields using tissue culture derived plantlets, two percent observed BBTD symptoms about three months after planting.

Forty eight percent of the farmers said that the disease becomes more severe during dry season, although the disease is present throughout the year.

In terms of BBTD management efforts, only five percent of growers particularly in Rwanda reported having attended training on how to manage the disease. Seventy eight percent of farmers believed that selecting asymptomatic suckers would reduce disease incidence.

An estimated 43% of the farmers were aware that the disease can be transmitted with infected planting material, while only 13% of the farmers reported having informed that the disease can be transmitted by insect vectors. Thirty six percent of farmers were not aware about disease transmission and believed that the disease can be spread through the soil, while eight percent attributed new infections to the use of contaminated garden tools (Fig. 3.4).

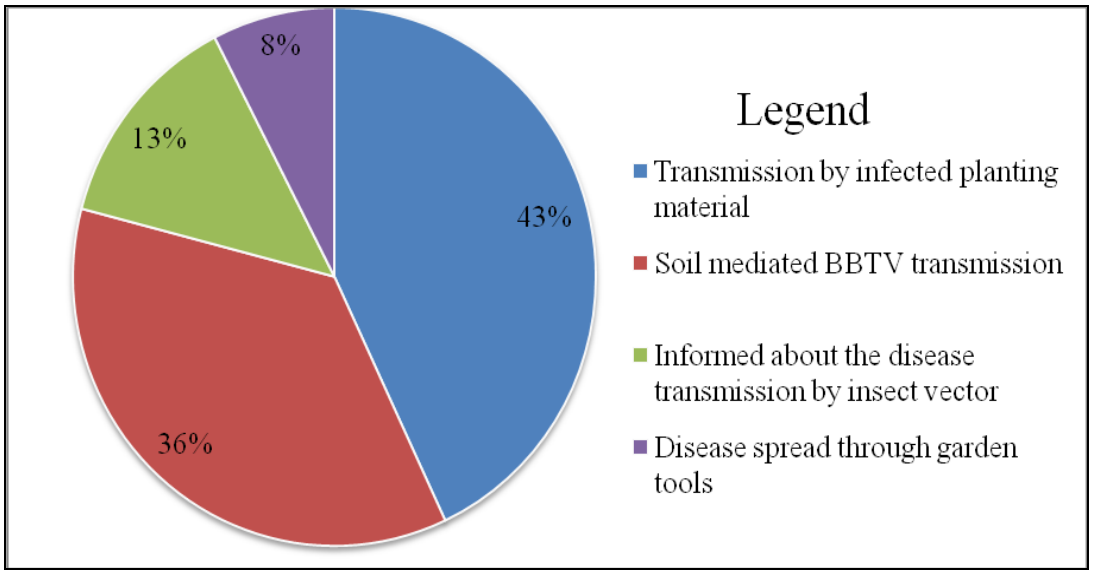

Fig. 3.2. Farmers' knowledge on BBTV transmission in African Great Lakes region. Values are proportion of 261 interviewed farmers.

Seventy seven percent of farmers reported to have been cutting single diseased plants at the pseudostem base without removing the whole mat, while only 15% stated to have been uprooting the entire mat when at least one plant showed typical BBTD symptoms. Lack of measures to quarantine BBTD-affected from non affected areas was reported by 98% of farmers. In all of the surveyed localities, the use of chemicals to control *P. nigronervosa* was not reported by farmers.

3.3.4. BBTV status of collected samples

BBTV was confirmed in the samples collected on BBTD symptomatic plants in the three countries of the GLRA. PCR results on 110 sampled PhytoPass kits varied with respect to the period between sampling and extraction of sample extract.

The highest positives rates of 73% and 60% were reported on samples extracted 10 - 12 days after sampling, while the least PCR positives results were observed with 13% of samples extracted after 37 days of PhytoPass kits conservation prior to PCR tests (Appendix 3.4).

3.4. Discussion

'Yangambi Km5' (AAA-brewing) and 'Kayinja' (ABB-brewing) varieties were reported to have been widely grown across large parts of the Rusizi valley encompassing Burundi, Eastern DR Congo and Western province of Rwanda. These two banana brewing varieties are easy to multiply and this could be the main reason for their preference by farmers in the Rusizi valley region (Nsabimana *et al*., 2008).

The disease incidence of more than 23% was observed in five surveyed localities. However, the lowest BBTD incidence of 14% was reported at Rumonge in Burundi. This implies that Rumonge was later infected with the introduction of infected plants in around 2000 unlike in the other five high-BBTD incident areas. In addition, the difference in the severity of the disease per location can be attributed to the seemingly poor maintenance of banana plantations (Smith *et al*., 1998*).*

P. nigronervosa is the only vector known to be transmitting BBTV and reproducing efficiently on banana (Ferreira *et al.*, 1997; Yasmin *et al*., 2001; Foottit *et al.*, 2010). This BBTV vector was found in all the surveyed banana fields at the six localities. The occurrence of aphids was observed to be high in the region with simple colonies and harbouring winged aphids which contribute to the spread of the disease. Winged aphids were observed to range from 4% to 18% across the six localities. The lowest

numbers of winged aphids (4%) observed in Bujumbura Rural may be attributed to lower temperatures at the higher altitudes (Ferreira *et al.*, 1997). On the other hand, poor maintenance of the perennial banana crop and its dense canopy might also help to increase the aphid vector population. The dense canopy also partially prevents rainfall from reaching the leaves and pseudostem of banana suckers, and thereby favouring the aphids' multiplication (Young and Wright, 2005).

Among *Musa* varieties, the BBTD incidence was observed to be varying from 5 to 48% with the highest incidence being observed in 'Indarama' (AAA, EA-brewing) and 'Yangambi Km5'(AAA-brewing); and the lowest incidence being observed in plants sourced from tissue culture and 'imizuzu'(AAB-plantain). Aphid populations were higher on 'Yangambi Km5' (AAA, brewing) and 'imizuzu' (AAB, plantain) unlike in the other banana cultivars. However, the aphid population did not have a direct correlation with BBTD incidence. The high aphid populations on 'imizuzu' (AAB, plantain) could be due to green yellow pseudostem colour that may attract aphids (Simmonds, 1966; Kumar and Hanna, 2008). The dense leaf canopy of 'Yangambi Km5' (AAA, brewing) mats may also favour a build up of aphids' population. On the other hand, the reason that some banana cultivars were observed to have lower aphid numbers but higher BBTD incidence, such as 'Indarama' (AAA, EA-brewing), could be attributed to the fact that such banana cultivars are highly susceptible to the disease. Previous studies have also indicated that banana cultivars with at least one B genome are more tolerant to BBTD as opposed to those with only A genomes (Espino *et al.*, 1993; Hooks *et al.*, 2009). This could be the reason for the relatively lower BBTD incidence in 'Kayinja' (ABB, brewing) and the 'imizuzu'

(AAB, plantain). In contrast, the lower BBTD incidence reported for the highland genotypes ‘Igisahira’ (AAA, EA-cooking) and ‘Igitsiri’ (AAA, EA-brewing) can be attributed to their occurrence at higher altitudes where fewer winged aphids, which transmit BBTV from plant to plant (Nelson, 2004) were reported, resulting in subsequent less BBTV-infection.

Although farmers could recognize and had local names for BBTD-symptoms, they were not well informed about how the disease can be managed. Several factors played an important role in the disease spread; these include lack of knowledge of using virus-free planting materials and long periods of insecurity in many parts of the GLRA which resulted into mass movement of people and planting materials. The most recent appearance of BBTD in Rumonge could have resulted from mass movements of people across Burundi during the political crisis of 1993 to 2003. The infected planting materials of ‘Yangambi Km5’ (AAA, brewing) and ‘Indarama’ (AAA, EA-brewing) may have been brought to Rumonge during that period.

The BBTD was confirmed in the samples from the three countries with PCR results ranging from 13 to 73% of positives among the samples collected on symptomatic plants using the PhytoPass system. Although it was reported that the PhytoPass kit may be conserved, in ambient conditions, 138 days pending molecular assays (Busogoro *et al.*, 2009), the period between sampling and crude extraction affected the success rates of diagnostic tests. Such results can be attributed to the banana extract quantity on PhytoPass membrane which may influence the success of PCR assays due to the likelihood of inhibition of phenolic compounds contained in the banana leaves (Dale, 1987; Wu and Su, 1989).

Overall, BBTD incidence was high in BBTD-affected regions of the GLRA, while *P. nigronervosa* was reported within all the surveyed locations. However, the BBTD incidence and aphid occurrence were not significantly correlated. Fluctuations in winged aphid numbers, mainly due to climatic conditions, may explain the subsequent none significant correlation between occurrence of *P. nigronervosa* colonies and BBTD incidence across the 3 countries.

Unfortunately, once established BBTD has never been eradicated from countries where it occurred (Jones, 2009), though in some areas in Australia, BBTD was managed by early detection of the diseased stools and immediate removal. This required partnership between communities and government working together for a common purpose (Magee, 1938; Brooks, 1999; Robson *et al.*, 2006; Daniells, 2009). Furthermore, in American Samoa, the BBTD incidence was reduced to about 5% using a public information campaign on rapid removal of infected mats as a method of disease control (Brooks, 1999). These examples may be adapted to small-scale agriculture of the GLRA and contribute in reduction of BBTD incidence. This entails raising awareness among stakeholders at all levels (policy makers, extension services, NGOs and farmers), promoting the use of disease-free planting materials, reduction of inocula by collective eradication of BBTV-infected mats, implementing quarantine measures in order to prevent the spread of the disease between affected and non affected areas in African Great Lakes region.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF BANANA BUNCHY TOP VIRUS ISOLATES FROM THE GREAT LAKES REGION OF AFRICA

Abstract

The genetic variability of *Banana bunchy top virus* (BBTV) isolates from the three countries of the GLRA was assessed. DNA-R and DNA-S components of the GLRA virus genome were amplified by polymerase chain reaction (PCR) and sequenced in this study. These two BBTV components were previously used to classify isolates between the South Pacific and the Asian groups. Pairwise comparisons and phylogenetic analysis based on nucleotide sequences involving GLRA isolates and those representing the two groups obtained from the GenBank database were carried out. Sequence similarity for both DNA-R and DNA-S components ranged between 99.1 to 100% among the GLRA isolates, 96.2 to 100% and 89.7 to 94.3% between the GLRA isolates and those previously clustering in the South Pacific and the Asian groups, respectively. These results showed that GLRA isolates belong to the South Pacific group and they were closely related to the Indian isolate. The genetic diversity of DNA-R segment revealed around 40 years of the BBTD presence in GLRA. The fact that similar banana cultivars and BBTV isolates were found across Burundi, DR Congo and Rwanda, implies that the disease may have mainly spread through exchange of suckers, in addition to the vector spread of the disease. Therefore, in addition to cultural practices aimed at control of aphid vectors,

quarantine measures should be adopted as a sustainable way to avert BBTD spread between affected and unaffected areas in African Great Lakes region.

4.1. Introduction

Banana and plantain are a staple food crop for approximately 400 million people worldwide (Islam *et al.*, 2010) and nourish over 70 million people in sub-Saharan Africa (AATF, 2003). This crop is ranked the first in terms of contribution to the total annual production in Burundi and Rwanda while it is the second after cassava in DR Congo (FAOSTAT, 2009). The perennial nature of banana crop, compared to other staples, allows households to access food all-year round, providing significant amounts of micronutrients (Fungo, 2009; Kumar *et al.*, 2011). Among banana varieties grown in Africa, plantain types (AAB genome) are mainly found in the humid lowlands of West and Central Africa, while the highland cooking and beer banana (EA) which contribute 30% of world banana production, are common in the Eastern African highlands (Tenkouano *et al.*, 2003). Eastern Africa including the Great Lakes zone is considered as a secondary center of diversity for the highland banana (Karamura, 1998; Tenkouano *et al.*, 2003) where smallholder farmers grow a mixture of 5 to 10 varieties around their homesteads (AATF, 2003). Banana plantations are subjected to various natural calamities, but diseases, in particular, viral diseases limit their potential. Among viral infections, banana bunchy top disease (BBTD) is the most destructive worldwide (Dale, 1987; Su *et al.*, 2003; Islam *et al.*, 2010).

BBTD was first reported from the Fiji Islands in 1889, but its causal agent was identified 100 years later (Magee 1927; Wardlaw 1961; Harding *et al.*, 1991; Kumar *et al.*, 2011), and was given the name, *Banana bunchy top virus* (Karan, 1995; Vetten *et al.*, 2005). BBTV is a member of genus *Babuvirus*, family *Nanoviridae* belonging to a group of circular single-stranded DNA (cssDNA) viruses (Allen, 1987;

Burns *et al.*, 1995; Karan, 1995; Vishnoi *et al.*, 2009). This group of Nanoviruses includes *Abaca bunchy top virus* (ABTV*)* which was identified as a member of genus *Babuvirus* causing 'bunchy top' symptoms such as BBTV (Sharman *et al.*, 2008).

BBTV is an isometric virus with a genome consisting of at least 6 components of circular single-stranded DNA (cssDNA) (Horser *et al.*, 2001b; Su *et al.*, 2003; Hu *et al.*, 2007; Sharman *et al.*, 2008). BBTV components initially named DNA-1 to 6 were renamed to better represent the function of the encoded protein (Vetten *et al.*, 2005). DNA-R encodes the replication initiation, DNA-S encodes the capsid protein (CP), DNA-M encodes a movement protein, DNA-C encodes a cell cycle link protein and DNA-N encodes a nuclear shuttle protein, while the function of the DNA-U3 is not yet known (Harding *et al.*, 1993; Hafner *et al.*, 1995; Wanitchakorn *et al.*, 1997). Each of these components is approximately 1 kb in length and is individually encapsulated in icosahedral virion of 18-20 nm in diameter and share a common genome organization. In addition, each circular ssDNA encodes a single open reading frame (ORF) except BBTV DNA-R which encodes two ORF (Burns *et al.*, 1995; Beetham *et al.*, 1997). All components contain a conserved stem-loop common region (SL-CR) with a conserved nine nucleotides -TATTATTAC- loop sequence which likely marks the origin of DNA replication, a major common region

(CR-M), a potential TATA box and a polyadenylation signal associated with each gene (Burns *et al*., 1995; Beetham *et al*., 1997; Su *et al.*, 2003). In addition, the DNA-R component of BBTV contains conserved iterons which are the sequences involved in sequence-specific interaction (Herrera *et al*., 2006; Amin *et al*., 2008).

DNA-R has been identified in all BBTV isolates where it encodes the 'master' Rep (M-Rep) that directs self replication in addition to replication of other BBTV genome components (Harding *et al.,* 1993; Karan *et al,* 1994; Theresia, 2008). On the other hand, the coat protein (CP) is encoded by DNA-S for the integral BBTV component (Horser *et al*., 2001b). Based on sequence analysis of DNA-R and DNA-S (CP) components, respectively, Karan *et al*. (1994), Wanitchakorn *et al.* (2000) and Kumar *et al.* (2011) demonstrated that BBTV isolates can be clustered into two distinct groups. The 'South Pacific' group comprising isolates from Australia, South pacific region, South Eastern Asia such as India, Pakistan and Africa; while the 'Asian' group comprises isolates from China, Indonesia, Japan, the Philippines, Taiwan and Vietnam (Wanitchakorn *et al.,* 2000; Horser *et al.,* 2001b; Kumar *et al*., 2011).

Although BBTD has long been recognised (Magee, 1927), molecular characterisation of BBTV only began in the early 1990s (Harding *et al*., 1993; Sharman *et al*., 2008). In Africa, only a handful of BBTV isolates from sub-Saharan Africa (SSA) have been characterized by Kumar *et al*. (2011), while a single isolate originating from Burundi (accession AF148943) has been considered earlier by Wanitchakorn *et al.* (2000). To date, significant molecular characterization using a substantial number of samples from the African Great Lakes region is lacking to generate accurate

strategies aimed at BBTD management. In this study, molecular analysis based on BBTV DNA-R (core region of replicase ORF) and DNA-S (CP) segments of the virus genome of the GLRA isolates was carried out to assess genetic diversity of BBTV within that region of Africa.

To determine the interrelationships and possible origin of BBTV occurring in GLRA, the nucleotide sequences of GLRA isolates and those representing the South-Pacific and Asian groups obtained from the GenBank database were pairwise compared.

4.2. Material and Methods

4.2.1. Sampling

Banana leaf samples were collected from among BBTD-affected regions in three countries namely Burundi, DR Congo and Rwanda in April to May 2010. Duplicate pieces of banana leaves of approximately 4 cm^2 each were taken from the youngest leaf of a banana plant exhibiting advanced BBTD symptoms. Leaf pieces were placed in individual Petri dishes lined with silica gel for the duration of the transport and transferred to the laboratory, where they were extracted and stored at -20°C pending use (Chase and Hills, 1991). In all, 37 samples collected from five Provinces of Burundi (Bubanza, Bujumbura Rural, Bururi, Cibitoke and Makamba), 22 from three districts in the Eastern South Kivu DR Congo (Kabare, Nyangezi and Kamanyola) and 20 from the Rusizi district of the Western Province in Rwanda, giving a total of 79 samples. These samples were collected from diverse banana genotypes namely AAA-EA, ABB, AAB and AABB types cultivated at different altitudes across the three countries. Diagnostic tests confirming the viral status of

samples were performed using previously described PCR analysis (Harding *et al*., 1993; Thomson and Dietzgen, 1995).

4.2.2. Crude extraction and BBTV detection by PCR

Leaf pieces preserved in silica gel were placed in mesh plastic bags (Agdia Biofords, France) and 2 ml of extraction buffer at 4°C was added (Busogoro *et al.*, 2009). The crude extract was obtained for each sample by thoroughly crushing banana leaves with the help of a vortex before being dispensed in three 200µl aliquots and stored at -20°C pending PCR analyses. PCR amplifications were carried out using diluted extracts (1:100 in distilled water) as described by Busogoro *et al.* (2009). PCR amplification and sequencing were performed using specific primer pairs previously described for CP and DNA-R components (Harding *et al*., 1993; Thomson and Dietzgen, 1995; Amin *et al*., 2008). All 79 samples were first subjected to the PCR amplification of a 349 bp fragment of the putative BBTV replicase gene using primer pairs BBT1 forward (5'-CTCGTCATGTGCAAGGTTATGTCG-3') and BBT2 reverse (5'-GAAGTTCTCCAGCTATTCATCGCC-3') for detection of the virus in all banana leaf samples (Harding *et al*., 1993; Thomson and Dietzgen, 1995). Primer pairs MREPF forward (5'-GAATTCAAGAATGGAATAATTC-3') and MREPR reverse (5'-GAATTCCTCTAATAACCC-3') described by Amin *et al*. (2008) were used to amplify a 1111 bp fragment of the BBTV DNA-R component, whereas primer pairs CPXI.PRI forward (5'-GCTAGGTATCCGAAGAAATCC-3') and BBTV3C.EXP reverse (5'-ATAAAGCTTTCAAACATGATATGT-3') were used to amplify a 550 bp fragment of the BBTV coat protein (Wanitchakorn *et al*., 2000).

PCR reactions were set up in a final volume of 50µl comprising 5µl of crude extract (diluted 1:100 in distilled water), 5µl of 10x PCR buffer (Roche), 6µl of $MgCl_2$, 1.2 µl (200µM/each) of dNTPs mix, 1µl of each primer (0.5µM), 0.25µl (1.25u/50µl) of *Taq* DNA polymerase (Fermentas) and sterile distilled water (30.55µl) was added to make the final volume (Burns *et al.*, 1995; Amin *et al.*, 2008).

The PCR procedure was performed using MyCycler (BIO-RAD, Belgium). The thermo-cycling scheme consisted of denaturation at 94°C for 4 min; 40 cycles of 30s to 1min at 94°C, 1 min at 52°C and 2 min at 72°C followed by a final elongation step at 72°C for 10 min. The amplified products were visualized by electrophoresis in a 1% (w/v) agarose gel using ethidium bromide staining along with 100bp ladder (Fermentas, France). Gels were then photographed on a digital gel documentation system (BioRad, Belgium). PCR products were quantified in ng/µl using NanoDrop ND-1000 spectrophotometer machinery with a limit of 1.80 values at A260/280 absorbance ratio. Amplified products specific to each of the DNA-R and CP components were then shipped for subsequent sequencing at Macrogen in South Korea.

4.2.3. Sequence analysis

The obtained nucleotide sequences of BBTV DNA-R and CP components of the GLRA isolates were compared in pairwise matrix with existing BBTV sequences from the GenBank database using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) (Theresia,

2008; Vishnoi *et al*., 2009). Multiple alignments for sequence comparison were performed using CLUSTALW (Thomson and Dietzgen, 1995; Amin *et al*., 2008).

The genetic diversity of BBTV isolates was determined between GLRA isolates and those representing reference isolates from the previously described Asian and South Pacific groups. The previously determined Burundian isolate (AF148943) and other isolates from sub-Saharan Africa (Kumar *et al*., 2011) were also included. In addition, *Abaca bunchy top virus* (ABTV) isolate Q1108 segment of DNA-S and DNA-R (Sharman *et al*., 2008), the closest relative to BBTV belonging to the same genus *Babuvirus* was used as an out group for the comparison (Table 4.1 and Table 4.2). The consensus trees were generated using neighbour-joining algorithms with 100 bootstrap replications using Sea View Version 4.2.9 (Gouy *et al*., 2010).

Table 4.1. BBTV and ABTV isolate sequences based on coat protein component obtained from the GenBank database with their accession numbers, origin and references.

N°	Accession N° (isolate)	Origin	References
1	AF148943 (Burundi)	Burundi	Wanitchakorn *et al*. (2000)
2	AF148944-CP-Fj	Fiji	Wanitchakorn *et al*. (2000)
3	AF148068-Ph-CP	Philippines	Wanitchakorn *et al*. (2000)
4	AF148942-Tw-CP	Taïwan	Wanitchakorn *et al*. (2000)
5	AF148945- Vt-CP	Vietnam	Wanitchakorn *et al*. (2000)
6	AF330706-CP-CZ	China	Cai and Mao, 2000*
7	AF238877-NSP-CP	China	He *et al*. (2001)
8	AB108451-Jk3-CP	Japan	Furuya *et al*. (2005)
9	AB113662-V14-CP	Vietnam	Furuya *et al*. (2005)
10	EF546804 (Q1108)#	Philippines	Sharman *et al*. (2008)
11	EF584544-CP-In	India	Islam *et al*. (2010)
12	JF755978	Cameroon	Kumar *et al. (*2011)
13	JF755979	Cameroon	Kumar *et al. (*2011)
14	JF755980	Malawi	Kumar *et al. (*2011)
15	JF755981	Gabon	Kumar *et al. (*2011)
16	JF755982	Gabon	Kumar *et al. (*2011)
17	JF755983	Angola	Kumar *et al. (*2011)
18	JF755984	DR Congo	Kumar *et al. (*2011)
19	JF755985	DR Congo	Kumar *et al. (*2011)
20	JF755986	DR Congo	Kumar *et al. (*2011)
21	JF755987	DR Congo	Kumar *et al. (*2011)

#: One isolate of *Abaca bunchy top virus* (ABTV). *: Submitted (20-December-2000), laboratory of Plant Biotechnology, Institute of Microbiology, Zhongguancun Street, Beijing 100080, China.

Table 4.2. BBTV and ABTV isolate sequences based on DNA-R component obtained from the GenBank database with their accession numbers, origin and references.

N°	Accession N° (isolate)	Origin	References
1	S56276-Au-Rep	Australia	Harding *et al*. (1993)
2	AF416469-Ph-Rep	Philippines	Karan *et al*. (1994)
3	AF416468-Tw-Rep	Taiwan	Karan *et al*. (1994)
4	AF416465-Eg-Rep	Egypt	Karan *et al*. (1994)
5	AF416470-In-Rep	India	Karan *et al*. (1994)
6	AF416466-Fj-Rep	Fiji	Karan *et al*. (1994)
7	AF416467-Tn-Rep	Tonga	Karan *et al*. (1994)
8	U18077-Hw-Rep	Hawai	Xie and Hu (1995)
9	AF110266-CZ-Rep	China	La *et al*. (1998)*
10	AF238875-CNSP-Rep	China	He *et al*. (2000)
11	AF416478-HCM-Rep	Vietnam	Bell *et al*. (2002)
12	AF416472-SL	Vietnam	Bell *et al*. (2002)
13	AB108456-JY1-Rep	Japan	Furuya *et al*. (2005)
14	AB113660-V14-Rep	Vietnam	Furuya *et al*. (2005)
15	AM418538-Pk-Rep	Pakistan	Amin *et al*. (2008)
16	EF546807 (Q1108)#	Philippines	Sharman *et al*. (2008)
17	JF755990- SSA	Gabon	Kumar *et al. (*2011)
18	JF755991-SSA	Gabon	Kumar *et al. (*2011)
19	JF755992-SSA	Gabon	Kumar *et al. (*2011)
20	JF755997-SSA	Angola	Kumar *et al. (*2011)
21	JF755996-SSA	DR Congo	Kumar *et al. (*2011)
22	JF755995-SSA	Malawi	Kumar *et al. (*2011)
23	JF755994-SSA	Malawi	Kumar *et al. (*2011)
24	JF755993-SSA	Malawi	Kumar *et al. (*2011)
25	JF755989-SSA	Cameroon	Kumar *et al. (*2011)
26	JF755988-SSA	Cameroon	Kumar *et al. (*2011)

#: One isolate of *Abaca bunchy top virus* (ABTV). *: Submitted (01-December-1998), laboratory of Plant Biotechnology, Institute of Microbiology, Zhongguancun Street, Beijing 100080, China.

4. 3. Results

4.3.1. BBTV detection by PCR and sequencing

BBTV was confirmed in all 79 samples collected from symptomatic banana plants using a primer pair targeting the putative replicase gene (A) of the BBTV genome. Among these positive samples, BBTV DNA-R (B) and CP (C) components of representative samples were amplified using corresponding primer pairs of each fragment (Fig. 4.1).

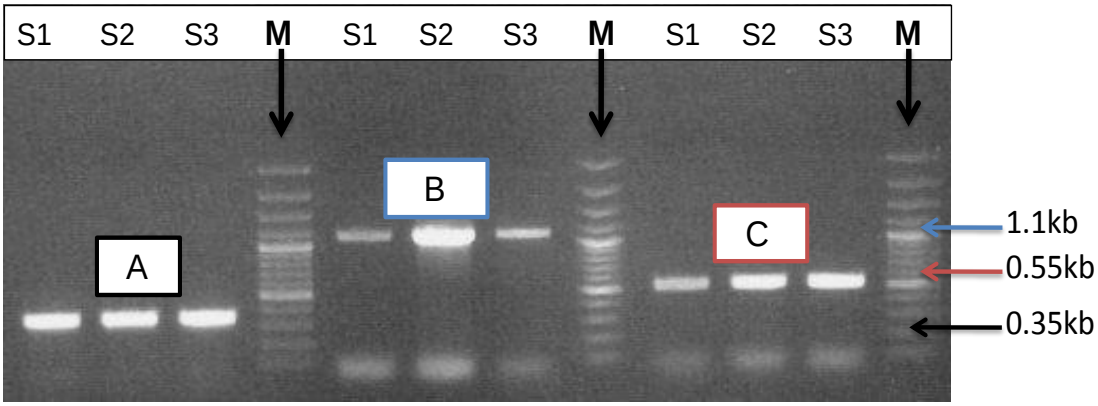

Fig. 4.1. Molecular detection profile of Banana bunchy top virus putative replicase gene (A=349bp), DNA-R (B=1111bp) and coat protein (C=550bp) components. Lane M: 100-bp ladder (Fermentas, France). S1, S2, S3 are examples of amplified samples from the African Great Lakes region.

Among tested positive samples, representative samples covering the different localities and banana varieties were considered for both BBTV DNA-R and Coat protein amplifications. PCR products of DNA-R and CP components obtained from 22 and 19 representative isolates, respectively, were sequenced and used in nucleotide sequence pairwise comparisons (Table 4.3 and Table 4.4).

Table 4.3. BBTV isolates for which sequences based on coat protein component were determined in this study with their accession numbers and origin.

N°	Accession number	Country	Province/ District	Altitude (masl)	Variety
1	JN204219 (1Bdi)	Burundi	Bubanza	1191	'Indarama'-AAA, EA
2	JN204220 (2Bdi)	Burundi	Bubanza	1090	'Yangambi Km5'-AAA, EA
3	JN204221 (9Bdi)	Burundi	Cibitoke	1015	'Yangambi Km5'-AAA, EA
4	JN204222 (10Bdi)	Burundi	Cibitoke	886	'Yangambi Km5'-AAA, EA
5	JN204223 (15Bdi)	Burundi	Bujumbura Rural	1754	'Ikigurube'-AAA, EA
6	JN204224 (25Bdi)	Burundi	Bujumbura Rural	774	'Indarama'-AAA, EA
7	JN204225 (30Bdi)	Burundi	Bururi	783	'Kayinja'-AAA,EA
8	JN204226 (36Bdi)	Burundi	Makamba	794	'Yangambi Km5'-AAA,EA
9	JN204227 (BK4Bdi)	Burundi	Cibitoke	893	'Yangambi Km5'-AAA,EA
10	JN204228 (2Rda)	Rwanda	Rusizi	1071	'Yangambi Km5'-AAA, EA
11	JN204229 (5Rda)	Rwanda	Rusizi	1060	'FHIA03'-AABB
12	JN204230 (15Rda)	Rwanda	Rusizi	1418	'Barabesha'-AAA, EA
13	JN204231 (18Rda)	Rwanda	Rusizi	1675	'Yangambi Km5'-AAA,EA
14	JN204232 (138Rda)	Rwanda	Rusizi	924	'Mitoke'-AAA, EA
15	JN204233 (4DRC)	DR Congo	Kabare	1717	'Yangambi Km5'-AAA, EA
16	JN204234 (10DRC)	DR Congo	Nyangezi	1589	'Yangambi Km5'-AAA, EA
17	JN204235 (18DRC)	DR Congo	Kamanyola	1252	'Yangambi Km5'-AAA,EA
18	JN204236 (20DRC)	DR Congo	Kamanyola	1221	'Kayinja'-AAA, EA
19	JN204237 (21DRC)	DR Congo	Kamanyola	913	'Yangambi Km5'-AAA, EA

Table 4.4. BBTV isolates for which sequences based on DNA-R component were determined in this study with their accession numbers and origin.

N°	Accession number	Country	Province/ District	Altitude (masl)	Variety
1	JN204197 (2Bdi)	Burundi	Bubanza	1090	'Yangambi Km5'-AAA, EA
2	JN204198 (7Bdi)	Burundi	Cibitoke	814	'Igisahira'-AAA, EA
3	JN204199 (9Bdi)	Burundi	Cibitoke	1015	'YangambiKm5'-AAA, EA
4	JN204200 (10Bdi)	Burundi	Cibitoke	886	'YangambiKm5'-AAA, EA
5	JN204201 (15Bdi)	Burundi	Bujumbura Rural	1754	'Ikigurube'-AAA, EAHB
6	JN204202 (25Bdi)	Burundi	Bujumbura Rural	774	'Indarama'-AAA, EA
7	JN204203 (30Bdi)	Burundi	Bururi	783	'Kayinja'-AAA,EA
8	JN204204 (33Bdi)	Burundi	Makamba	794	'Igisahira'-AAA,EA
9	JN204205 (35Bdi)	Burundi	Makamba	794	'Indarama'-AAA,EA
10	JN204206 (36Bdi)	Burundi	Makamba	794	'YangambiKm5'-AAA,EA
11	JN204207 (120Bdi)	Burundi	Bururi	785	'Kamaramasenge'-AAA, EA
12	JN204208 (BK4Bdi)	Burundi	Cibitoke	893	'Yangambi Km5'-AAA,EA
13	JN204209 (2Rwd)	Rwanda	Rusizi	1071	'Yangambi Km5'-AAA, EA
14	JN204210 (5Rwd)	Rwanda	Rusizi	1060	'FHIA03'-AABB
15	JN204211 (12Rwd)	Rwanda	Rusizi	924	'Kayinja'-ABB, EA
16	JN204212 (13Rwd)	Rwanda	Rusizi	924	'Yangambi Km5'-AAA, EA
17	JN204213 (15Rwd)	Rwanda	Rusizi	1418	'Barabesha'-AAA, EA
18	JN204214 (18Rwd)	Rwanda	Rusizi	1675	'Yangambi Km5'-AAA,EA
19	JN204215 (9DRC)	DR Congo	Nyangezi	1589	'Malaya'-AAA, EA
20	JN204216 (11DRC)	DR Congo	Nyangezi	1589	'Yangambi Km5'-AAA, EA
21	JN204217 (13DRC)	DR Congo	Nyangezi	1560	'Malaya'-AAA, EA
22	JN204218 (18DRC)	DR Congo	Kamanyola	1252	'Yangambi Km5'-AAA,EA

4.3.2. Sequence analysis of BBTV based on coat protein component

Sequence comparisons of a 475 bp product of each isolate representing the BBTV-CP component showed greater than 99% nucleotide sequence identity among the GLRA isolates (sequenced in this study). The nucleotide sequence identity ranged from 97.2 to 99.7% was observed between GLRA isolates and other South Pacific group isolates which include SSA, whilst they share only between 89.8 and 94.3% identity with Asian isolates.

Additionally, the nucleotide sequences of the CP components showed between 95.6 and 99.8% identity among SSA isolates described by Kumar *et al.* (2011), whereas they share higher levels of identity to isolates from other regions of the South Pacific group (95.2 to 98.5%) than to those from the Asian group (81 to 94.8%). In overall, these results suggest that South Pacific isolates share 95 to 100% similarities and only 81 to 94.8% identity when they are compared with isolates from the Asian group. On the other hand, pairwise comparisons between CP nucleotide sequences from the Asian and South Pacific groups showed the higher sequence variability among isolates from Asian group (0.7% to 8%) than among isolates from the South Pacific group (0% to 4.8 %).

Further comparisons between BBTV and *Abaca bunchy top virus* (ABTV) CP nucleotide sequences showed a low nucleotide sequence similarity ranging from 66.8 to 67.5% with isolates from the GLRA, whilst it ranged from 51 to 68.4% when compared with other isolates from the South Pacific group including SSA.

The comparison of ABTV isolate with isolates from the Asian group was ranged from 49.6 to 56.7% (Table 4.5).

Table 4.5. BBTV coat protein nucleotide sequence similarities (%) between isolates from the African Great Lakes region and those belonging to the South pacific and Asian groups*

CP isolates from	**GLRA of this study**	**SSA**	**SP-group**	**Asian-group**
Great Lakes Region of Africa(GLRA)	99.3-100			
Sub Saharan Africa (SSA)	97.2-98.5	95.6-99.8		
Other regions of South Pacific group	97.4-99.7	95.2-98.5	97.7-99.8	
Asian group	89.8-94.3	81.2-94.8	82.3-93.9	92-99.3
An out-group '*Abaca bunchy top virus*' (ABTV)	66.8-67.5	51-56.2	52.4-68.4	49.6-56.7

* : Min and max values of nucleotide sequence similarities (%) of BBTV coat protein sequences of the African Great Lakes region (GLRA) isolates compared with other isolates obtained in GenBank database from, other countries of sub-Saharan Africa, the South Pacific group out of Africa (India and Fiji), the Asian group and *Abaca bunchy top virus* (ABTV) used as an out group.

The CP nucleotide sequence comparisons with a consensus sequence derived from 19 GLRA isolates showed five haplotypes. The first haplotype includes isolates corresponding to the consensus sequence, while other 4 haplotypes differed by a single nucleotide substitution with regards to the consensus sequence. The following nucleotide sequence substitutions were observed in four distinct sites (C/T, at 256nt, in JN204222-10Bdi isolate and JN204227-BK4Bdi isolate which are from the same banana variety 'Yangambi Km5' but obtained at different altitudes), (G/C, at 258nt, in JN204225-30Bdi isolate), (T/C, at 276nt, in JN204232-138PR isolate) and (A/G, at 405nt, in JN204230-15Rw isolate) (Fig. 4.2).

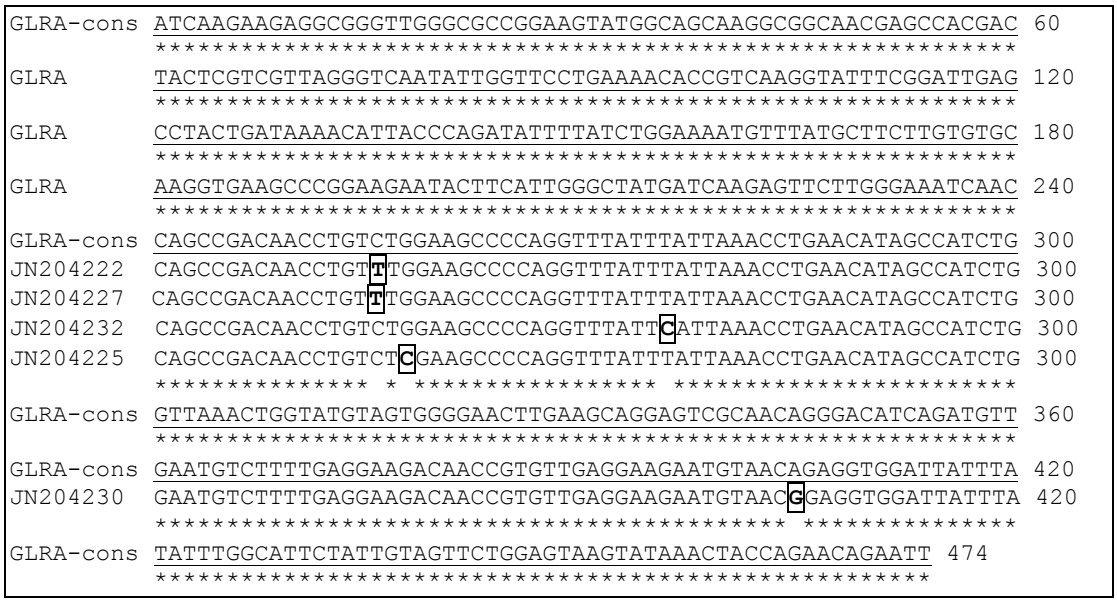

Fig. 4.2. BBTV-CP nucleotide sequences alignment among the Great Lakes Region of Africa (GLRA) isolates as compared to their consensus nucleotide sequences. Consensus sequence is underlined, asterisks indicate absence of mutation and nucleotide substitutions are indicated in bold and boxed.

Phylogenetic analysis based on the BBTV CP nucleotide sequences using the neighbour-joining method confirmed the clustering of BBTV isolates into two major groups, the Asian and the South Pacific groups (Karan *et al*., 1994; Wanitchakorn *et al*., 2000). The South Pacific group consists of all GLRA isolates (sequenced in this study) including Burundian isolate (AF148943) that was deposited earlier in GenBank by Wanitchakorn *et al.* (2000), Indian isolate, isolates from the sub-Saharan Africa described by Kumar *et al.* (2011) and Fiji (Fig. 4.3). Within the South Pacific group, three sister subgroups with high bootstrap support (99%) were distinguished. The first subgroup includes all GLRA isolates (sequenced in this study), the Burundian isolate (AF148943) previously reported and the Indian isolate (EF584544).

The second subgroup includes all sub-Saharan isolates, while the third subgroup contains a single isolate from Fiji (AF148944). On the other hand, the Asian group was divided into two main subgroups, the subgroup which includes isolates from China, Japan, Philippines and Taiwan and the subgroup of Vietnam's isolates with bootstrap support of 92% and 56 %, respectively.

The ABTV isolate used as an out group of BBTV was classified below all the BBTV isolates from both Asian and South Pacific groups (Fig.4.3).

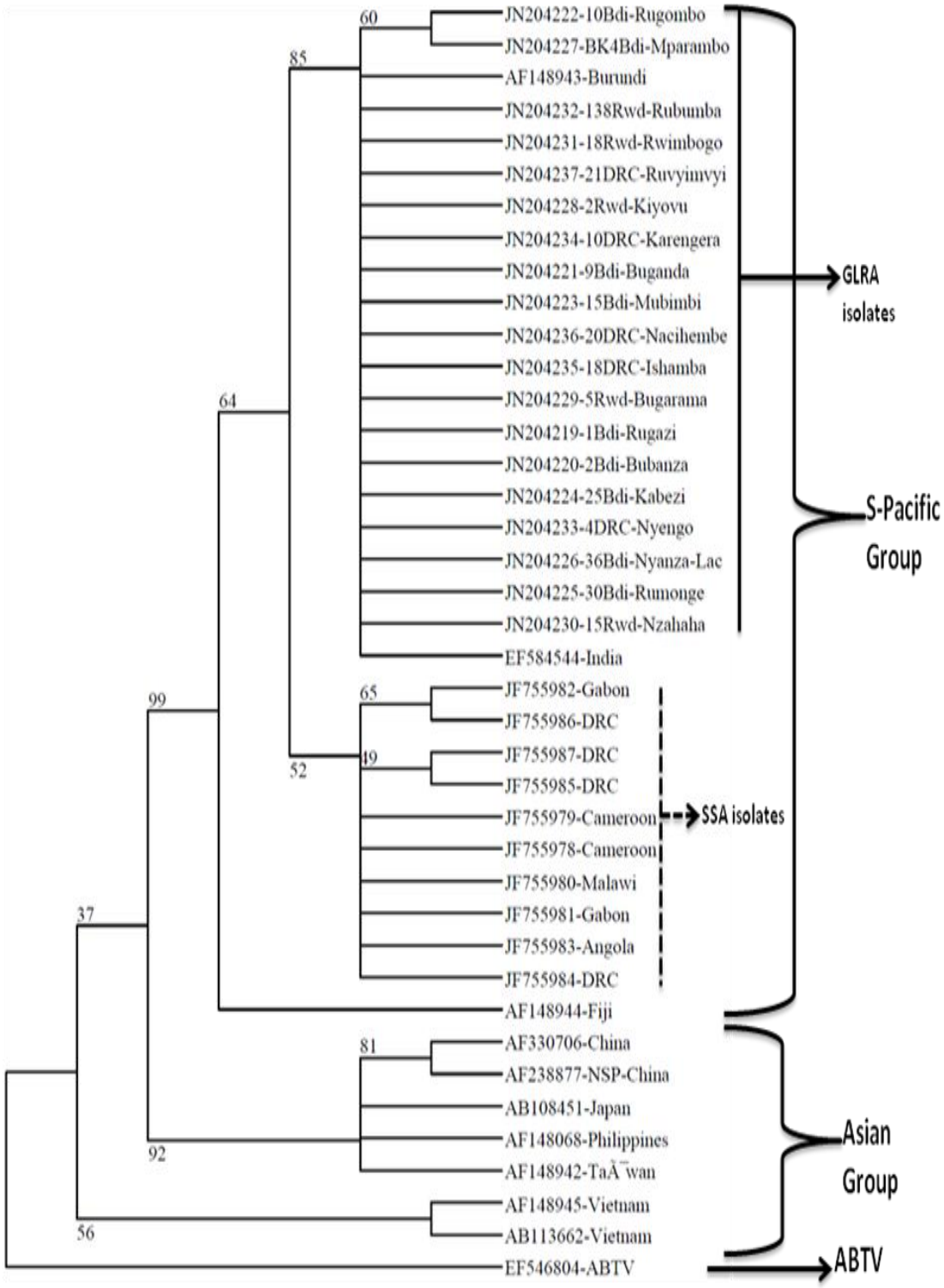

Fig. 4.3. Dendrogram showing relationships based on BBTV CP nucleotide sequences of 19 isolates collected in the African Great Lakes Region compared with representative BBTV isolates and one ABTV used as an out group, both obtained from the GenBank database.

4.3.3. Sequence analysis of BBTV based on DNA-R genome component

Sequence analysis was carried out using a 238 bp DNA fragment for each of the 22 isolates, corresponding to the core region of the BBTV DNA-R gene. Nucleotide sequence comparisons showed greater than 99% identity among GLRA isolates. These GLRA isolates (sequenced in this study) showed high level of nucleotide sequence similarity to the South Pacific group isolates (96.2 to 100%) than to the Asian group isolates (89.7 to 93.4%). On the other hand, nucleotide sequences variability was reported to be higher within the Asian group (0.7% to 10%) than among those of the South Pacific group (0% to 4.2%). Pairewise comparisons between BBTV isolates from GLRA and ABTV, the closest relative out group, showed low sequence identities ranging from 72.8 to 73.2% (Table 4.6).

Table 4.6. BBTV DNA-R nucleotide sequence similarities (%) between isolates from African Great Lakes region and those belonging to the South pacific and Asian groups*

DNA-R isolates from	GLRA of this study	SSA	S Pacific group	Asian-group
Great Lakes Region of Africa (GLRA)	99.1-100			
Sub-Saharan Africa (SSA)	96.2-100	97.4-100		
Other regions of South Pacific group (SP)	97.3-100	95.8-99.2	97.5-99.3	
Asian group	89.7-93.4	84.1-91.6	88.9-94	89.3-99.3
An out-group '*Abaca bunchy top virus*' (ABTV)	72.8-73.2	72.5-73.7	72.4-73.4	69.7-73.7

* Min and Max values of nucleotide sequences similarities (%) of BBTV DNA-R fragment from pairwise comparisons among African Great Lakes region isolates and between isolates obtained in database from the sub-Saharan Africa, the South Pacific group out of Africa (India and Fiji), the Asian group and *Abaca bunchy top virus* used as an out group.

Ten haplotypes were identified among nucleotide sequences compared to the consensus sequence derived from 22 isolates, based on their core regions of BBTV DNA-R replicase gene. The first haplotype includes nucleotide sequences of 11 isolates which were homologous to the consensus sequence, covering the three countries. Interestingly, the JF755989-TV13.1 Cameroun isolate was similar to the consensus sequence of the GLRA isolates with 100% of nucleotide sequence similarity. A second haplotype includes three isolates from Nyanza-Lac, Burundi (JN204204-33Bdi, JN204205-35Bdi and JN204206-36Bdi) which were collected at a similar altitude (794 masl) from distinct varieties. The remaining 8 isolates comprise a unique haplotype which differ by 1 to 2 base pairs from the consensus sequence (Fig.4.4).

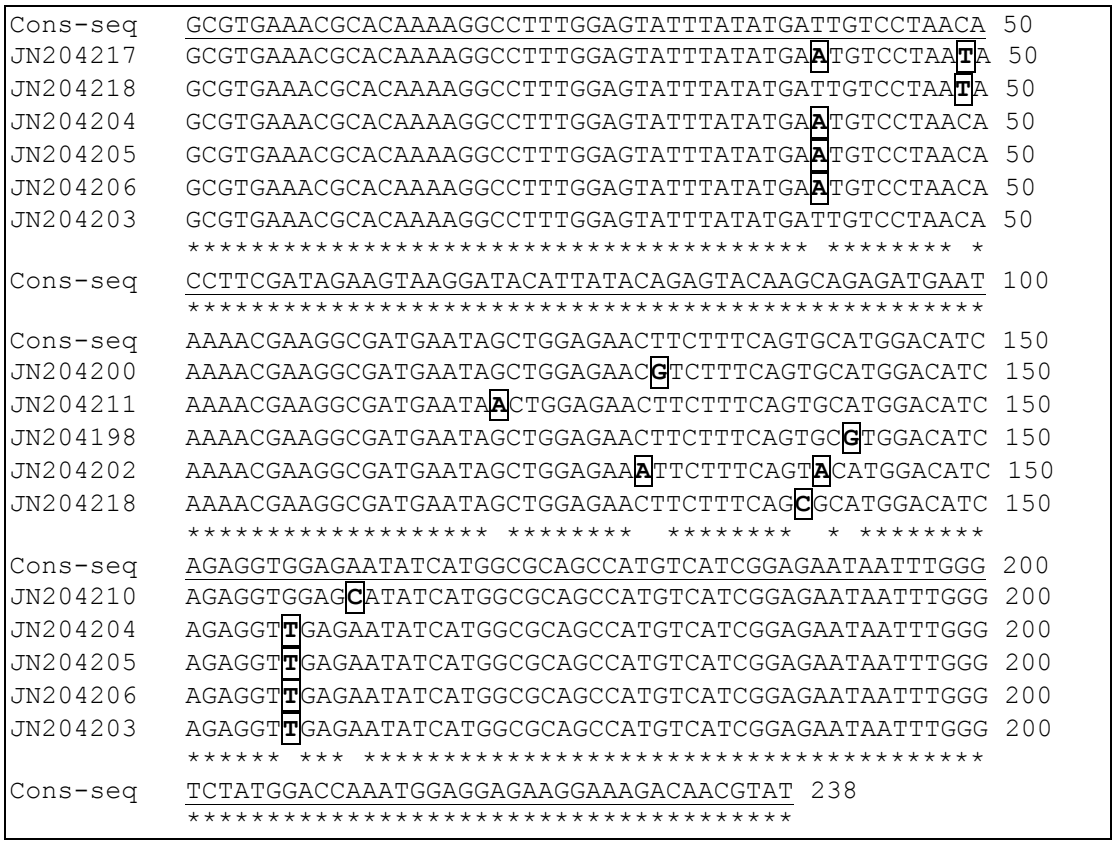

Fig. 4.4. BBTV DNA-R (core region) nucleotide sequences alignment among the African Great Lakes Region isolates as compared to their consensus nucleotide sequences. The consensus sequence is underlined; asterisks indicate absence of nucleotide substitutions which are indicated in bold and boxed.

Phylogenetic analysis based on Rep sequences using the neighbour-joining method has confirmed the previous reports (Karan *et al*., 1994; Wanitchakorn *et al*., 2000) clustering BBTV isolates into two major groups with high bootstrap support (100%) between the Asian and the South Pacific group.

The latter consists of all GLRA isolates of this study in addition to other 10 sub-Saharan Africa isolates described by Kumar *et al.*(2011) and other isolates from India (AF 416470-In), Australia (S56276-Au), Fiji (AF416466-Fj), Tonga (AF 416467-Tn), Hawaï (U18077-hw), Pakistan (AM 418538-pk) and Egypt (AF 416465) obtained from GenBank database (Fig. 4.5).

Four subgroups were distinguished among South Pacific group isolates, the first includes together all GLRA and SSA isolates followed by the Indian isolate (AF 416470-In); the third subgroup includes isolates from Australia, Fiji, Tonga, Hawaï and Pakistan, while Egypt is in its own subgroup. The GLRA and other SSA isolates show closest relationship with an isolate from India rather than other South Pacific isolates.

Among the GLRA isolates, three subgroups were distinguished based on phylogenetic analysis. The 2 isolates collected in DR Congo (JN204218-18DRC and JN204217-13DRC) were strongly similar although collected at different altitudes and from different banana varieties. The closest 4 isolates (JN204206-36Bdi, JN204204-33Bdi, JN204205-35Bdi, JN204203-30Bdi) from the south of Burundi were collected in the similar locations but from different banana varieties. Interestingly, among SSA isolates described by Kumar *et al.* (2011), the Cameroun isolate (JF755989-TV13.1) grouped together with the GLRA isolates, while 9 other isolates from SSA belonged to a different subgroup (Fig. 4.5).

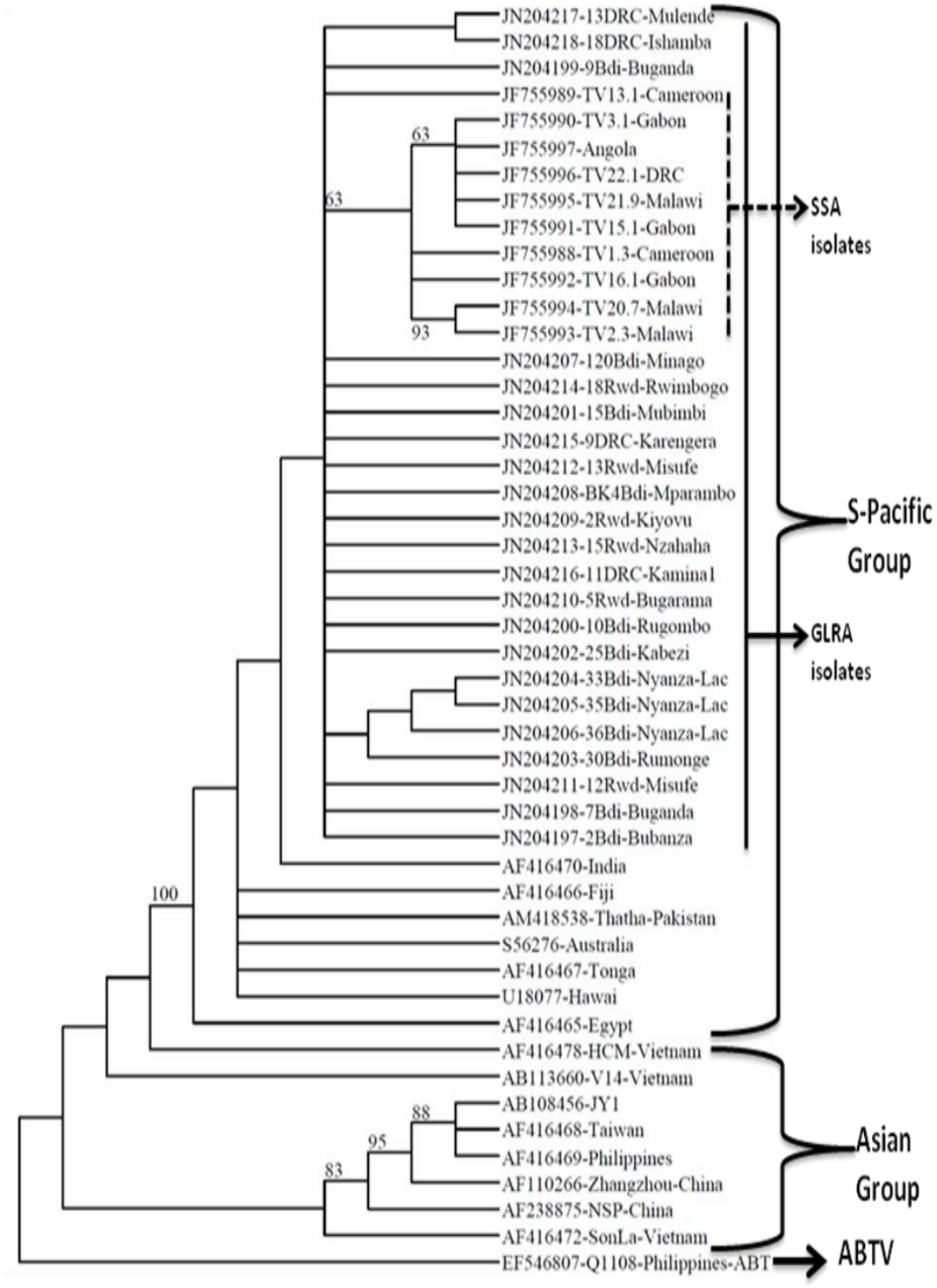

Fig. 4.5. Neighbour-joining tree illustrating the BBTV DNA-R sequence relationships among 22 isolates from the Great lakes region of Africa and isolates from GenBank database, 10 from other sub-Saharan Africa, 7 of South Pacific group out of Africa, 9 of Asian group and 1 ABTV used as an out group.

4.4. Discussion

The nucleotide sequences of BBTV DNA-R (Rep) component and the Coat Protein (CP) encoded by ORF of DNA-S (Wanitchakorn *et al.*, 2000; Horser *et al*., 2001b; Furuya *et al.*, 2005, Kumar *et al.,* 2011) were used to characterize BBTV isolates from the GLRA. The nucleotide sequences of GLRA isolates obtained in this study and of isolates earlier deposited in the GenBank database were compared to assess the interrelationships of BBTV isolates from GLRA with those from the two geographic clusters Asian and South Pacific, the likely origin of BBTV in the GLRA, the possible BBTV introduction period in the GLRA, the genetic variability among GLRA isolates and the likely influence of banana variety and altitude on nucleotide sequence mutations.

The nucleotide sequence similarities of DNA-R (Rep) and DNA-S (CP) segments of BBTV genome, between BBTV GLRA and ABTV isolates ranged from 66.8 to 73.2% are comparable to 54 to 81% previously reported across all the six BBTV components (Sharman *et al.*, 2008). Based on BBTV CP gene of GLRA isolates compared with isolates of the South Pacific and the Asian groups showed the nucleotide differences ranging from 0.7 to 2.8% and 5.7 to 10.2%, respectively. The later corroborate with previous estimates of 3% variability among the South Pacific group isolates and more diversity of around 6% across the Asian group isolates (Wanitchakorn *et al.,* 2000). On the other hand, using BBTV DNA-R component (rep) of GLRA isolates, a range of 0.9 to 3.8% and 6.6 to 10.3% of nucleotide differences are comparable to the previous averages of 3.8% among South Pacific group isolates and approximately 10% between the two groups (Karan *et al*., 1994).

Additionally, the phylogenetic analysis using these two genome components strongly confirmed that all the GLRA isolates belong to the South Pacific group.

Among South Pacific isolates, based on the CP nucleotide sequences, the Indian isolate (EF584544) showed closest relationship with the GLRA isolates than the other SSA isolates. Using the core region of DNA-R fragment, all BBTV isolates from sub-Saharan Africa were grouped together followed by the Indian isolate. The interrelationships among the SSA isolates which include the GLRA isolates may suggest the possible multiple introductions of the virus into sub-Saharan Africa as previously reported (Wardlaw, 1961; Fouré and Manser, 1982; Dale, 1987), either with the introduction of new cultivars by African between countries and other migrant workers (Kumar *et al.*, 2011), or throughout the traditional traders travelling between the Indian sub-continent and Africa (Purseglove, 1972; Price, 1994; Kumar *et al.,* 2011). Karan *et al.* (1994) had suggested that BBTV infections should have two major sources, one in Asia and another in the South Pacific. Irrespective of the means of the first BBTV introduction, the African virus isolates are likely to have the South Pacific origin and may have acted as the cause of epidemics through either the traditional farmers' practices of intra-and inter-regional exchange of suckers or introduction of infected plants from research stations, while aphids may have spread the virus between plantations at local level (Kumar *et al.,* 2011). The two gene sequence-based phylogenies results suggest that the virus isolates from the GLRA could have originated from India rather than from other countries of the South Pacific group.

In addition, BBTD could have been spread into GLRA through exchange of banana cultivars, which has been confirmed by the presence of BBTV susceptible variety (‘indarama-AAA’/ ‘Pome’) in the region, originated from India according to Edmond De langhe (Pers. Com).

Previous analysis of sequence variations based on BBTV DNA-R component showed a maximum of 8% variation among isolates from Vietnam within the Asian group (Bell *et al.*, 2002). This higher degree of diversity in the nucleotide sequences of DNA-R (rep) within the Asian group has been reported by Wanitchakorn *et al.* (2000) and Furuya *et al.* (2005), whereas in other countries within the south Pacific group such as Pakistan, the sequence variations of 0.45% was reported 20 years after disease identification compared to 2% reported over 80 years in Australia (Karan *et al*., 1994; Amin *et al*., 2008). In this study, the DNA-R sequence variation was around 0.9% among GLRA isolates. From these results, it is possible that BBTD might have been present in Rusizi valley region of Africa from 1970s. This is in line with the previous report of a survey carried out by Sebasigari and Stover (1988) which suggested in 1987 that BBTD might have been present for 20 years in Rusizi valley encompassing Burundi and Rwanda. Although, BBTD was confined in a small area of Rusizi valley in 1990s, at that time, the lack of farmers’ awareness about BBTD transmission and management practices could be the main factor explaining the continuous spread of BBTV in the GLRA.

Based on nucleotide sequences of the core region of DNA-R, 9 nucleotide differences among the GLRA isolates were observed in about 40 years compared to only 5 nucleotide differences, using the entire DNA-R component, reported among Australian isolates in around 80 years (Karan *et al.*, 1994).

Among the Asian group isolates which are known to have higher genetic variability, 12 nucleotide substitutions were noted by Vishnoi *et al.* (2009) in a Chinese isolate while only 2 nucleotide substitutions were reported among Indian isolates which belong to the South Pacific group. These nucleotide substitutions of DNA-R (rep) among BBTV isolates from the GLRA showed a relatively higher genetic variability as compared to other South Pacific isolates. In addition, the BBTV coat protein, known to be a BBTV component highly conserved (Furuya *et al.*, 2005; Vishnoi *et al.*, 2009) showed four nucleotide substitutions within the GLRA isolates, whereas Vishnoi *et al.*, (2009) noted none among Indian isolates. These mutations could be influenced by wheather conditions, since Drew *et al.* (1992) and Hooks *et al.* (2008) had noted that climate affects the disease evolution as symptoms are expressed after 3 weeks in summer against 4 to 5 months in winter. In addition, the multiple introductions of the virus across regions may have influenced the genetic variability among GLRA isolates (Wanitchakorn *et al.*, 2000; Furuya *et al.*, 2005).

The BBTV isolates across the three countries of the GLRA were grouped together. This suggests similarity in origin of GLRA isolates which were most likely distributed through exchange of planting materials. It is likely that the virus was introduced in Rusizi valley and its surrounding regions of Burundi, Eastern South-Kivu DR Congo and Rwanda by the exchange of 'Yangambi Km5-AAA genome'

variety originated from Yangambi agricultural research station in the central DR Congo where BBTV was reported as early as in 1950s (Wardlaw, 1961; Fouré and Manser, 1982; Kavino *et al*., 2007).

The BBTV isolates from the same location obtained from different varieties and at different altitudes were grouped together. This suggests the likely virus mutation according to the introduction period regardless of the banana variety or altitude, corroborating with previous reports that sequence variation of the virus is strongly depend on the period of time it exists in a region (Wanitchakorn *et al.*, 2000; Amin *et al.*, 2008).

In general, although GLRA isolates were genetically identical and grouped together within the South Pacific group, nucleotide substitutions among GLRA isolates revealed higher genetic variability compared to other South Pacific isolates. Previously, the BBTV genetic variation was reported to be related to its pathogenecity (Su *et al.*, 2003). This may explain the observed severe BBTV symptoms on the BBTV-infected banana plants in GLRA. Consequently, this genetic variability could be the factor which causes tolerance breakdown of all cultivars (Amin *et al.*, 2008). This suggests that the use of tolerant varieties alone is not enough and should be associated with collective eradication of BBTV-infected mats in order to reduce virus inocula for long term BBTV management.

Moreover, the disease spread across the three countries has been attributed to the exchange of planting materials between farmers. Hence, in GLRA there is a need of quarantine measures between BBTD affected areas and non affected areas to prevent spread of the virus in regions not yet BBTD-affected.

Further research should be undertaken on the virus genome using all the six BBTV components in order to confirm these findings on the relatively higher level of sequence variation and likely evolution of GLRA isolates.

CHAPTER FIVE

SCREENING *MUSA* GENOTYPES FOR RESISTANCE TO BANANA BUNCHY TOP DISEASE

Abstract

Banana bunchy top disease (BBTD) is one of the most devastating diseases affecting banana and plantain cultivation in African Great Lakes region. In order to identify putative sources of resistance, a cultivar screening trial comprising 40 *Musa* genotypes was established in March, 2007 until September, 2010 at the ISABU Mparambo research station located, Rusizi valley, in north western Burundi (893 masl). Dessert banana (AAA group), East African highland banana (AAA, EAHB), plantains (AAB), cooking banana (ABB), a tetraploid hybrid and wild diploid banana (*Musa acuminata* and *Musa balbisiana*) were assessed. Ten plants per genotype were planted in a field of 10 blocks. Each plant per genotype was a replicate within a completely randomised block with border rows consisting of BBTV-infected 'Yangambi Km5' (AAA) plants. Colonies of aphid vector, *Pentalonia nigronervosa* were collected from BBTD-affected fields and released in the plot to enhance virus spread. The first symptoms appeared 80 days after the trial establishment on 'Yangambi Km5', while the results indicate that genotypes containing B genomes tend to be more tolerant to BBTD. Forty-two months after the trial establishment, there were only five genotypes ['FHIA-03' (AABB), 'Highgate' (AAA, Gros Michel subgroup), 'Kayinja' (ABB), *Musa balbisiana* type Tani (BB), and 'Saba' (ABB)] which did not manifest typical disease symptoms on any of the 10 plants per

genotype. Further diagnosis carried out using PCR tests at the *Université de Liège-Gembloux Agro-Bio Tech* in Belgium on leaf samples of the five asymptomatic genotypes has confirmed the absence of the virus in all the 10 plants of these five genotypes. However, suckers from 'FHIA03' (AABB), 'Highgate' (AAA) and 'Saba' (ABB) planted in farmers' fields under higher pressure of the disease showed BBTD symptoms. These genotypes can be considered to be BBTD tolerant. Therefore, based also on their potential production, five BBTV-tolerant cultivars ['Corne plantain' (AAB-plantain), 'FHIA-03' (AABB), 'Highgate' (AAA, Gros Michel subgroup), 'Igjindi' (AAB-plantain) and 'Saba' (ABB)] were selected for farmers' adoption. Although these tolerant cultivars could potentially act as a reservoir for the virus, they may be the best option for BBTD integrated management for small-scale farms.

5.1. Introduction

Banana and plantain (*Musa* spp.) are cultivated in over 120 countries in tropical and subtropical regions worldwide (INIBAP, 2000; Hu *et al*., 2007). Fungal, bacterial and viral diseases have been threatening banana production in Central Africa, as has been the case elsewhere. BBTD is spread through infected planting materials and transmitted by an aphid vector, *Pentalonia nigronervosa* Coquerel (Magee, 1940; Allen, 1987; Hu *et al.*, 2007). The incubation period of the disease is approximately 4 weeks (Allen, 1987; Drew *et al.*, 1989), with symptom development being a function of external temperatures (Sun, 1961; Dale *et al.*, 2000). The occurrence of symptomless infections on certain genotypes complicates disease management and

increases the risk of pathogen spread since symptomless plants may act as reservoirs for the virus (Allen, 1987; Drew *et al.*, 1989).

Resistant genotypes do not exist and the recommended strategy for controlling the disease includes prompt uprooting of symptomatic plants and replanting with virus-free tissue culture plantlets. This eliminates BBTV-infected plants that are the likely source of secondary infection and spread of the pathogen (Dale, 1987; Hooks *et al*., 2008; Jones, 2009). Virus-free planting material is ascertained by indexing of plantlets using serological and molecular tools such as triple antibody sandwich enzyme-linked immuno sorbent assay (TAS-ELISA) and polymerase chain reaction (PCR), respectively (Geering, 2009; Ikram *et al*., 2009).

Although no *Musa* genotype is known to be resistant to BBTV, numerous studies report differences in susceptibility (Magee, 1948; Daniells, 2009). Cultivars in the AA and AAA genomic groups are highly susceptible to BBTD with the exception of 'Gros Michel', whereas cultivars containing two B genomes are regarded as less susceptible if not tolerant (Magee, 1948; Ariyatne and Liyanage, 2002). The deployment of resistant cultivars may be an option for the integrated management of BBTD in the regions where small-scale agriculture dominates.

The present study evaluated 40 genotypes, belonging to a wide range of *Musa* groups, for their reaction to *Banana bunchy top virus*.

5.2. Materials and Methods

A BBTV screening trial of one hectare with 40 genotypes was established in March, 2007 at the Mparambo agricultural research station of the *Institut des Sciences Agronomiques du Burundi* (ISABU). Mparambo is located at 893 masl (S 2°50'220, E 29°04'375) in the province of Cibitoke in North-Western Burundi, bordering Western province of Rwanda and the Eastern South Kivu DR Congo.

According to climatic data collected at Mparambo station from June 2006 to September 2007, the average daily temperature was 32°C (min 14°C, max 36°C), while the average relative humidity was 73% (min 27%, max 97 %).

Thirty-five of the genotypes screened were established using lateral shoots (i.e., suckers) obtained from IRAZ banana collection known to be in BBTD-free zone located in Gitega (1,645masl), Burundi. The remaining five genotypes ('FHIA-03', 'Isha', 'Nakitembe', 'Poyo' and 'Yangambi Km5') were planted using tissue-culture derived plantlets obtained from AGROBIOTECH, a private tissue-culture laboratory based in Bujumbura, Burundi. One hundred *in vitro* plantlets of 'Yangambi Km5' (AAA) commonly cultivated in the region and susceptible to BBTV were used as a control and planted at random amongst the 39 test genotypes which belong to a wide range of *Musa* groups. The experimental layout has 10 repetitions (blocks**).** Each plant per genotype was a replicate within a completely randomised block. Plants were established at a spacing of 3 by 2 m. The plot was located at around 1 km from the closest banana fields infected by BBTV and had previously not been cultivated with banana. Five kilograms of decomposed farmyard manure was applied in each

planting hole prior to planting. No mineral fertilizer was applied. Desuckering and manual weeding were carried out when necessary, and three to four suckers were maintained within each mat. Old and dead leaves were pruned at two-week intervals and used as mulch.

The inoculum source for the trial consisted of BBTD-symptomatic 'Yangambi Km5' plants obtained from farmers' fields in Cibitoke where the presence of BBTV had been previously confirmed using TAS-ELISA. Each row of test genotypes was planted adjacent to a row of diseased 'Yangambi Km5' plants (i.e. 55 plants per Block) which contained aphid (*P. nigronervosa*) populations.

After trial establishment, the source of BBTV inocula and the infected plants which had reached their final stage (i.e. death of the plant), were regularly replaced with infected 'Yangambi Km5' suckers.

At the same time, the aphids collected in the nearby farmers' fields were released on the diseased 'Yangambi Km5' border row plants to enhance the virus spread.

The occurrence of aphids on trial plants was also assessed using a scale of 0 to 5 as previously explained (Chapter 3). BBTD symptoms were recorded on the mother plants and lateral shoots at 2-week intervals, and the incidence per cultivar was calculated as the percentage of the infected plants over the total number of plants for a given cultivar in the trial. In addition, the time from trial establishment to the first BBTD symptom appearance was recorded for each genotype. The incidence of panama disease (*Fusarium oxysporum* f. sp. *cubense*) and black leaf streak (*Mycosphaerella fijiensis*) was also recorded. Plant height at flower emergence and

pseudostem circumference at 20 cm above the soil level were measured. Additionally, the data on the number of bunches produced and their bunch weights were recorded.

PCR analyses were carried out at the *Université de Liège Gembloux* Agro-Bio Tech in Belgium using samples collected by PhytoPass kits on symptomless genotypes. Additionally, the data collected were subjected to analysis of variance (ANOVA) using statistical software COSTAT 6.4 (Cohort software, Minneapolis, USA, 2006) (Dynarski *et al.,* 2007). All tests were carried out at 5% level of significance. Treatment mean comparison was done using Student-Newman-Keuls.

5.3. Results

5.3.1. Occurrence of BBTD and *P. nigronervosa* in the screening field

The first visible disease symptoms appeared on the control genotype 'Yangambi Km5' 80 days after the trial establishment.

Moreover, among six genotypes which were BBTV-infected at their first ratoon, five genotypes were composed by A genome; these were ['Akondro Mainty' (AA), 'Barabeshya' (AAA, EA), 'Ingumba y'imbihire' (AAA, EA), 'Intamakamwe' (AAA, EA), 'Isha' (AAA, EA)]. In contrast, the genotypes such as the plantains 'Corne plantain' (AAB) and 'Igjindi' (AAB) which showed BBTD symptoms after 18 months of the trial may be considered as tolerant. The tolerant cultivars could produce at least on their 3 stems within a mat prior to the appearance of the disease symptoms.

This was observed from the number of cropping cycles (i.e. first crop = first cycle; first ratoon = second cycle; second ratoon = third cycle, etc.) that can be achieved at first appearance of the disease respective to the genotype and disease incidence. BBTD incidence across the 40 genotypes ranged from 0 to 90%. Five genotypes including one wild banana (BB) [*Musa balbisiana* type 'Tani' (BB)] and four edible cultivars ['FHIA-03' (AABB), 'Highgate' (AAA), Kayinja (ABB) and 'Saba' (ABB)] did not show any visible disease symptoms at 42 months after trial establishment. On the other hand, seven genotypes ['Akondro Mainty' (AA), 'Fougamou1' (ABB), 'Inyabubere' (AAA, EAHB), 'Kisubi' (AB), 'Mumbulu' (AAB-plantain), '*Musa balbisiana* type Butuhan' (BB) and 'Prata' (AAB)] distinguished themselves from the control cultivar 'Yangambi Km5' by a lower (< 27%) disease incidence. The highest disease incidences (60 to 90%) were observed on the first and the second ratoons of three genotypes 'Figue sucree' (AA), 'Nakitembe' (AAA, EA) and 'Poyo 2' (AAA, Cavendish) (Table 5.1).

The banana aphids were observed on all genotypes and higher aphids' populations were frequently reported on plantain genotypes such as 'Mumbulu' (AAB, plantain). This genotype was classified as the first containing more aphids following by other plantain cultivars (Table 5.1).

Table 5.1. *Musa* genotypes screened for resistance to BBTV with time (months) and cycle number at first appearance of BBTD symptom, disease incidence (i.e, at least one plant within a mat is infected) and aphids' occurence at 42 months after trial establishment[#].

Genotype	Genome group / Subgroup	Type	Months to first symptoms	Cycle number at time of first symptoms	BBTD incidence	Aphids occurrence
Akondro Mainty	AA	beer	15	1	10	2.6 bcd
Figue Sucrée	AA	dessert	13.33	1.66	90	2.7 bcd
Musa balbisiana 10852	BB	wild	8	1	30	2.2 cde
Musa balbisiana type Butuhan	BB	wild	11	2	10	2.5 bcd
Musa balbisiana type Tani	BB	wild	NA	NA	0	1.7 de
Kingala	AB	dessert	14.5	3	50	2.2 cde
Kisubi	AB	beer	18	4	20	2.5 bcd
Ney Poovan	AB	beer	12.5	3	40	2.4 bcd
Americani	AAA/Cavendish	dessert	16.83	3.83	70	2.5 bcd
Barabeshya	AAA/EAHB	cooking	10	1	40	2.9 bcd
Chibulangombe	AAA/EAHB	beer	13.2	4.2	60	2.1 cde
Gisahira Uganda	AAA/EAHB	cooking	17	4.5	50	2.5 bcd
Guineo Negro	AAA/EAHB	cooking	14.66	2.33	30	2.3 cde
Highgate	AAA/Gros Michel	dessert	NA	NA	0	2.4 bcd
Ingaju	AAA/EAHB	cooking	25	5	33	2.8 bcd
Ingumba y'imbihire	AAA/EAHB	cooking	8.5	1	40	2.2 cde
Intamakamwe	AAA/EAHB	beer	8	1	30	2.4 bcd
Intare	AAA/EAHB	cooking	21	4	44	2.6 bcd
Inyabubere	AAA/EAHB	beer	17.5	4	20	2.3 cde
Isha	AAA/EAHB	beer	6	1	33	1e
Nakitembe	AAA/EAHB	cooking	24	2	67	2.1 cde
Nyakibuzi	AAA/EAHB	beer	14	3	50	2.6 bcd
Poyo	AAA/Cavendish	dessert	11	2.16	87	2 cde
Yangambi Km5	AAA/Ibota	beer	2.7	1.2	27	2.8 bcd
Corne Plantain	AAB/plantain	cooking	18.66	4	70	3.2 abc
Guindi	AAB/Pome,	beer	16.2	3.2	78	2.5 bcd
Igjindi	AAB/plantain	cooking	24.5	3.5	50	3.7 ab
Kamaramasenge	AAB, dessert	dessert	25	8	50	2.3 cde
Mumbulu	AAB/plantain	cooking	13	4	10	3.9 a
Pisang Ceylan (Mysore)	AAB, dessert	dessert	12	3	50	2.6 bcd
Pisang raja	AAB/Nendra padaththi	beer	11.5	3.5	40	2.6 bcd
Prata	AAB	dessert	30	6	10	2.4 bcd
Cacambu	ABB/Bluggoe	cooking	22	7	30	2.5 bcd
Fougamou1	ABB/Pisang Awak	beer	25	6	10	2.5 bcd
Gisandugu	ABB/Pisang Awak	beer	29	6	30	2.5 bcd
Kayinja	ABB	beer	NA	NA	0	3 a-d
Monthan	ABB	cooking	18.25	3	40	3.3 abc
Pisang Awak	ABB	beer	29	8	30	2.7 bcd
Saba	ABB	cooking	NA	NA	0	2.1 cde
FHIA-03	AABB	beer	NA	NA	0	2.3 b-e
CV (%)			51.13	68.79		30.04
F-test			*	NS		***
LSD (5%)			19.74	5.62		0.86

#: NS, *: not significant and significant at $P < 0.05$, respectively. Cycle number (mother plant = 1st cycle; first ratoon = second cycle; second ratoon = third cycle, etc…) when disease symptoms first appeared. NA - no BBTD symptom was observed, in boxe is the control 'Yangambi Km5' and lines separate different genotypes. Means followed by the same letter in each column are not significantly different from each other according to Student-Newman-Keuls test at $P < 0.05$.

5.3.2. Influence of BBTD incidence on bunch weight of first five ratoons

Overall disease incidence gradually increased in the screening field from 20.4% at 24 months to 33.3% at 42 months after trial establishment. This increase in BBTD incidence inversely affected bunch weight which decreased from 16.7 kg to 3 kg in average from the first to the 5^{th} ratoon (Table 5.2). The lower averages resulted from the BBTV-infected mats which could either produce small bunches or not until the mat is entirely destroyed by the disease.

Table 5.2. Average bunches weight (Kg) according to five consecutive ratoon cycles on edible genotypes [#].

Genotype	Genome group / Subgroup	Type	Average bunches weight of five consecutive ratoons (Kg)				
			1	**2**	**3**	**4**	**5**
Akondro Mainty	AA	beer	8.30 e-j	5.37h-l	3.72h-m	0.87m-p	1.75d-j
Figue Sucrée	AA	dessert	5.23i-l	4.07i-m	3.17h-m	3.55f-p	2.15d-j
Kingala	AB	dessert	7.35f-k	4.61h-m	3.14h-m	1.72j-p	1.13f-j
Kisubi	AB	beer	5.01i-l	6.2g-k	2.84i-m	2.5i-p	0.48g-j
Ney Poovan	AB	beer	6.25g-l	3.94i-m	2.89i-m	1.8j-p	1.65e-j
Americani	AAA/Cavendish	dessert	15.63 bc	12.39def	11.78b	8.53b-e	1.27f-j
Barabeshya	AAA/EAHB	cooking	14.39 b-d	9.46d-i	7.42b-j	2.4i-p	1.75d-j
Chibula ngombe	AAA/EAHB	beer	13.03 b-e	9.75d-i	8.64b-h	6.1d-l	3.75d-j
Gisahira Uganda	AAA/EAHB	cooking	12.85b-e	13.38cde	10.73b-f	9.21a-d	6.04cd
Guineo Negro	AAA/EAHB	cooking	8.96e-i	7.65e-j	3.68h-m	4.4d-o	3.72d-j
Highgate	**AAA/Gros Michel**	**dessert**	**14.37b-d**	**5.54h-l**	**1.38k-m**	**0.15n-p**	**0.03hij**
Ingaju	AAA/EAHB	cooking	14.07 b-d	13.67bcd	11.53bc	7.56b-h	4.55c-g
Ingumba-y'imbihire	AAA/EAHB	cooking	16.09b	9.79d-i	3.98h-m	2.97g-p	2.54d-j
Intamakamwe	AAA/EAHB	beer	14.68 b-d	12.65def	10.89b-e	7.89b-g	8.7bc
Intare	AAA/EAHB	cooking	15.04 b-d	8.81d-i	5.47e-l	1.21l-p	1.08f-j
Inyabubere	AAA/EAHB	beer	11.15 c-g	11.82d-g	9.94b-g	6.31d-j	4.21d-h
Isha	AAA/EAHB	beer	6.41g-l	0.9lm	0.05m	0.07p	0.04j
Nakitembe	AAA/EAHB	cooking	1.80l	0.12lm	0.06lm	0.13 op	0.15ij
Nyakibuzi	AAA/EAHB	beer	11.54 b-f	8.99d-i	7.35b-j	6.39d-j	3.37d-j
Poyo	AAA/Cavendish	dessert	3.99j-l	2.25j-m	2.68j-m	1.32k-p	0.44g-j
Yangambi Km5	AAA/Ibota	beer	6.13h-l	5.26h-l	5.46e-l	2.85h-p	2.65d-j
Corne Plantain	**AAB/plantain**		**14.91 b-d**	**19.490ab**	**19.22a**	**12.5ab**	**14.96a**
Guindi	AAB/Pome,	beer	4.49i-l	6.32g-k	5.17f-l	4.89d-n	2.91d-j
Igjindi	**AAB/plantain**	**cooking**	**16.33b**	**10.01d-h**	**5.23f-l**	**2.77h-p**	**1.09f-j**
Kamaramasenge	AAB, dessert	dessert	6.74f-k	2.51j-m	2.04j-m	1.57j-p	1.94d-j
Mumbulu	AAB/plantain		5.46i-l	5.45h-l	4.67g-m	2.69h-p	1.85d-j
Pisang Ceylan	AAB, dessert	dessert	3.25kl	5.66h-l	5.79d-k	3.65e-p	3.56d-j
Pisang raja	AAB/Nendra padathi	beer	10.43d-h	13.44cde	8.44b-i	6.86c-i	2.46d-j
Prata	AAB	dessert	7.87f-k	6.34g-k	8.62b-h	5.39d-m	5.18c-f
Cacambu	ABB/Bluggoe	cooking	16.32b	19.28abc	18.12a	11.52abc	11.41ab
Fougamou1	ABB/Pisang Awak	beer	10.60d-h	11.99d-g	6.13c-k	2.58i-p	3.98d-i
Gisandugu	ABB/Pisang Awak	beer	4.73i-l	1.34k-m	0.7k-m	0.88m-p	0.05hij
Kayinja	ABB	beer	7.49f-k	9.64d-i	7.13b-j	4.46d-o	1.05f-j
Monthan	ABB	cooking	24.24a	14.68bcd	11.13bcd	8.32b-f	2.7d-j
Pisang Awak	ABB	beer	8.11e-k	7.28f-j	6.2b-k	6.25d-k	5.69cde
Saba	**ABB**	**cooking**	**14.98 b-d**	**23.810 a**	**22.34a**	**13.62a**	**12.73ab**
FHIA-03	**AABB**	**beer**	**16.39b**	**5.86h-l**	**5.9c-k**	**6.48d-j**	**2.98d-j**
Average weight			**16.7**	**6.2**	**6.1**	**6.8**	**3.0**
CV (%)			14.3	30.4	28.8	39.0	34.9
F-test			***	***	***	***	***
LSD (5%)			4.92	5.935	5.607	4.954	4.357

#: ***: highly significant at P < 0.001. Means followed by the same letter in each column are not significantly different from each other according to the Student-Newman-Keuls test at $P < 0.05$; lines separate different genotypes, in boxe is the control 'Yangambi Km5', in bold: five tolerant cultivars retained out of 40 genotypes screened for resistance to BBTV.

5.3.3. Characteristics of BBTD tolerant cultivars selected

Five cultivars [‘Corne plantain’ (AAB-plantain), ‘FHIA-03’ (AABB), ‘Highgate’ (AAA, Gros Michel subgroup), ‘Igjindi’ (AAB-plantain) and ‘Saba’ (ABB)] were selected based on their tolerance to BBTV and their potential bunches yield for farmers’ adoption. In addition, the status of these cultivars face with the main banana diseases (i.e, Sigatoka and *Fusarium* wilt) was considered in comparison with the control ‘Yangambi Km5’. The ‘Saba’, ‘Highgate’ and ‘FHIA03’ were highly tolerant to BBTD, without disease symptoms up to 42 months of the screening field trial. Banana production of ‘Saba’ variety was not affected by other diseases, while the potential bunches’ yield of ‘Highgate’ and ‘FHIA03’ was reduced from the second ratoon by black leaf sigatoka disease. On the other hand, ‘Corne plantain’ (AAB-plantain) and ‘Idgindi’ (AAB-plantain) became BBTV-infected later, after 18 months of the screening field trial. These two plantains have produced large banana bunches on their first 3 to 4 ratoons (Table 5.2) before they became BBTV-infected, and were not infected with other diseases.

The girth of healthy plants at 20 cm from the soil of banana plants of these five tolerant genotypes was the largest as compared to the control ‘Yangambi Km5’ (AAA). Similarly, the four tolerant cultivars (i.e, with at least one B genome) showed a higher plant height of their healthy plants at flower emergence of their healthy banana plants compared to the control ‘Yangambi Km5’ (AAA), with the exception of the ‘Highgate’ (AAA, Gros Michel subgroup). These tolerant genotypes were giants, while the susceptible ‘Poyo’ (AAA-cavendish) had shown the lowest girth and height at flower emergence of its healthy plants in the trial. Moreover, all 11

genotypes containing two B genomes showed the largest girth and longest plant height at flower emergence on their healthy banana plants. These results showed that, tolerant varieties (i.e, B genome varieties and Gros-Michel) were more giants comparing to the susceptible varieties (Table 5.3).

Table 5.3. Plant height at flower emergence and girth at twenty centimeter on healthy plants of the trial [#]

Genotype	Genome group/ Subgroup	Type	Plant height (cm)	Plant girth at 20cm (cm)
Akondro Mainty	AA	beer	318b-f	68.65def
Figue Sucrée	AA	dessert	276c-g	98.90b-e
Musa balbisiana 10852	BB	wild	411ab	118.35bc
Musa balbisiana type Butuhan	BB	wild	400ab	84.70b-f
Musa balbisiana type Tani	BB	wild	413ab	125.95b
Kingala	AB	dessert	285b-g	67.95def
Kisubi	AB	beer	291b-g	65.45def
Ney Poovan	AB	beer	291b-g	61.85ef
Americani	AAA/Cavendish	dessert	266c-g	69.25def
Barabeshya	AAA/EAHB	cooking	340b-e	72.00def
Chibula ngombe	AAA/EAHB	beer	255c-g	66.05def
Gisahira Uganda	AAA/EAHB	cooking	331b-f	75.45def
Guineo Negro	AAA/EAHB	cooking	283b-g	64.55def
Highgate[#]	**AAA/Gros Michel**	**dessert**	**229e-g**	**80.55def**
Ingaju	AAA/EAHB	cooking	333b-f	74.66def
Ingumba y'imbihire	AAA/EAHB	cooking	318b-f	80.15def
Intamakamwe	AAA/EAHB	beer	277c-g	65.25def
Intare	AAA/EAHB	cooking	318b-f	76.22def
Inyabubere	AAA/EAHB	beer	292b-g	73.40def
Isha	AAA/EAHB	beer	246c-g	49.83ef
Nakitembe	AAA/EAHB	cooking	227e-g	80.55c-f
Nyakibuzi	AAA/EAHB	beer	313b-f	69.00def
Poyo	AAA/Cavendish	dessert	191g	50.75ef
Yangambi Km 5	**AAA/Ibota**	**beer**	**266c-g**	**61.55ef**
Corne Plantain#	**AAB/plantain**		**281b-g**	**78.95def**
Guindi	AAB/Pome	beer	238d-g	65.55def
Igjindi#	**AAB/plantain**	**cooking**	**321b-f**	**74.70def**
Kamaramasenge	AAB, dessert	dessert	282b-g	71.85def
Mumbulu	AAB/plantain		307b-f	72.70def
Pisang Ceylan	AAB, dessert	dessert	224fg	46.20f
Pisang raja	AAB/Nendra padaththi	beer	326 b-f	105.7bcd
Prata	AAB	dessert	411ab	87.40b-f
Cacambu	ABB/Bluggoe	cooking	372 abc	84.25 b-f
Fougamou1	ABB/Pisang Awak	beer	401 ab	91.60 b-e
Gisandugu	ABB/PisangAwak	beer	373 abc	122.60 b
Kayinja	ABB	beer	298 b-g	82.75 b-f
Monthan	ABB	cooking	455 a	153.90 a
Pisang Awak	ABB	beer	402 ab	87.61 b-f
Saba[#]	**ABB**	**cooking**	**350 bcd**	**79.70 def**
FHIA-03[#]	AABB	beer	**278 b-g**	**82.60 b-f**
CV (%)			20.94	30.87
F-test			***	***
LSD (5%)			81.97	30.82

#: ***: highly significant at $P < 0.001$; Means followed by the same letter in each column are not significantly different from each other according to the Student-Newman-Keuls test at $P < 0.05$; lines separate different genotypes and in bold: growth parameters of the five tolerant cultivars as compared to the control in boxe 'Yangambi Km5'.

5.4. Discussion

At 42 months after trial establishment, only 5 genotypes were still asymptomatic. Four out of the 5 genotypes contain 2B genomes, while 'Highgate' is an AAA dessert banana. Dela Cruz *et al.* (2008) also reported high levels of tolerance to BBTV for the following genotypes which contain at least one B genome ['Cachaco' (ABB), Cardaba (ABB), 'Saba' (ABB), 'Pisang Ceylan' (AAB, Mysore), the tetraploids 'FHIA-01' (AAAB) and 'CRBP 39' (AAAB)]. In agreement with our results, Magee (1948) and Geering (2009) had reported the low susceptibility of 'Highgate' (AAA-subgroup 'Gros Michel') to BBTV. In addition, the tolerant cultivars were the largest cultivars in terms of height at flower emergence and/or girth at 20 cm from the soil. These results support the hypothesis that structural and physiological status might contribute to the tolerance, as had been suggested for 'Gros Michel' (Magee, 1948). Magee (1948) also reported high susceptibility of Cavendish genotypes, which corroborates with our findings of rapid symptom expression on 'Poyo'.

Among the five symptomless genotypes, the wild unedible diploid BB banana and the Fusarium wilt susceptible variety such as 'Kayinja' were not suitable for large-scale dissemination in BBTD affected zones, as these areas are also highly affected by *Fusarium* wilt.

PCR results of the samples collected on asymptomatic genotypes up to the end of the experiment all tested negatives. However, the three genotypes 'Saba', 'FHIA03,' and

‘Highgate’ showed disease symptoms when planted in farmers’ fields. Hence, they are the only tolerant genotypes and could potentially act as virus reservoirs.

However, the use of tolerant varieties should be the best and the only choice in BBTD affected regions with small-scale agriculture. Five genotypes were eventually selected for farmers’ adoption in BBTD-affected areas of the Rusizi valley region. These include three genotypes [‘Saba’ (cooking), ‘FHIA03’ (cooking/ dessert), ‘Highgate’ (dessert)] which had no visible BBTD symptoms at the end of the screening trial. The ‘Saba’-ABB (BIZ 204-256 or ITC 0116-0026) was obtained from banana field collection of IRAZ. It is a cooking variety with higher BBTD tolerance, large number of suckers per mat and higher yield. In addition, ‘Saba’ was resistant to black sigatoka (*Mycosphaerella Fijiensis*) and *Fusarium* wilt (Sebasigari and Stover, 1988). The ‘FHIA03’-AABB was obtained from AGROBIOTEC, the *in vitro* laboratory based in Bujumbura-Burundi. It showed high yield during the first cycle, but was infected by black leaf streak from the second cycles. The ‘Highgate’-AAA (BIZ 198 or ITC 0263) obtained from banana field collection of IRAZ, is a dessert banana, highly tolerant to BBTD but susceptible to banana black streak. ‘FHIA03’ and ‘Highgate’ are suitable in areas where banana black streak are not a major threat to banana production.

In addition, two plantains cultivars [‘Corne plantain’ and ‘Igjindi’] with a high level of disease tolerance, high yields and which were in high demand in the local markets were also selected. The ‘Corne plantain’-AAB-plantain (BIZ 282 and ITC 0210) was obtained from banana field collection of IRAZ. It showed BBTD symptoms on 4th ratoon cycle after 18 months of the field trial. This ‘corne plantain’ variety is used

for dessert and brewing purposes, producing the biggest bunches as compared to that of the control 'Yangambi Km5'.

The 'Igjindi'-AAB-plantain (BIZ 172) was also obtained from banana field collection of IRAZ and showed BBTD symptoms at 24 months of the trial on the third ratoon cycle. This plantain variety is used for cooking and produces the biggest bunch.

In the field trial, large aphid colonies were observed on plantain genotypes confirming Kumar and Hanna's (2008) observations in West Africa. Robson *et al.,* (2006) reported that aphids prefer the base of plantlets and plantains due to the clear colour of their pseudostem. The plantains contain one B genome and this could be the reason for their relative tolerance to the disease despite the large numbers of aphids which were observed. This may be one of the factors explaining no direct correlation observed between aphid numbers and disease incidence. The BBTD incidence within the field trial increased around 12% per year resulting onto a gradual decrease in banana yields. The lower averages of buches' weight observed might be due to low photosynthesis rate of chlorotic leaves of BBTV-infected plants affecting the banana production (Dale, 1987; Hooks *et al.,* 2008).

In overall, these five tolerant cultivars need to be introduced in farmers' conditions for evaluation and adoption as a component of BBTD integrated management. Additionally, sanitation practices which include eradication of infected mats, source of BBTV inocula, should be associated and subsequently prevent rapid breakdown of these tolerant genotypes.

CHAPTER SIX

SPATIAL AND SEASONAL OCCURRENCE OF PENTALONIA NIGRONERVOSA AND ITS INFLUENCE ON BANANA BUNCHY TOP VIRUS SPREAD IN BURUNDI

Abstract

Banana bunchy top disease (BBTD) management strategy in large-scale banana plantations consists of prompt uprooting of infected mats treated with insecticides/soapy solutions to prevent spread of the vector when uprooting and use of disease-free planting material when establishing plantations. The use of insecticides is not accessible to small-scale farmers in Burundi and a clear policy based on collective teamwork to uproot infected mats is lacking. This study on BBTD epidemiology was carried out in the context of small-scale farms in Burundi for an integrated management approach. Banana trials were established in farmers' fields considering different plot locations, while spatial and seasonal occurrence of aphid vectors was evaluated at three different altitudes. In addition, serological tests were performed on banana leaf samples to confirm the presence and titer of the virus. The results show that BBTD incidence varies among grown banana cultivars and locations. At nine months after plot establishment, BBTD incidence ranging from 21.8 to 56.4% was observed in plots established within affected fields, while 0 to 12.3% was reported in plots located between 5 and 30 m away from affected banana fields. Aphid numbers were higher in the dry season. These aphids were able to acquire and transmit the virus irrespective of altitude. A mean incubation period of

21 and 84 days was observed at low (780masl) and high (2090masl) altitudes, respectively. Thus, a holistic approach taking into account banana variety, plot location, disease-free planting material and regular field sanitation should be promoted for long term BBTD management.

6.1. Introduction

Banana bunchy top disease (BBTD) is one of the most economically important diseases in many banana producing areas of Africa, Asia and the South Pacific (Furuya *et al*., 2005; Hooks *et al*., 2009). Between 1913 and 1920, the banana growing industry in Australia was almost completely destroyed by the disease (Magee, 1927; Hooks *et al*., 2009). In the 1990s, the first severe outbreak of BBTD in Africa was estimated to have reduced the banana production in the Nkhatabay and Nkhotakota districts of Malawi from 3,500 ha to about 800 ha (Soko *et al.*, 2009; Kumar *et al.,* 2011). Although accurate estimates of yield losses are lacking for the Great Lakes countries of Africa, about 90% yield loss has been reported in severely BBTD-infected plants of susceptible varieties such as 'Poyo', AAA-Cavendish in a screening trial conducted in the Rusizi valley, in Burundi (Niyongere *et al*., 2011a). Due to the high destructive potential of the disease, banana bunchy top virus (BBTV) was listed as one of the world's 100 worst invasive species and the International Plant Protection Convention included it as a pathogen to be subjected to rigorous quarantine measures (IPPC, 2010; Kumar *et al.*, 2011).

BBTD is dispersed over long distances through the exchange of infected suckers and/or through non-virus indexed tissue culture (TC) derived plantlets (Thomas and

Caruana, 2000; Kumar and Hanna, 2008). Through these propagules, the virus is introduced into new areas where *Pentalonia nigronervosa* occurs (Almeida *et al*., 2009). The banana aphid, *P. nigronervosa* Coquerel (Hemiptera, Aphididae), which is the known vector of BBTV transmits the virus while feeding on banana plants.

The aphid was initially reported to have occurred on member species of the Zingiberaceae and Araceae families, but morphological and morphometric studies confirmed that these aphids were in fact a separate species (*P. caladii* van der Goot) (Bhadra and Agarwala, 2010; Foottit *et al.*, 2010). *P. nigronervosa* Coquerel was, therefore, confirmed to have high host specificity to *Musa* spp., and has been found in almost all banana growing countries (Kumar *et al.*, 2011). The life span of an aphid ranges from 19 to 26 days and during this period the aphid will produce up to 20 offsprings in optimal conditions of 24 to 28°C (Völkl *et al.*, 1990; Yasmin *et al.*, 1999). The aphid acquires the virus after at least 4 hours of feeding on an infected plant and retains BBTV throughout its adult life but does not transmit it to its progeny (Nelson, 2004). The winged aphids, which often develop after 7 to 10 generations of wingless individuals, are most likely responsible for the spread of the virus (Nelson, 2004; Young and Wright, 2005). The winged aphids can transmit the virus to a healthy banana plant by feeding on it for as little as 15 min to about 2 hours (Dale, 1987, Hu *et al*., 1996). Ambient temperatures above 14°C enable aphid flights (Jones *et al.*, 2010). Aphids are more frequently observed near the base of banana plants, in between leaf sheaths, and at the base of the youngest unfurled leaf (Robson *et al.*, 2006). In addition, Young and Wright (2005) reported a spatial edge-effect, with larger aphid colonies observed at the edge of plantations. Yellow traps filled

with soapy water are generally used to collect and monitor winged aphids (McCartney and Fitt, 1985).

In contrast to other banana diseases such as Xanthomonas wilt (Karamura and Tinzaara, 2009); BBTV cannot be transmitted mechanically through garden tool use (Wardlaw, 1961; Kumar *et al.*, 2011).

The disease incubation period (i.e. till appearance of dark-green leaf streaks on the leaf lamina) varies from 19 days in the summer (25 to 33°C) to 125 days in the winter (June to August with -2 to 11.5°C) in Australia (Magee, 1927; Allen, 1987). An incubation period of 25 to 85 days (23 to 29°C) has also been reported in Hawaii (Hooks *et al.,* 2008). Previous research showed that this incubation period, after screenhouse inoculation, is positively correlated with the age of the host plant and negatively correlated with the number of virulent aphids feeding on the plant (Robson *et al.*, 2006; Hooks *et al*., 2008). The incubation period is also influenced by banana variety, rate of leaf emergence or genetic differences between BBTV strains (Wu and Su, 1989; Robson *et al.*, 2006; Hooks *et al*., 2008). Madden *et al.* (2006) has defined an epidemic as "a change in disease intensity in a host population over time and space". In addition, McCartney and Fitt (1985) suggested that disease management needs an understanding of host plant (i.e. cultivar, type of planting material, growth); epidemic environments (i.e. temperature, rainfall, wind), and pathogen availability and dispersibility.

The exponential increase of BBTD was reported by Smith *et al.* (1998) and *P. nigronervosa*'s high transmission efficiency suggests that disease incidence can

increase rapidly in the absence of aphid population control (Hu *et al*., 1996, Robson *et al.,* 2006). The recommended strategies for controlling BBTD in different regions of the world include identifying and destroying virus-infected plants as early as possible, replanting with virus-free TC plants in isolated fields and controlling aphid-vector populations associated with banana plants year-round irrespective of the presence of the disease (Young and Wright 2005; Robson *et al.*, 2006; Hooks *et al.*, 2009). BBTD was reported to be more prominent at elevations below 1,300 meters above sea level (masl) in Eastern Democratic Republic of the Congo (DR Congo) (Walangululu *et al.* 2010). Although most BBTV infected mats can be found at the lowest elevations in the Rusizi valley (encompassing parts of Burundi, Rwanda and Eastern DR Congo), some infected banana mats were observed up to 1,700 masl in the hills surrounding the valley (Niyongere *et al.*, 2011b). Information is however lacking on disease epidemiology at lower and higher elevations in central Africa. Indepth knowledge of BBTD epidemiology is required for developing an effective management strategy in the small-scale farming system context of the Great Lakes region of Africa. The purpose of the current study was to understand BBTD epidemiology across different agro-ecologies and altitudes in Burundi. On farm and greenhouse trials were established to assess: BBTD epidemiology at different altitudes, the aphids' ability to acquire and transmit BBTV at low and high altitudes and the optimal plot location taking into account distance from a diseased field and wind direction for establishing new plantations with minimum risk of early reinfection.

6.2. Materials and Methods

6.2.1. Assessing influence of altitude on BBTD spread at Benga and Kagazi

The possible effect of altitude on BBTD spread was assessed at two BBTD-affected locations in Burundi. The first site was at Benga in Isale commune, Bujumbura rural western province located in a hilly region at 1,268 masl, while the second site was at Kagazi, Cibitoke commune, north-western province which is a flat valley region located at 893 masl. 'Yangambi Km5' (*Musa* AAA genome, beer) is mainly intercropped with beans and maize in Kagazi, while more dense plantations of East African highland banana (AAA-EA, beer type) and 'Gros Michel' (AAA, dessert) in association with fruit trees dominate in Benga. Host farmer selection criteria included: those located in an affected area, easy access to selected plots and willingness to collaborate in the trials. Four plots were established at each location and monitored over a 24-month period (December, 2007 to December, 2009).

Each plot had 3 banana varieties namely the cooking variety 'Nyambururu' (AAA-EA), and the dessert varieties 'Kamaramasenge' (AAB) and 'Grande Naine' (AAA). Three tissue culture (TC) plantlets and 3 suckers per variety were planted in each plot. Hence a total of 72 TC plantlets and 72 suckers were established across both sites. These planting materials were obtained from the *Institut de Recherche Agronomique et Zootechnique* (IRAZ) nursery (where TC plantlets were hardened) and from the IRAZ banana field collection (source of suckers). The IRAZ Mashitsi research station, Gitega, central province is known to be located in a BBTV-free zone. Plant spacing was 3 and 2 m inter and intra-row, respectively. Trial plots were

established at Benga and at Kagazi in sites which were approximately 5 to 10 m from an existing field containing BBTV-infected plants. Weeding was regularly carried out using a hand hoe, while no organic or inorganic fertilizers were applied to the plants during the trial. BBTD-infected plants displaying symptoms were monitored at weekly intervals. The monthly average incidence levels were calculated as proportion of infected plants over a total number of tested banana plants per location.

Preliminary results showed that the distance between existing infected plants and new established plants influences reinfection rates in newly established plots. Therefore, further studies were undertaken to determine the influence of the distance between a new established plot and an existing affected banana field on disease spread.

6.2.2. Influence of plot location on disease incidence and aphid population

The BBTD spread and aphid populations were assessed for a period of 9 months between April and December 2009 in two rural villages (890-895 masl) located along the third and fourth roads of Rugombo village, Cibitoke commune, north-western province, Burundi. Six treatments were established according to the distance and the location (south or north, which predominantly corresponds to upwind or downwind) from existing BBTD-affected banana plantations. The treatments comprised plots located (i) inside an affected banana plantation where infected mats had not been uprooted, (ii) inside an affected banana plantation where infected mats had been uprooted and all plant debris was removed from the field immediately after uprooting, (iii) approximately 5 m outside and north from an affected banana

plantation, (iv) at 30 m outside and north from an affected banana plantation, (v) approximately 5 m outside and south from an affected banana plantation and (vi) at 30 m outside and south from an affected banana field. The first two treatments are the most common farmers' practices in the region.

Two plots were established at each of the 6 locations, and there were thus a total of 12 plots in the trial. In each plot, banana varieties 'Americani' (AAA, Cavendish), 'Gisandugu' (ABB), 'Kamaramasenge' (AAB) and 'Yangambi Km5' (AAA) were planted. These varieties were in the form of TC plantlets sourced from the IRAZ nursery and corresponding suckers were obtained from the banana field collection of IRAZ. In addition, suckers of local 'Yangambi Km5' (AAA), sourced from BBTD-free fields at Rugombo, Cibitoke north-western province, were confirmed to be BBTV free after ELISA testing and were used as a local control for the disease incidence. Each plot measured 15 by 12 m and plant spacing was 3 and 2 m inter and intra-row, respectively, while planting holes measured 40 x 40 x 40 cm. Three 3-month old TC plantlets and three suckers of each test variety, as well as six suckers of the local control 'Yangambi Km5', were planted per plot. Thirty six TC plantlets and 36 suckers per test variety plus 72 suckers of the control 'Yangambi Km5' were planted, making a total of 144 TC plantlets and 216 suckers. The fields were maintained according to farmers' practices (i.e. no organic or inorganic fertilizer was used; a hand hoe was used for weeding).

The BBTD spread was assessed weekly, based on disease symptoms using the scale (chapter three), for a period of 9 months. In addition, aphids were counted on all plants bi-monthly throughout the trial. The BBTV presence was confirmed using the

commercial AGDIA kit (France) for triple antibody sandwich enzyme-linked immuno sorbent assays (TAS-ELISA) in symptomatic plants and surrounding asymptomatic plants from the six plot locations.

6.2.3. Seasonal and spatial distribution of banana aphids

Winged aphid populations were monitored using yellow water traps in order to determine their distribution in three different banana growing ecologies in Burundi. The possible seasonal effect on aphid counts over a one year period (January 2009 to December 2009) was analysed using available data on temperature and rainfall. Two yellow water traps were positioned within banana plots at each of the three sites namely (i) Kagazi, Cibitoke north-western province (890 masl), (ii) Benga, Bujumbura Rural western province (1,268 masl) and (iii) the Mashitsi-IRAZ regional banana field collection, Gitega central province (1,645 masl).

The yellow water traps were also placed in a banana plantation at the Mparambo (898 masl) ISABU agricultural research station, Rusizi valley, Cibitoke province. Two traps were positioned inside the banana field, while another two traps were positioned southwards at both 30 and 50 m away from the banana field, respectively, to assess the spatial distribution of aphids in and around the banana field. The aphids were collected during two consecutive years (September 2007 to September 2009).

At all sites, the trapped winged aphids were collected from traps three times per week on Mondays, Wednesdays and Fridays. These aphids were then preserved in bottles containing 70% ethanol pending formal identification and enumeration using

a dissecting microscope at the Entomology laboratory of ISABU, Bujumbura, Burundi.

6.2.4. Assessing the effect of altitude on BBTV transmission rates

The BBTV transmission rates were assessed at two different altitudes in Burundi. The first location was the ISABU headquarters in Bujumbura (780 masl; with average min and max temperatures of 17 and 32°C, respectively), while the second location was at Gisozi, ISABU research station (2,090 masl; with average min and max temperatures of 6 and 25°C, respectively).

Banana aphids were collected in farmers' fields at Rugombo, Rusizi valley, Cibitoke, north-western province (890 masl, average temperature of 25°C) which is highly affected by bunchy top and at the IRAZ banana field collection at Mashitsi (1,645 masl, average temperature of 19°C) in Gitega, central province, located in a BBTD-free zone.

Hardened three-month old TC plantlets of 'Intobe' (AAA-EA) variety were obtained from the IRAZ nursery. Six virus transmission trials were conducted. The first two direct transmissions were carried out as follows:

(i) Using aphids from IRAZ-Gitega on TC plantlets at Bujumbura to verify if the Gitega aphids carry the virus and

(ii) Using aphids from Cibitoke on TC plantlets at Bujumbura to confirm if the Cibitoke aphids carry the virus.

For the indirect virus transmission, aphids were fed in both Bujumbura and Gisozi for 24 hours on BBTV-infected plants prior to their introduction to the test plantlets. This experiment was conducted in order to assess if aphids are able to acquire and transmit the virus at different altitudes. These indirect transmissions were carried out as follows:

(iii) Using aphids from Cibitoke on TC plantlets at Bujumbura;

(iv) Using aphids from Cibitoke on TC plantlets at Gisozi;

(v) Using aphids from IRAZ-Gitega on TC plantlets at Bujumbura and

(vi) Using aphids from IRAZ-Gitega on TC plantlets at Gisozi.

All aphids used in the virus transmission trials were killed, after an inoculation access period of 48 hours, using a systemic insecticide (dimethoate) in order to avoid aphid spread.

Six 'Intobe' TC plantlets were used in each of the trials, giving a total of 24 TC plantlets in four trials carried out at Bujumbura, while 12 TC plantlets were used in two trials conducted at Gisozi. At each location, 6 control plants (no inoculation) were added. The transmission trials entailed the placing of twenty aphids in between the two youngest leaf petioles of each test TC plantlet, making a total of 720 aphids.

Visible BBTD symptoms and leaf emergence rates were recorded at weekly intervals over a four-month period (April-July, 2009) in order to determine the incubation period at different altitudes. In addition, 36 banana leaf samples were collected on BBTV-infected (i.e all 6 leaf samples for each of the 5 treatments making a total of 30 samples) and control (i.e 6 leaf samples; 1 leaf sample per treatment) TC plantlets for BBTV detection using TAS-ELISA.

6.2.5. Detection of BBTV using TAS-ELISA

The triple antibody sandwich enzyme-linked immuno sorbent assay (TAS-ELISA) was used to detect the presence of BBTV in banana leaf samples and determine the virus concentration. Virus detection was carried out using coating IgG polyclonal antibodies, general extract buffer (GEB), AB conjugate antibodies and substrate buffer 1X (1mg of pNPP/ml of substrate buffer) as per the instructions supplied with the kit. The characteristic yellow coloration was observed for positive samples, while virus concentration of each sample was determined at 405 nm using a spectrophotometer. These experiments were conducted in the pathology laboratory, Department of Crop Production, ISABU headquarters, Bujumbura. The optical densities (OD) at 405 nm of absorbance were obtained using a spectrophotometer based in the biotechnology laboratory of the *Faculté des Sciences Agronomiques du Burundi* (FACAGRO). The optic density (OD) of 0.1 was considered as a limit between positive and negative samples.

6.2.6. Climate data

Temperature and rainfall data for the period 2007 to 2009 were obtained from the meteorological stations of the *Institut Géographique du Burundi* (IGEBU) located at Bujumbura airport, Gitega airport and the Mparambo ISABU research station which are representative for the study sites located at Bujumbura rural, Gitega and Cibitoke provinces, respectively. Monthly averages were calculated (Table 6.1). These data were used in analysis of seasonal effect on aphid counts along a 12 month-period.

Table 6.1. Average monthly rainfall and temperature data (calculated with 2007, 2008 and 2009 data) collected at Bujumbura airport, Gitega airport and at the ISABU Mparambo research station in Burundi.

	Bujumbura airport		**Gitega airport**		**Mparambo**	
Months	**[a]Rainfall(mm)**	**[b]T°C**	**Rainfall(mm)**	**T°C**	**Rainfall(mm)**	**T°C**
January	134.3	24	108.3	19.79	122.5	24
February	137.1	24.3	132.8	20.08	132.4	24.3
March	196	24.2	196	20.35	115.5	24.2
April	212	24	78.3	19.58	117.2	24.3
May	110	24	83.5	19.86	44.1	23.9
June	20.9	24	0	19	33.8	23.1
July	7.2	23.5	1.8	18.89	22.5	23.4
August	14.4	24	0	20.06	4.7	23.1
September	53.5	25	55.8	20.83	22.3	25
October	116	27	68.3	20.2	83.4	25
November	172	24.6	222.5	19.77	125.8	24.6
December	181.6	24.1	90.5	20.12	119.2	24.1

[a]Rainfall measured in mm and [b]T°C: average temperatures in degrees Celsius per month for each locality. The rainfall and temperature data were obtained from the *Institut Géographique du Burundi* (IGEBU) stations located at the Bujumbura airport and the Gitega airport. In addition, climatic data was also collected at the *Institut des Sciences Agronomiques du Burundi* (ISABU) Mparambo research station. These climatic data collection sites are representative of our study sites located in Bujumbura rural, in Gitega and in Cibitoke provinces, respectively.

6.2.7. Data analysis

BBTD spread was evaluated by assessing the effect of location, variety and type of planting material (TC plantlets or suckers). The data collected on BBTD symptoms were converted into 1 for presence or 0 for absence. A logistic regression model was then fitted to assess the effect of these variables on disease occurrence.

The model fitted was

$$\log_e\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots$$

Where p is the probability that the event occurs (that is disease presence): 1-p is the probability that the event does not occur; β_0 is intercept and $\beta 1_,\ \beta_{2,\ldots,}$ are regression coefficients of $x_{1,}\ x_{2\ldots}$ explanatory variables, respectively.

The counts of aphids were analyzed using the Poisson regression model which expresses the natural logarithm of the event as a linear function of predictors. The model was $\log_e (Y) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 \ldots$ where Y is the mean of the response variable and $\beta_{0,}\ \beta_{1,}\ \beta_{2,\ldots}$ are the regression coefficients and $x_{1,}\ x_{2\ldots}$ are the predictors. These models take randomly references per each category of parameters in comparison.

Data analyses were carried out using GenStat 12th edition VSN Int (VSN International ltd., 2009), while figures were drawn using Microsoft office Excel 2007.

6.3. Results

6.3.1. BBTD spread within banana farms at Benga and Kagazi in Burundi

Regression analysis indicated that BBTD incidence significantly varied ($p<0.001$) according to trial location, banana variety and planting material type (i.e suckers and tissue culture-derived plantlets) (Appendix 6.1). Visible disease symptoms were first observed at two to three months after trial establishment at Benga compared to four months at Kagazi. Two years after trial establishment, tissue culture–derived plants of all tested varieties showed a disease incidence ranging from 35 to 40% at Benga and 10 to 35% at Kagazi. At 24 months, sucker-derived plants showed a BBTD incidence at Benga ranging from 30 to 50% and from 20 to 50% at Kagazi. All tested genotypes showed disease symptoms although 'Nyambururu' (AAA-EA) was least infected for both planting material types in Kagazi and for sucker-derived plants at Benga (Fig. 6.1).

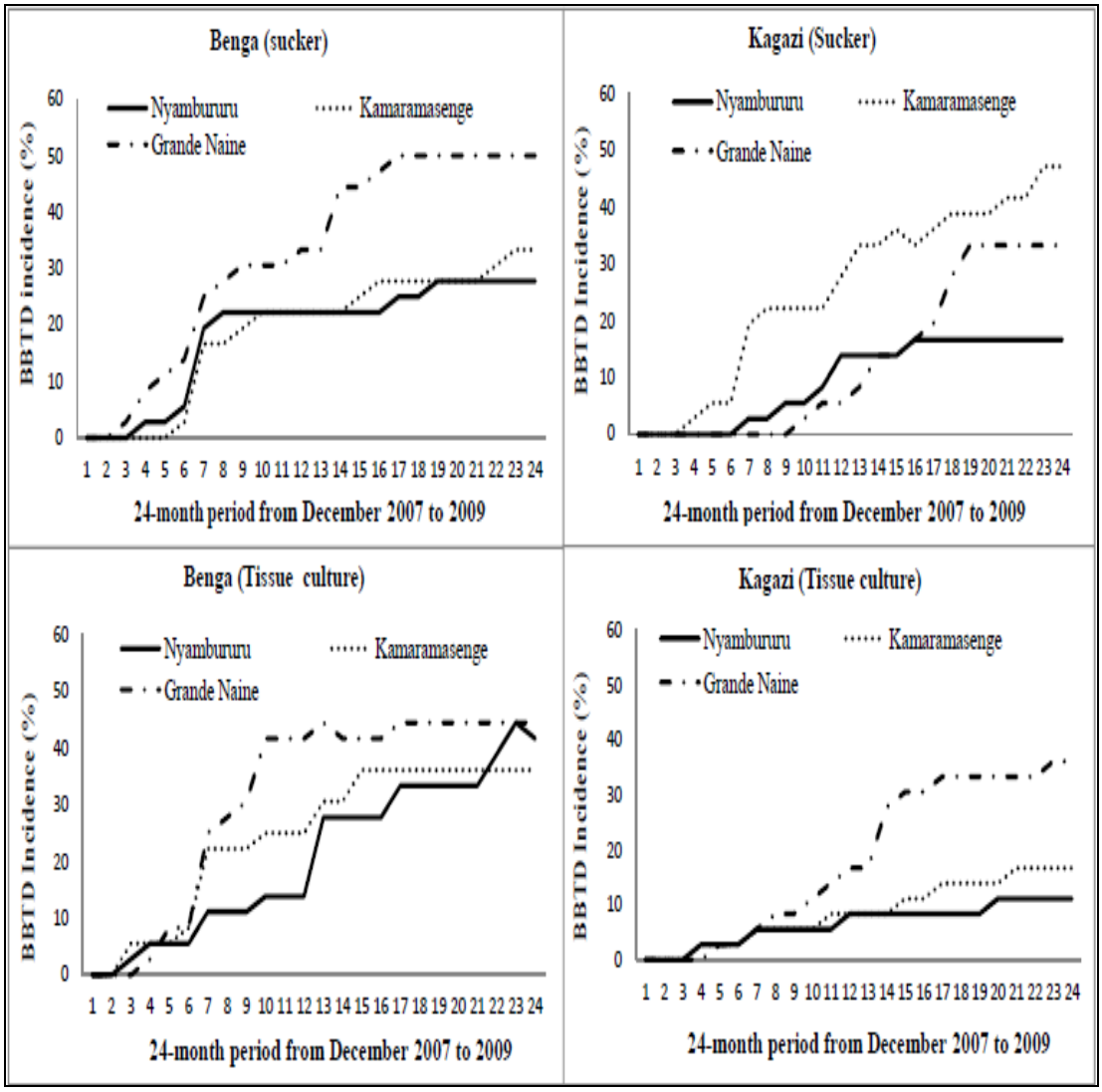

Fig. 6.1. Banana bunchy top disease incidence as influenced by type of planting material for the three banana varieties planted at Benga and Kagazi in Bujumbura rural and Cibitoke provinces in Burundi, respectively, over a 24 month-period.

6.3.2. Influence of plot location on BBTD spread and aphid occurrence

BBTD spread as influenced by plot location

During the first two months, the plots located inside the infected banana fields attained a disease incidence of about 10%, whereas no BBTD symptoms were reported within the plots located outside the affected banana fields. At nine months after trial establishment, BBTD incidence had increased and was higher in the plots

located inside a banana plantation where infected mats had been uprooted (56.4 %), while a 21.8% incidence was observed in plots located inside affected banana fields where infected mats had not been uprooted (Table 6.2). BBTD incidence was 6.0% and 12.3% in plots established outside and north, at 5 m and at 30 m, respectively, from an affected banana plantation. On the other hand, a disease incidence of 9.9% was observed in plots located at 5 m outside and south from an affected banana field. The banana plants established at 30 m outside and south from affected plantations did not show any disease symptom up to nine months after trial initiation. Among the grown *Musa* varieties, 'Americani' (AAA) showed a BBTD incidence ranging from 0% to 15.9%, while 'Gisandugu' (ABB) and Kamaramasenge (AAB) showed a disease incidence ranging from 0% to 7.3% at 9 months after trial establishment. The TC-derived 'Yangambi Km5' (AAA) showed a disease incidence ranging from 0% to 19.9%, while the sucker-derived 'Yangambi Km5' plants (control) had a disease incidence level ranging from 0% to 7%. This difference could be explained by a higher susceptibility of young 'Yangambi Km5' TC plantlets to BBTV infection as compared to the more robust sucker-derived 'Yangambi Km5' plants (Table 6.2).

Table 6.2. BBTD incidences observed in six different treatments at 9 months after trial establishment.

Genotypes	Disease incidence (%) according to plot location[#]					
	i	**ii**	**iii**	**iv**	**v**	**vi**
Americani (AAA,Cavendish)	13.2	15.9	2	1.3	6	0
Gisandugu (ABB)	0.7	7.3	0	0	0	0
Kamaramasenge (AAB)	3.3	7.3	0	4	2.6	0
Yangambi Km5 (AAA)	2.6	19.9	2	0	0	0
Sucker-derived Yangambi Km5 (AAA)	2	6	2	7	1.3	0
Total BBTD incidence	21.8	56.4	6.0	12.3	9.9	0

Plot located (i) inside a BBTD-affected field where BBTV infected mats had not been uprooted, (ii) inside a BBTD-affected field where BBTV-infected mats had been uprooted, (iii) at 5 m outside and downwind from a BBTD-affected field, (iv) at 30 m outside and downwind from a BBTD-affected field, (v) at 5 m outside and upwind from a BBTD-affected field and (vi) at 30 m outside and upwind from a BBTD-affected banana plantation.

BBTV was confirmed, using TAS-ELISA, in 85 out of 225 leaf samples collected from banana plants in all the six plot locations. The tests confirmed the presence of BBTV in all plot locations. In addition, 24% of leaf samples collected in plots located at 30 m outside and south of an existing infected field, where no BBTD symptoms had been reported, were BBTV-infected (Appendix 6.2). Intense yellow coloration of positive samples, with OD ranging from 0.8 to 2.18, was obtained for samples collected on plants showing marginal leaf chlorosis to bunchy top appearance (i.e disease severity stages 3 to 5), while OD values ranging from 0.1-0.8 were measured on samples taken from plants showing dark green streaks on their leaf lamina (i.e: disease severity stage 1 or 2) (Niyongere *et al*., 2011a).

Aphid counts in different plot locations relative to existing infected fields

Plot location and season affected the aphid numbers on plants in the six treatments. In the first month (April 2009) of the trial, aphid numbers were highest in plots established inside a banana plantation and north from an existing banana plantation.

On the other hand, few aphids were reported during the first five months of the trial in plots located outside and south from a banana plantation. The aphid counts were also lower in September and October which coincides with the beginning of the rainy season with highest rainfall in Burundi (Table 6.3).

Table 6.3. Monthly aphid numbers counted on all plants in the six different plot locations.

Monthly aphid counts	**Aphid numbers per month and plot location[#]**					
	i	**ii**	**iii**	**iv**	**v**	**vi**
April	736	198	209	98	0	0
May	529	105	51	0	41	4
June	237	61	26	65	0	0
July	185	1	11	155	0	0
August	286	247	198	72	0	96
September	31	22	79	42	16	13
October	24	5	2	127	50	53
November	2	6	125	33	121	112
December	11	4	26	1	408	164
Total	2041	649	727	593	636	442

Plot located (i) inside an affected field where BBTV infected mats had not been uprooted, (ii) inside an affected field where BBTV-infected mats had been uprooted, (iii) at 5 m outside and downwind from a BBTD-affected field, (iv) at 30 m outside and downwind from a BBTD-affected field, (v) at 5 m outside and upwind from a BBTD-affected field and (vi) at 30 m outside and upwind from BBTD-affected banana plantation.

Regression analysis showed that aphid numbers significantly varied ($P<0.05$) with plot location and season. The low aphid counts in September and October depends on the increased rainfall and corresponding lower temperatures which negatively affect the dynamics of aphid populations (Table 6.4).

Table 6.4. Aphid numbers as influenced by plot location and weather conditions.

Parameter	Estimate	s.e.	t(40)	t pr.	Antilog of estimate
Constant	6.210	0.368	16.86	<.001***	497.8
Plot location (ii)	-1.146	0.501	-2.29	0.027*	0.3180
Plot location (iii)	-1.236	0.518	-2.38	0.022*	0.2905
Plot location (iv)	-1.032	0.480	-2.15	0.038*	0.3562
Plot location (v)	-1.530	0.583	-2.62	0.012*	0.2166
Plot location (vi)	-1.166	0.504	-2.31	0.026*	0.3116
May	-0.531	0.518	-1.02	0.312NS	0.5882
June	-1.160	0.646	-1.80	0.080NS	0.3135
July	-1.260	0.671	-1.88	0.068NS	0.2836
August	-0.322	0.487	-0.66	0.511NS	0.7244
September	-1.810	0.841	-2.15	0.037 *	0.1636
October	-1.559	0.757	-2.06	0.046 *	0.2103
November	-1.135	0.639	-1.77	0.084NS	0.3215
December	-0.704	0.548	-1.28	0.206NS	0.4948

Plot location-i and April were randomly taken as references among locations and season (months) factors, respectively. NS, *, ***: Not significant, significant (p=0.05) and highly significant at P=0.001 (Fisher's test), respectively.

6.3.3. Seasonal and spatial distribution of banana aphids

Aphids sampled in yellow traps at three different agro-ecological zones: Banana aphids were observed in all the three study regions in Burundi, irrespective of altitude and the presence of the disease. The highest aphid numbers (average yearly count of 904 winged aphids) were reported at Benga (1,268 masl), where dense AAA-EA banana plantations are located. Far lower aphid numbers (average yearly count of 256 winged aphids) were observed at Kagazi which is located at 890 masl and where 'Yangambi Km5' is cultivated in a less dense intercropping system. The lowest aphid numbers were reported in the well maintained *Musa* collection at IRAZ, Mashitsi (1,645 masl) with an average yearly count of 234 winged aphids**.**

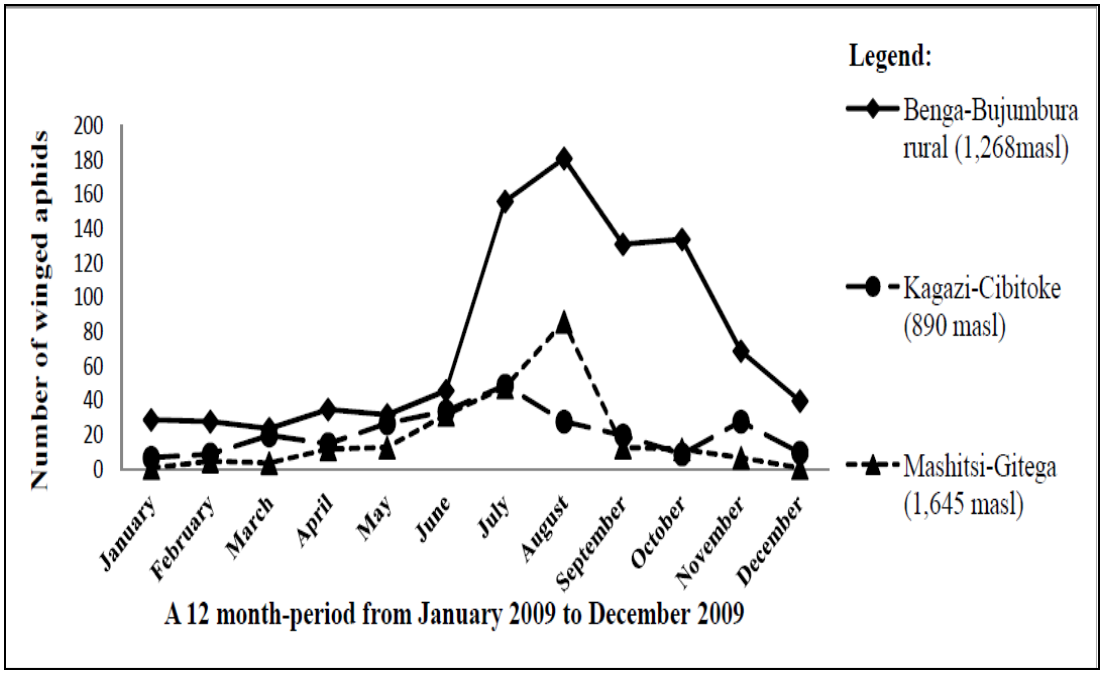

Fig. 6.2. Seasonal and spatial distribution of *Pentalonia nigronervosa* based on monthly averages of winged aphids, collected in six yellow water traps (two per location), positioned within banana fields, at three different altitudes of Benga, Kagazi and Mashitsi in Burundi, during a 12 month-period from January 2009 to December 2009.

The winged aphid numbers fluctuated throughout the year within plantations and were significantly ($p<0.05$) influenced by the prevailing temperature (Table 6.5). The highest winged aphid numbers were recorded in the period June to August which corresponds to the dry season in Burundi (Fig. 6.2). This period is characterised by higher average temperatures (24 to 32°C) in addition to the absence of rainfall. In addition, banana management activities come to a standstill during the dry season. Deleafing and desuckering are not practiced leading to bushy banana mats and clusters, creating an ideal breeding ground for aphids. In contrast, deleafing and desuckering are carried out during the rainy season to provide more light for the intercropped legumes.

Table 6.5. The effect of rainfall and temperature on aphid numbers collected in yellow water traps at three different ecologies in Burundi.

Parameter	Estimate	s.e.	t(20)	t pr.	Antilog of estimate
Constant	-7.94	5.31	-1.50	0.150NS	0.00035
February	0.008	0.61	0.01	0.989 NS	1.008
March	0.281	0.628	0.45	0.66 NS	1.324
April	0.609	0.577	1.06	0.303 NS	1.839
May	0.566	0.564	1	0.327 NS	1.761
June	0.934	0.687	1.36	0.189 NS	2.544
July	1.859	0.698	2.66	0.015*	6.416
August	1.786	0.678	2.64	0.016*	5.968
September	0.779	0.623	1.25	0.226 NS	2.18
October	0.238	0.737	0.32	0.75 NS	1.269
November	0.911	0.553	1.65	0.115 NS	2.488
December	0.317	0.588	0.54	0.596 NS	1.373
Rainfall	-0.00288	0.00413	-0.7	0.494 NS	0.9971
Temperature	0.477	0.217	2.2	0.040*	1.611

January was randomly taken as reference among months. NS, *: not significant and significant at P=0.05 (Fisher's test), respectively.

Aphids sampled in yellow water traps located inside and outside a banana field

Yearly aphid numbers collected in yellow water traps which were positioned inside a banana field, and at 30 and 50 m outside and south from a banana plantation were 207, 69 and 27, respectively. The number of aphids was inversely proportional to the distance from a banana plantation, with an estimate coefficient of 0.33 at 30 m compared to 0.13 at 50 m from a banana plantation (Fig. 6.3)

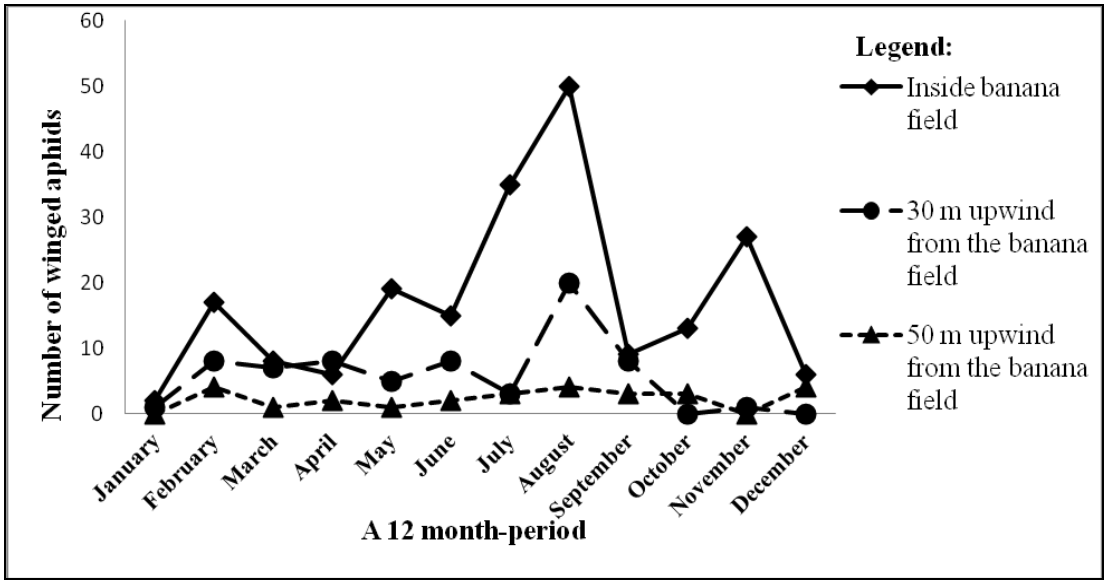

Fig. 6.3. Monthly average numbers of winged aphids, collected in six yellow water traps (with two traps per location) positioned inside a banana field, and at 30 and 50 m outside and upwind from a banana field at the ISABU Mparambo research station in Burundi.

The aphid numbers varied over a period of a year and the differences were highly significant ($p<0.001$) at 30 m as well as at 50 m from a banana plantation. This may be explained by the significant ($P<0.05$) influence of rainfall on winged aphid numbers (Table 6.6).

Table 6.6. Effect of distance from a banana plantation and rainfall on aphid numbers at the ISABU Mparambo research station.

Parameter	estimate	s.e.	t(31)	t pr.	Antilog of estimate
Constant	10.46	4.89	2.14	0.041*	34890
Traps located at 30m	-1.099	0.279	-3.94	<.001***	0.3333
Traps located at 50m	-2.037	0.411	-4.96	<.001***	0.1304
Rainfall	-0.00691	0.00297	-2.33	0.027*	0.9931

*, ***: Significant and highly significant at $P<0.05$ and $P<0.001$(Fisher's test), respectively.

6.3.4. Assessing the effect of altitude on BBTV transmission rates

BBTV transmission using aphids from non-affected banana field

A BBTD incidence of 0% was reported at Bujumbura on TC plantlets when aphids collected from the *Musa* collection at IRAZ were allowed to feed on them. This proves the absence of BBTV in the IRAZ banana field collection. However, virus transmission trials using aphids collected at the IRAZ banana field collection and allowed to feed for 24 hours on BBTV-infected plants, resulted in BBTD incidence levels of 83% and 50% at Bujumbura and Gisozi, respectively. Although at different rates, these aphids were thus able to acquire and transmit the virus independently of altitude.

BBTV transmission using aphids from BBTD-affected banana fields

A BBTD incidence of 100% was observed at Bujumbura on TC plantlets infected with aphids obtained from affected farmers' fields at Cibitoke. This confirmed that aphids collected at Cibitoke carry the BBTV. The transmission rate when aphids collected from farmers' fields at Cibitoke had been allowed feeding access of 24 hours on BBTV-infected plants was 83% and 67% at Bujumbura and at Gisozi, respectively.

BBTD incubation period at Bujumbura and at Gisozi

The mean incubation period of the disease was 21 days at Bujumbura (780 masl; average min and max temperatures: 17-32°C) versus 84 days at Gisozi (2,090 masl;

average min and max temperatures: 6-25°C). BBTD symptoms were consistently observed on the second newly emerging leaf. The observed slower growth of banana plants and corresponding reduced leaf emission rate at higher elevations translates to a longer incubation period. The first observed symptoms on BBTV-infected TC plantlets at Bujumbura as well as at Gisozi were dark green dots and dashes of variable length on the leaf veins and dark green streaks on leaf midribs and petioles. This is in line with previous observations made by Magee (1927) and Thomas *et al.* (1994). More advanced symptoms included leaf marginal chlorosis, dwarfing of leaves and upright and crowded leaves at the apex of the plant giving the plant a bunchy top appearance (Magee, 1927).

All the 30 samples obtained from infected TC plantlets tested positive, while the 6 samples collected from the control plants tested negative in TAS-ELISA.

6.4. Discussion

In this chapter, we determined the epidemiology of BBTD at different altitudes, the aphids' ability to acquire and transmit BBTV at low and high altitudes, as well as the effect of plot location relative to an existing diseased field and wind direction on the rates of re-infection.

The higher disease incidence observed at Benga can be attributed to the presence of a large number of winged aphids (904) compared to Kagazi (256 trapped aphids). The larger number of winged aphids observed in Benga could result from the more densely cultivated banana (AAA-EA) production system, compared to the less dense 'Yangambi Km5' dominated system in Kagazi.

BBTD incidence also varied according to plot location with a higher disease incidence (56.4%) observed in plots located inside an affected banana plantation where infected mats had been uprooted. The observed high disease incidence could be explained by winged aphids which were disturbed during diseased mat removal and which eventually migrated to the newly established young plantlets. Young and Wright (2005) also reported the preference of aphids for newly established young plants. In addition, the removal of diseased mats resulted in an overall lower number of young suckers (i.e. lateral shoots) in the field and a less dense plantation which may have made it easier for winged aphids to locate the new young plantlets.

A low disease incidence (21.8%) was observed in plots located inside an infected banana plantation where infected mats had not been uprooted. Winged aphid populations may have been less disturbed in this plot type compared to plots where

all the infected mats were uprooted. Additionally, an overall higher number of lateral shoots which attract also winged aphids may have contributed to the observed lower incidence on the newly established plantlets. In addition, aphid spread was found to be influenced by plot position (i.e. north or south from an existing field) and distance between newly established plots and existing affected banana plantations. For example, no banana plants showed BBTD symptoms (0%) in plots located south and at 30 m away from an affected banana field at 9 months after trial establishment.

In both study areas, susceptibility to the disease varied according to the genotype used, which is in agreement with reports from India and the Philippines (Thomas and Caruana, 2000; Geering, 2009). Moreover, Bhadra and Agarwala (2010) reported that under field conditions, the population of aphid vectors grows in response to phenology of their respective host plants. Banana pseudostem color influences aphid preference (Robson *et al.,* 2006) which in turn contributes to variations in infection level.

In terms of seasonal and spatial distribution of the vector, winged aphids were found in all studied regions irrespective of altitude and the presence of BBTD. However, aphid colonies were significantly influenced by climate conditions. The highest winged aphid numbers were reported during the dry season in Burundi. Cultural practices such as deleafing and desuckering, usually practiced during the rainy season, should preferably be continued during the dry season (C. Niyongere and G. Blomme, person. commun.).

In addition, winged aphid numbers were significantly but negatively influenced by rainfall, which is in agreement with Young and Wright (2005) who stated that in Hawaii, aphid numbers were negatively correlated with rainfall in less dense canopy conditions. It is postulated that reduced leaf canopy coverage will allow raindrops and runoff water to negatively affect the aphid populations on the banana pseudostems. In our study, the aphids were observed in traps located up to 50 m from an existing banana plantation. Although, Allen (1987) stated that more than 60% of new infections occur within 20 m from the nearest source of infection, the vector could potentialy spread the BBTD way further from the source of infection.

In the present study, BBTV transmission trials carried out at two different altitudes revealed that aphids collected in healthy fields and subsequently reared on BBTV-infected plants were able to acquire and transmit the virus at Bujumbura (780 masl) as well as at Gisozi (2,090 masl). Thus, although BBTD is currently mainly reported at below 1,300 masl (Walangululu *et al*., 2010), our study indicates the possibility for disease spread to higher altitude regions. The TC plantlets infected at Bujumbura (780 masl) showed the first BBTD symptoms after 21 days, while for those infected at Gisozi (2,090 masl) the mean incubation period was 84 days. The first BBTD symptoms were observed on the second newly emerging leaf after inoculation and a lower leaf emergence rate was observed at the higher altitude site, which may be one of the factors that led to a delayed symptom expression. Although the observed virus transmission rate was lower at the high altitude site, climate change (i.e. higher temperatures) and the existence of dense and poorly maintained banana fields in Burundi may enhance the spread of the disease to higher elevation regions.

The TAS-ELISA tests confirmed BBTV in banana leaf samples and indicated virus presence in asymptomatic plants. These infected but asymptomatic plants could serve as a source of inoculum for further virus spread (Allen, 1987). Higher virus concentrations were reported in samples displaying advanced BBTD symptoms. As such, an effective management strategy is dependent on early visual detection and prompt uprooting of symptomatic plants as has been suggested by Magee (1938), Dale (1987) and Hooks *et al.* (2008).

Under suitable conditions, aphid vector populations rise progressively resulting in a gradual increase of diseased mats and corresponding inoculum levels (Magee, 1938; Hooks *et al.*, 2009). Once the disease has been introduced into an area, Robson *et al.* (2006) reported that eradication is very difficult. Hence, the use of virus-free planting material of tolerant varieties and new plot establishment at at least 30 m away from an existing diseased field should be adopted to prevent the early establishment of the virus and possible inoculum build up. In parallel, regular cultural practices (e.g. prompt uprooting of diseased mats, regular deleafing, desuckering and weeding) need to be enforced in order to maintain aphid colonies under the threshold at which winged aphids are produced and spread the virus (Kumar *et al.*, 2011).

In addition, quarantine measures should be established between BBTD-affected and non-affected areas (Thresh, 2003). Training of extension staff and farmers is paramount for an integrated management/control strategy under small-scale farmer conditions in Burundi.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

Banana bunchy top disease (BBTD) is the most important viral disease affecting banana production worldwide (Dale, 1987; Geering, 2009; IITA, 2010, Kumar *et al.*, 2011). In the GLRA, the disease was reported in 1958 in DR Congo and 1987 in Burundi and Rwanda (Wardlaw, 1961; Sebasigari and Stover, 1988). Since then, no studies have been conducted to determine the disease status in the region and therefore no management strategies have been developed.

The current study aimed to establish the occurrence of banana bunchy top virus (BBTV), the causal agent of BBTD, its vector, farmers' knowledge and source of resistance among banana and plantain cultivars grown in the GLRA. Such information is an important prerequisite before any remedial measures can be taken to prevent further spread of the disease (Daniells, 2009). The results showed high BBTD incidence and severity (>20%) in 5 out of the 6 surveyed localities in GLRA (Chapter three). High populations of winged and wingless aphids (*Pentalonia nigronervosa)*, the vectors of BBTV were found in all the surveyed banana fields. The high aphid populations could be attributed to poor maintenance of the banana plantations, where a mat had more than 10 suckers. The dense crop canopy presents suitable conditions for aphid multiplication resulting to higher spread of the disease (Young and Wright, 2005; Robson *et al*., 2006). Although farmers could recognize BBTD symptoms (90%), the majority (95%) were not aware of any disease management strategies including use of virus free planting material. In addition, they

reported that no cultivar was resistant to BBTD. The lack of awareness in using virus-free planting material and the common practice of uncontrolled movement of suckers across the region have greately contributed to the spread of the disease. Raising farmers' awareness on crop and disease management practices should be promoted in the GLRA.

The genetic status of BBTV isolates prevailing in GLRA (Chapter four) was also determined. The nucleotide sequence comparisons revealed that GLRA isolates belong to the South Pacific group which comprises isolates from Africa, India, Australia, Egypt and Fiji (Wanitchakorn *et al.*, 2000). Furthermore, the GLRA isolates were closer to the Indian isolate, suggesting that the most likely origin of the GLRA isolates could be India rather than other South Pacific countries. The presence of BBTD in GLRA was estimated at around 40 years. These GLRA isolates showed a higher number of nucleotide sequence substitutions as compared to other South Pacific isolates. Su *et al.* (2003) found that this genetic variability is related to the virus pathogenecity, while Amin *et al.* (2008) showed that this could affect negatively the resistance or tolerance of banana cultivars.

Although there are no known BBTD resistant banana cultivars, considerable variation among banana cultivars in their susceptibility to the virus has been reported (Daniells, 2000). Hence, in chapter five of this study, the varietal screening for BBTD resistance using 40 genotypes of different *Musa* subgroups was carried out. The results confirmed previous reports that genotypes with at least one B genome are less susceptible to BBTD (Dale, 1987; Stover and Simmonds, 1987). Except 'Highgate' (AAA- dessert) with A genome, other four varieties containing B genome

which include ‘Saba’ (ABB-cooking), ‘FHIA03’ (AABB-cooking/ dessert), and two plantains ‘Corne plantain’ (AAB-beer/ desert) and ‘Igjindi’ (AAB-cooking) showed some degree of tolerance to the disease. These five tolerant varieties were selected for adoption by farmers as component of an integrated BBTD management in context of small-scale farming system. The use of tolerant genotypes will however require the adoption of cultural practices in order to limit fast breakdown of resistance. Thus, as previously reported, a combination of varietal tolerance and a good sanitation programme may contribute towards an effective control of this virus (Ploetz and Pegg, 2000).

Furthermore, BBTD spread as influenced by the occurrence of the *P. nigronervosa* was assessed in the present study (Chapter 6). The results revealed that aphid populations increase significantly during the dry season of July to August. In parallel, previous findings by Wu and Su (1989) in Taiwan confirmed that virus concentration was highest in BBTV-infected leaves collected during the dry season and lowest in the leaves obtained during cold season. Therefore, it is important to adopt cultural practices which consist in control of aphid populations especially during the dry season.

Wind direction and distance from the source of inocula were noted as factors which influence the spatial spread of aphids. This could be attributed to the origin of aphid in the vicinity of the plantation, as Young and Wright (2005) also reported large numbers of banana aphids closest to the plantations’ edges. The banana plants located at 30 m upwind away from BBTD-affected banana fields were not displaying BBTV symptoms at nine months from plots establishment. Hence, small-scale

farmers should establish new banana plot at least 30 m upwind from affected banana plantations. In addition, it is important to practise good field sanitation in newly established plots and surrounding banana plantations such as reducing the number of plants at 3-4 per mat and regularly removing old leaves. This maintains aphid populations at low level and reduces BBTD spread between banana plantations.

The present results confirmed that BBTV can spread irrespective of altitudes and *P.nigronervosa* is associated with banana plantations. Moreover, higher virus concentration was observed in samples from banana plants displaying advanced BBTD symptoms. Therefore, the prompt eradication of BBTV-infected plants and the control of movement of banana suckers between areas growing banana should be collectively adopted in GLRA.

The BBTD remains a serious threat to banana production in most areas of the GLRA and persist to spread in the absence of any meaningful control measures. Integrated management of BBTD needs to take into account many factors including agro-ecological practices such as use of tolerant varieties, collective reduction of disease pressure, climatic factors; and socio-economic aspects such as availability and cost of indexed virus-free planting materials, as they relate to the general plant health (Blomme *et al.,* 2003; Mgenzi *et al.,* 2003). Therefore, farmers' awareness raising and the use of tolerant varieties campaigns should be carried out at the local community level. Additionally, quarantine measures ought to be established by policy makers to prevent virus spread in new areas not yet BBTD-affected. The proposed tolerant cultivars and these technologies which consist of aphids' control and plot's locations should be introduced for adoption and use by farmers.

From these results, future activities may be suggested to achieve effective integrated management of bunchy top virus in African Great Lakes region, such as (i) Raising awareness of all stakeholders including farmers with monitoring and effective management practices; (ii) data collection in different ecologies by public services (Every two weeks for at least 5 years) on primary infection and secondary importance of disease, farmers' practices, vector dynamic, weather conditions, disease intensity and yield loss relationships, for developing BBTD predictive models in small-scale agriculture system; (iii) molecular characterization using all the six BBTV components in order to confirm these findings on BBTV variability in African Great Lakes Region; and (iv) advocate for quarantine regulations and monitoring their application across countries and beween affected and non affected locations within African Great Lakes region.

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APPENDICES

Appendix 3.1. Questionnaire used during the survey

Objectives:

- To determine incidence and severity of BBTV in major banana growing areas in the three countries (Burundi, Rwanda and Eastern DR Congo).
- To assess the cultivars grown and existence of BBTD resistant cultivars
- To evaluate the knowledge of farmers on BBTD management

Data targeted

- BBTD incidence of the disease: proportion of infected mats over the total of surveyed mats
- BBTD severity: scored using the scale of BBTD from 1-5.
- Importance of *P.nigronervosa* populations: according the scale from 1-5
- Distribution of the disease (using GPS data)
- Farmers' knowledge on BBTD identification and management

Data collection: Number of the survey paper: |____|____||____|____|

1. Coordinates of the investigator

Name: …….

Address of Institution: …….

Date: [| |]

Hour of filling the questionnaire: Beginning.....Finish time..... Nationality:……..

2. Producer / interlocutor

Name and first name: …….

Commune/ district :.....

Zone/ sector:.....

Hill/ Cell:.....

Province:.....

GPS co-ordinates: Altitude:..... Longitude: Latitude:.....Country:.....

3. Evaluation of BBTD incidence and severity

Considering 30 mats randomly selected, 100 m between two farms and 5 km between villages

30 mats by farm	Variety name	**Incidence evaluation**: At least a plant within a mat show BBTD symptom **1 = yes** or **0 = Not**	**Severity quotation** upon infected plants according to scale BBTV (0-5): **0** without symptom **1.** Scratches sunk on leaves **2.** Scratches sunk to the pseudo-trunk **3.** Discolouration of the sheets with normal size **4.** Reduced size of faded leaves **5.** Bunchy top aspect at the top of banana plant	Presence of the vector on each mat: **1 = yes** and **0 = Not**	Importance of the vector on the mat according to the scale (1-5):**1.** A simple colony without winged insects, **2.** Several simple colonies **3.** A large colony with winged insects **4.** Several large colonies **5.** Colonies generalized at the level of the leaves and the pseudo-trunk
1 to					
2					
-					
-					
30					

4. Farming practices

4.1. Source of planting material

Each time put between blanket: 0 = Not or 1=Yes

1. Suckers from the same plantation [ ]

2. Suckers from close neighbours (less than 1 km) [ ]

3. Suckers obtained far (>10km) from your location [ ]

4. *In vitro*-planlets distributed by the state services (Extension, Research, NGOs) [ ]

5. *In vitro*-seedlings bought by farmers themselves [ ]

6. Others specify……..

4.2. Banana varieties cultivated in the location

Cooking	Beer	Dessert
1)	1)	1)
2)	2)	2)
3)	3)	3)
4)	4)	4)
5)	5)	5)

4.3. The age of banana plantation in the surveyed site: [ ]

Put between hooks the number corresponding to the answer given

1. Banana plantation of less than 3 years
2. Banana plantation of 3-5 years
3. Banana plantation of 5-7 years
4. Banana plantation of 7-9 years
5. Banana plantation of more than 10 years

4.4. Farming system

Put between hook: 0 = Not or 1=Yes

1. Banana crop in monoculture (without association) [ ]

2. Intercropping system with:

2.1. Colocasia [ ]

2.2. Cassava [ ]

2.3. Sweet potato [ ]

2.4. Beans [ ]

2.5. Maize [ ]

2.6. Others:

5. Farmer's knowledge about Banana Bunchy Top Disease

5.1. Identification of symptoms

Each time put between hook: 0 = Not or 1=Yes

1. The farmer can identify the symptoms of the disease: [ ]

2. Which symptoms observed according to the farmer?

2.1. Observation of the symptoms on the leaf [ ]

2.2. Observation of the symptoms on the pseudo-trunk [ ]

2.3. Bunchy top aspect of the infected plant [ ]

2.4. The rooting system become non functional [ ]

2.5. Others:

3. Which is the local name allotted to the BBTV in the location: …….;

5.2. When in the location BBTD was identified for the first time?

Put between hooks the number corresponding to the answer given

A. In its own plantation [ ]

0. Does not remember when

1. Less than one year ago

2. There is 2-3 years:

3. 3- 5 years ago:

4. There is more than 5 years

B. In the close neighbours fields [ ]

0. Does not remember when

1. Less than one year ago

2. There is 1- 3 years

3. There is 3-5 years

4. There is more than 5 years

C. In the remote villages [ ]

0. Does not remember when

1. Less than one year ago

2. There is 1-3 years

3. There is 3-5 years

4. There is more than 5 years

5.3. Likely origin of BBTV in the location

A. The place where the farmer observed BBTD symptom for the first time: [ ]

Put between hooks the number corresponding to the answer given

1 = in its own plantation

2 = in the neighbours plantations (less than 10km)

3 = in the remote villages (more than 10 km)

4 = not known by the farmer

B. The distance of the zone concerned with the disease according to the farmer

Each time put between hook: 0 = Not or 1=Yes

1 = in its own plantation [ ]

2 = in the neighbours (less than 10km) [ ]

3 = in the remote villages (more than 10 km) [ ]

C. Three varieties more susceptible to the disease

1.....

2.....

3.....

D. The origin of these susceptible varieties in the past

Each time put between hook: 0 = Not or 1=Yes

1. In the neighbours (less 10km) [ ]

2. In the remote villages (more than 10 km): [ ]

3. Coming from the in vitro laboratories: [ ]

4. In a nearby country (which): [ ]

5.4. BBTD spread in the location

Put between hooks the number corresponding to the answer given

A. Time after observation of the symptoms with continous banana production [ ]

0. Not known

1. Less than one year

2. Two years of production

3. Three years of production

4. Five years of production

5. More than 5 years

B. Time taken by BBTV to spread on banana plants located near infected plants [ ]

1. Weeks

2. Months

3. Years

4. Not known

C. How long do you observe BBTD symptoms in a newly established banana plantation?

Put between hooks the number corresponding to the answer given

1. For a plantation installed using suckers from the same field [ ]

1 = weeks

2 = month

3 = years

4 = not known

2. When they use suckers from neighbours [ ]

1 = weeks

2 = month

3 = years

4 = not known

3. When they set up the plantation using in vitro-seedlings: [ ]

1 = weeks

2 = month

3 = years

4 = no known

D. Distance between an infected field and newly established plantation [ ]

Put between hooks the number corresponding to the answer given

1 = the new suckers are planted near those infected

2 = new plantation in the same field after destruction of infected mats

3 = the new plantation is distant from that BBTD-affected

4 = No special precaution in establishing new plantation

E. The symptoms are there function with susceptible varieties in the zone?

0 = No or 1=Yes [ ]

F. Do you have resistant varieties without BBTD symptoms since the appearance of the disease in your location? 0 = No or 1=Yes [ ]

If yes which varieties:

□1.....

□ 2.....

□ 3.....

On these tolerant varieties, check if their petioles are closed like for varieties with B genotype or opened for standard AAA genotype. Look if there is influence of pseudo-stem shape on the frequency of *the* vector, *Pentalonia Nigronervosa*

1. Do you observe the vector in banana plants which have closed petiole (genotype B)? [ ]

2. Do you observe more population of the vector in banana with opened petiole? (Genotype A) [ ]

5.5. Influence of climatic conditions on BBTD spread

1. Existence of a weather station nearer [ ]

So yes which is the name of the station? :…….

2. Is there the influence of climatic conditions at the origin of the appearance of BBTV?

- Probable Influence of the great dry season (2005-2006) in the area on BBTV [ ]

3. Which is the season of great frequency of BBTD:[ ]

Put between hooks the number corresponding to the answer given

1 = More cases of disease in rain season

2 = More cases of disease in dry season

3 = It is the same irrespective of the season

5. 6. Farmers' knowledge on BBTV transmission

Each time put between hook: 0 = Not or 1=Yes

1. Transmission by the soil [ ]

2. Possible transmission by suckers [ ]

3. Possibility of transmission by a vector [ ]

4. Transmission by the tools (hoes, knife,) [ ]

5. Other modes of transmission suggested according to the farmer:

5.7. Evaluation of aphid population with the farmer

1. Did you observe that kind of aphids (*Pentalonia nigronervosa*) on other crop? [ ]

So yes which:

1. Colocasia [ ]
2. Cassava [ ]
3. Sweet potato [ ]
4. Beans [ ]
5. Maize [ ]
6. Others

2. Report of the investigator on the presence of *Pentalonia nigronervosa* upon other crops.

0 = No or 1=Yes [ ] If yes, which crops?

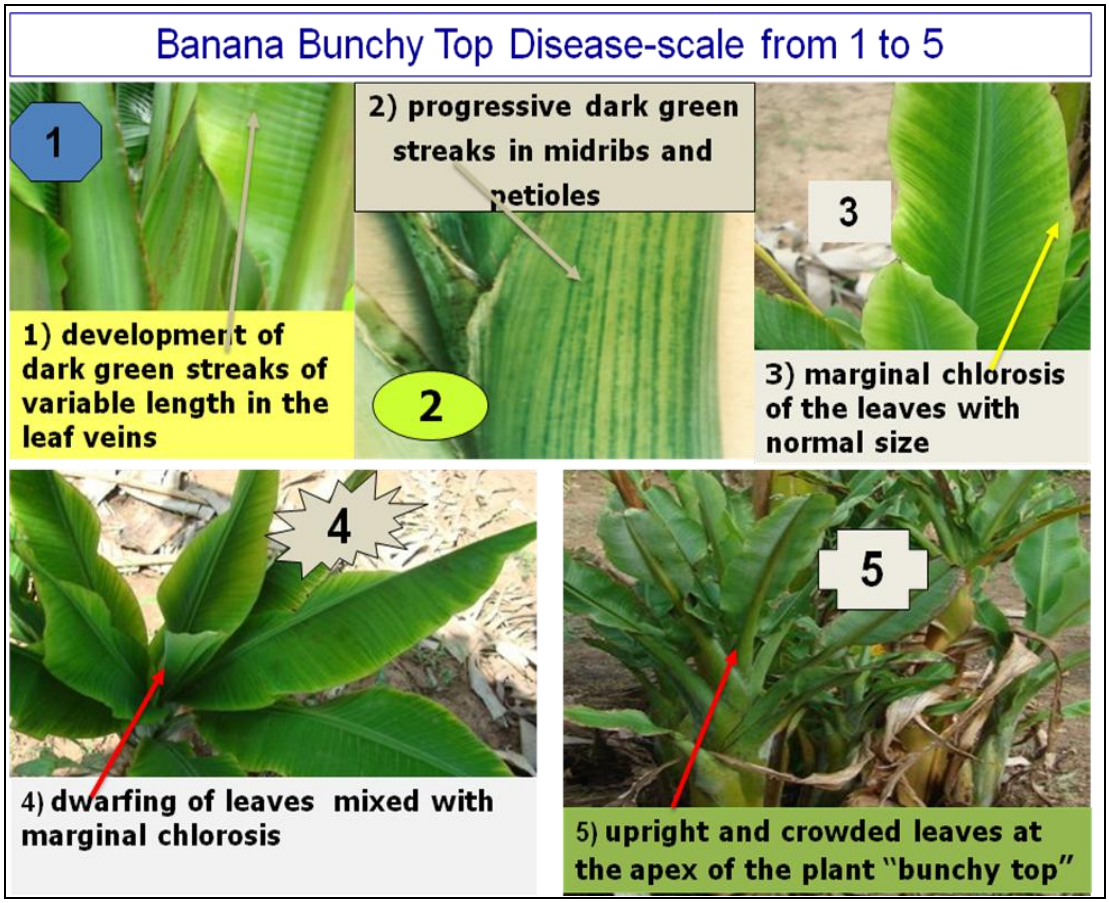
5.8. Farmer's practices aimed at BBTD management

Each time put between hook: 0 = Not or 1=Yes

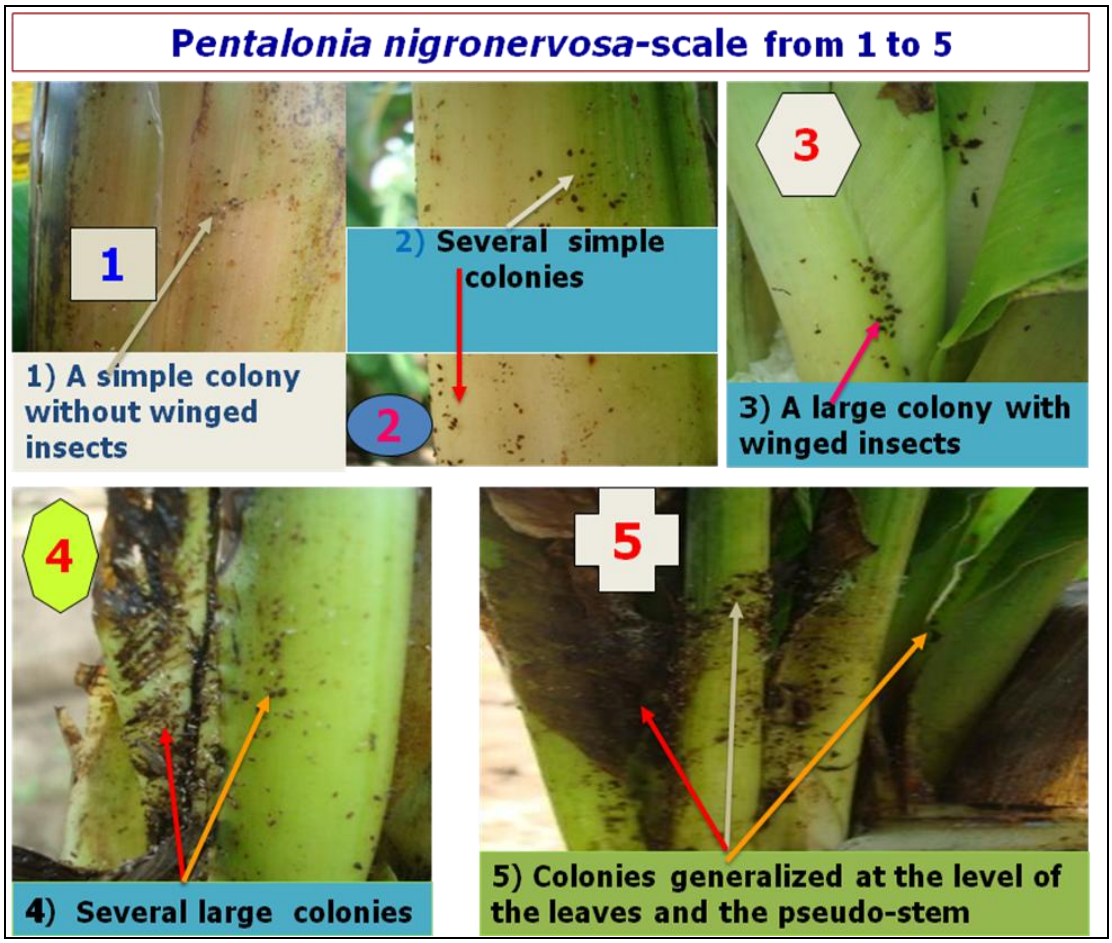
1. Are there organised farmer's training by the public extension services on how to manage BBTV? [ ]
2. Do you make choice of the healthy suckers taking into account the BBTD presence? [ ]
3. Are the plants showing the symptoms directly destroyed? [ ]
4. All the mats of banana crop infected by BBTV are there destroyed? [ ]
5. Is there a supervising authority on how to introduce suckers coming from the infected zones? [ ]
6. Do you use chemicals to control the disease? [ ]
7. Others proposed by farmers:

"End of the questionnaire"

Appendix 3.2. Photographic representations of rating scale (1-5) used to assess banana bunchy top disease (BBTD) severity.



Appendix 3.3. Photographic representations of rating scale (1-5) used to assess importance of banana aphids *(P.nigronervosa)* on banana plants.



Appendix 3.4. PCR results (+ is positive and – is negative) of samples collected using PhytoPass kits in three countries Burundi, DR Congo and Rwanda.

No Lab	Location/ Country	Sites	Period between sampling and extraction (days)	Variety	PCR results Dilution 100X
1	1. Bujumbura Rural/ Burundi	Ruhororo-Mubimbi	45	Indarama	-
2		Benga-Mubimbi	45	Indarama	-
3		Benga-Mubimbi	45	Indarama	-
4		Benga-Mubimbi	45	Indarama	-
5		Benga-Mubimbi	45	Pentalonia	-
6		Ruhororo: 1836m	44	Indarama	-
7		Benga	45	Indarama	-
8		Buhanada	45	Igisukari	-
9		Muhororo	45	Igisukari	-
10		Benga: 1340m	45	Igisukari	-
11		Benga	45	Igisukari	-
12		Buja rural: 1280m	45	Igisukari	+
13		Muhororo: 1548m	45	Igisahira	-
14		Ruce-bugarama	44	Igisahira	-
15		Ruce: 2096m	44	Indarama	-
16	2. Rumonge/ Burundi	Gitaza: 814m	40	Indarama	-
17		Gakungwe: 769m	40	Kayinja	-
18		Ruziba	40	Igisahira	+
19		Gitaza: 811 m	41	Kayinja	-
20		Minago	40	Igisukari	-
21		Gitaza: 814 m	37	Kayinja	-
22		Gitaza: 814 m	40	Igisahira	-
23		Gakungwe	40	Km5	-
24		Gakungwe	40	Indarama	-
25		Gakungwe	40	Indarama	-
26		Minago: 774m	40	Igisahira	+
27		Kigwena: 803 m	37	Igisahira	-
28		Kigwena: 803 m	37	Igitsirye	-
29		Kigwena: 805 m	37	Umusare	-
30		Rumonge: 805 m	37	Ikigurube	+
31	3. Cibitoke / Burundi	Mugina: 1028 m	41	Km5	-
32		Murwi : 1058m	41	Km5	-
33		Rugombo: 921m	40	Km5	-
34		Rugombo: E. rural	38	Gde Naine	+
35		Murwi: 1148m	38	Igisahira	-
36		Murwi: 1182m	38	Igisahira	-
37		Buganda: 1038m	38	Igisahira	-
38		Mugina	37	Igitsirye	-
39		Mugina: 1330 m	37	Igitsirye	+
40		Cibitoke	38	Igitsirye	-
41		Rugombo: E.rural	37	Gde Naine	-
42		Mugina: 1179m	37	Km5	-
43		Rugombo-ch.mère	38	Km5	-
44		Buganda : 1038 m	38	Km5	-
45		Mugina : 1028 m	37	G. Michel	-

46	Rusizi district/	Rebero: 1536m	14	Barabesha	-
47	Rwanda	Bugarama: 1022m	15	Poyo	+
48		Bugarama: 1022m	15	Km5	+
49		Bugarama: 1022m	15	Umushaba	-
50		Bugarama: 1022m	14	Km5	-
51		Bugarama: 1022m	15	Ikivuvu	+
52		Essai-ISAR	15	FHIA03	+
53		Bugarama	14	Km5	+
54		Rebero:1536m	13	FHIA25	-
55		Bugarama	15	Km5	+
56		Bugarama	15	Indarama	+
57		Bugarama	14	Km5	-
58		Muganza	15	Igisahira	-
59		ISAR-Bugarama	15	Igitsirye	-
60		Bugarama	14	Igisahira	-
61		Bugarama	15	Km5	-
62		Bugarama	15	Indarama	+
63		Bugarama	15	Km5	-
64		Rebero:1536m	14	Igisahira	+
65	Nyangezi and	Nyangezi: 1623m	13	Kamara	-
66	Kamanyola/	Nyangezi	13	Igisahira	+
67	Western	Nyangezi	13	Mushikazi	-
68	DR Congo	Nyangezi	13	Km5	-
69		Kamanyola	13	Km5	-
70		Nyangezi- Mazinzi	13	Gisukari	-
71		Nyangezi- Mazinzi	13	Kamara	-
72		Nyangezi- Mazinzi	14	Gisukari	-
73		Nyangezi- Mazinzi	13	Km5	+
74		Nyangezi- Mazinzi	13	Kamara	-
75		Nyangezi- Mazinzi	13	Igitsirye	-
76		Nyangezi-Munya	14	Kamara	-
77		Nyangezi-Munya	14	Km5	+
78		Nyangezi-Munya	14	Km5	-
79		Nyangezi-Munya	13	Malaya	+
80		Nyangezi-Munya	13	Kamara	-
81		Nyangezi-Munya	14	G.Michel	+
82		Nyangezi-Munya	13	Kayinja	-
83		Nyangezi-Munya	13	Igisahira	-
84		Nyangezi-Munya	14	Kayinja	-
85		Kamanyola: 964 m	12	Km5	+
86		Kamanyola	12	Igisahira	-
87		Nyangezi	13	Kayinja	+
88		Kamanyola	12	Gisukari	-
89		Nyangezi	13	Kamara	+
90		Nyangezi	12	Kamara	+

Samples from Burundi and Rwanda collected using two PhytoPass kits per sampled banana plant.

No Sample	No Lab	Country	sites	Period between sampling and extraction (days)	Variety	PCR results: d100X
91	119 119’	Burundi	Benga-Isare: 1285m	10	Indarama	+ +
92	120 120’	Burundi	Gatare, Minago, Rumonge: 785m	10	Kamaramasenge	+ +
93	121 121’	Burundi	Ruziba, Mugina,Cibitoke: 1191m	10	Yangambi Km5	+ +
94	122 122’	Burundi	Muhoro,Mageyo, Mubimbi: 1529m	10	Umugerantango	+ +
95	123 123’	Burundi	Mugendo, Bugarama, Rumonge: 781m	11	Indarama	- -
96	124	Burundi	Manege, Murwi, Cibitoke: 1087m	10	Yangambi Km5	+
97	125 125’	Burundi	Migera, kabezi : 801m	11	Mugomozi	+ +
98	126 126’	Burundi	Nyaruhongoka, Gitaza, Muhuta : 785m	11	Kamaramasenge	+ +
99	127 127’	Burundi	Manege, Murwi, Cibitoke: 1124m	10	Yangambi Km5	+ +
100	128 128’	Burundi	Gahongore, Gasenyi, Cibitoke: 1033	11	Yangambi Km5	+ +
101	129 129’	Burundi	Gasozo, Mageyo, Mubimbi: 1428m	11	Ikimaraya	- -
102	130 130’	Burundi	Mparambo, Rugombo,Cibitoke: 893m	10	Yangambi Km5	+ +
103	131 131’	Burundi	Butuhurana, Benga, Isare : 1241m	11	Indarama	- -
104	132 132’	Burundi	Muyange, Mugina, Cibitoke : 1128m	10	Yangambi Km5	- -
105	133 133’	Burundi	Butuhurana, Benga, Isare: 1301m	11	Indarama	+ +
106	134 134’	**Rwanda**	Bugarama-Rubumba	12	Plantain	+ +
107	135 135’	**Rwanda**	Bugarama-Rubumba	12	Yangambi Km5	+ +
108	136 136’	**Rwanda**	Bugarama-Rubumba	12	Intuntu (Igitsirye)	- -
109	137 137’	**Rwanda**	Bugarama-Rubumba	12	Yangambi Km5	- -
110	138 138’	**Rwanda**	Bugarama-Rubumba	12	Mitoke	+ +

+: represents a sample tested positive and -: is a samples tested negative using PCR tests.

Appendix 6.1. Effect of locality, variety and type of planting material on banana bunchy top disease incidence.

Parameter#	Estimate	s.e.	t(*)	t pr.	Antilog of estimate
Constant	-0.8859	0.0766	-11.57	<.001***	0.4124
Benga site	-0.7717	0.0539	-14.31	<.001***	0.4622
Kamaramasenge (AAB)	0.4433	0.068	6.52	<.001***	1.558
Grande Naine (AAA)	0.7608	0.0664	11.47	<.001***	2.14
Sucker-derived	-0.4579	0.0894	-5.12	<.001***	0.6326
TC plantlet	-0.6302	0.0918	-6.87	<.001***	0.5325

#: The Kagazi site and 'Nyambururu' variety were randomly taken as references.
NS, ***: not statistically different and highly significant at P=0.001 (Fisher's test), respectively.

Appendix 6.2. BBTV status based on TAS-ELISA tests carried out on banana leaf samples collected in six plot locations on plants showing BBTV-symptoms and plants suspected of being BBTV-infected.

Sampling among 60 plants per plot location	collected samples	positive samples	%disease incidence
Inside a BBTD-affected field where infected mats had not been uprooted	23	12	52
Inside a BBTD-affected field where infected mats had been uprooted	23	14	61
At 5 m outside and downwind from a BBTD-affected field	44	14	31
At 30 m outside and downwind from a BBTD-affected field	35	21	60
At 5 m outside and upwind from a BBTD-affected field	55	13	23
At 30 m outside and upwind from a BBTD-affected field	45	11	24
Total	225	85	-