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Detection and identification of viruses infecting *Musa* spp. using Polymerase Chain Reaction and High Throughput Sequencing technologies

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DETECTION AND IDENTIFICATION OF VIRUSES INFECTING *MUSA* SPP. USING POLYMERASE CHAIN REACTION AND HIGH THROUGHPUT SEQUENCING TECHNOLOGIES

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Abstract

Banana mild mosaic virus (BanMMV) (*Betaflexiviridae*, *Quinivirinae*, unassigned species) is a filamentous virus infecting *Musa* spp. with a very wide geographic distribution. It is defined as a significant risk for the banana sector in particular in the various DROMs, and seems to fulfil the criteria of a regulated non-quarantine pest. Therefore, it is important to establish appropriate control measures based on the setting-up of specific and sensitive diagnostic techniques. BanMMV displays a very high genome variability which makes its molecular detection by specific primers particularly challenging, and requires the development of diagnostic tests with high inclusivity.

BanMMV detection and/or sanitation remains labor- and time-consuming. The current BanMMV indexing process for an accession requires the testing of no less than four plants cultivated in a greenhouse for at least 6 months and causes a significant delay for the distribution of the germplasm.

The main objective of this thesis was to improve the diagnostic of BanMMV from *Musa* accessions. This study aimed to design new diagnostic primers in order to improve detection of BanMMV, following the identification of novel isolates of the virus. It also aimed to test the use of banana *in vitro* plants in order to accelerate the testing process and evaluate virus therapy success.

In this context, a discrepancy in test result was observed between electron microscopy and immunocapture (IC) reverse transcription (RT) polymerase chain reaction (PCR) test results for one asymptomatic banana accession. The absence of molecular detection and the presence of filamentous particles suggested the presence of a new variants or new virus. The accession underwent high throughput sequencing which allowed the identification of two complete genomes of BanMMV with high nucleotide identity that constitute the two novel isolates of BanMMV found in this accession. These findings triggered the development of a new diagnostic primer based on these two new sequences and the BanMMV CP sequences already published in GenBank. A retrospective analysis of 110 different germplasm accessions from diverse origins was performed in order to compare BanMMV CP9 and

BanMMCP2 primers. Five accessions showed contrasting results. The new primer missed the detection of BanMMV infection from three accessions.

Similarly, BanMMCP2 failed to detect BanMMV infection from two banana accessions. Interestingly, the analytical sensitivity was better with BanMMV CP9 comparing to BanMMCP2. Through this study, we recommended the use of the two primers successively to improve the inclusiveness of the protocol.

HTS technologies are one of the most important advances that have revolutionized molecular diagnostics. Their adoption in plant pest diagnostics, in particular plant virus diagnostics, has been growing steadily in the last decades. In same line, this study showed the improved performance of these technologies in BanMMV detection in comparison to the conventional molecular techniques from the same plants (in absence of therapy). These without *a priori* technologies were much more sensitive than RT-PCR from the same *in vitro* plants with 100% of diagnostic sensitivity (DSE) for HTS comparing to a DSE of 65% for RT-PCR. Interestingly, HTS technologies allowed the identification of a new species from these samples, with a genome of 7,364 bp, presenting a typical genome organisation of *Betaflexiviridae* members after annotation of its new contig. Thus, it could be interesting to consider the in-depth biological characterization of this new species. This would help to know more about the symptomatology, transmission, host range of the new species, and its potential impact on either banana industry and/or the environment

In summary, this study suggested two tests for the detection of BanMMV from *in vitro* plants. HTS technologies could be performed from RNA extracts of pooled leaves or bases. If these technologies are not available or too expensive, RT-PCR could be applied instead on individual RNA extracted from at least four plants. This proposed methodology helps to avoid the greenhouse cultivation and thus can save time and space.

Keywords: Diagnostic, BanMMV, High Throughput Sequencing (HTS), *Musa*, *in vitro* plants , therapy, PCR, RNA extraction

Résumé

Le virus de la mosaïque atténuée du bananier (BanMMV) (*Betaflexiviridae*, *Quinivirinae*, espèce non attribuée) est un virus filamenteux appartenant à la famille des *Betaflexiviridae*. Il infecte *Musa* spp. avec une très large répartition géographique. Il présente un risque important pour la filière bananière notamment dans les différents DROMs, et semble remplir les critères d'un organisme réglementé non de quarantaine. Par conséquent, il est important d'établir des mesures de contrôle appropriées basées sur la mise en place des techniques de diagnostic spécifiques et sensibles. BanMMV présente une variabilité génomique très élevée qui rend sa détection moléculaire par des amorces spécifiques particulièrement difficile, et nécessite le développement de tests diagnostiques à forte inclusivité.

La détection et/ou l'assainissement du BanMMV reste laborieux et chronophage. Le processus actuel d'indexage d'une accession vis-à-vis de ce virus nécessite de tester pas moins de quatre plantes cultivées en serre pendant au moins 6 mois, ce qui entraîne un retard important dans la distribution du matériel génétique.

L'objectif principal de cette thèse était d'améliorer le diagnostic de BanMMV à partir d'accessions de *Musa*. Cette étude visait à développer de nouvelles amorces de diagnostic afin d'améliorer la détection de BanMMV, suite à l'identification de nouveaux isolats de ce virus. Cette étude visait également à tester l'utilisation de plants de bananier *in vitro* afin d'accélérer le processus de test et d'évaluer le succès de la thérapie virale.

Dans ce contexte, une divergence dans les résultats des tests a été observée entre la microscopie électronique et les résultats des tests d'immunocapture (IC) de transcription inverse (RT) de réaction en chaîne par polymérase (PCR) pour une accession de bananier asymptomatique. L'absence de détection moléculaire et la présence de particules filamenteuses suggèrent la présence d'un nouveau variant ou d'un nouveau virus. L'accession a subi un séquençage à haut débit qui a permis l'identification de deux génomes complets de BanMMV avec une identité nucléotidique élevée qui constituent les deux nouveaux isolats de BanMMV trouvés dans cette accession. Ces découvertes ont permis le développement d'une nouvelle amorce de diagnostic basée sur ces deux nouvelles séquences ainsi que les séquences BanMMV CP déjà publiées dans GenBank. Une analyse rétrospective de 110 accessions différentes de matériel génétique d'origines diverses a été réalisée afin de

comparer les amorces BanMMV CP9 et BanMMCP2. Cinq accessions ont montré des résultats contrastés. La nouvelle amorce a échoué de détecter le BanMMV à partir de trois accessions. De même, BanMMCP2 n'a pas réussi à détecter l'infection par BanMMV à partir de deux accessions de bananiers. Par contre, la sensibilité analytique était meilleure avec BanMMV CP9 par rapport à BanMMCP2. A travers cette étude, nous avons recommandé l'utilisation des deux amorces successivement pour améliorer l'inclusivité du protocole.

Les technologies HTS constituent l'une des avancées les plus importantes qui ont révolutionné le diagnostic moléculaire. Leur adoption dans le diagnostic des ravageurs des plantes, en particulier le diagnostic des virus des plantes, n'a cessé de croître au cours des dernières décennies. Dans ce contexte, cette étude a montré l'amélioration des performances de ces technologies dans la détection de BanMMV par rapport aux techniques moléculaires conventionnelles issues des mêmes plantes (en absence de thérapie). Ces technologies sans *a priori* étaient beaucoup plus sensibles que la RT-PCR à partir des mêmes plantes *in vitro* avec 100% de sensibilité diagnostique (DSE) pour le HTS par rapport à une DSE de 65% pour la RT-PCR. Les technologies HTS ont permis l'identification d'une nouvelle espèce à partir de ces échantillons, avec un génome de 7,364 pb, présentant une organisation génomique typique des membres de *Betaflexiviridae* après annotation de son nouveau contig. Donc, il pourrait être intéressant d'envisager la caractérisation biologique approfondie de cette nouvelle espèce à l'aide de diverses expériences et investigations. Cela aiderait à en savoir plus sur la symptomatologie, la transmission, la gamme d'hôtes de la nouvelle espèce et son impact sur l'industrie bananière et/ou l'environnement.

En résumé, cette étude a proposé deux tests pour la détection de BanMMV à partir de plantes *in vitro*. Les technologies HTS pourraient être réalisées à partir d'extraits d'ARN de feuilles ou de bases regroupées d'au moins quatre plantes par accession. Si ces technologies ne sont pas disponibles ou trop chères, la RT-PCR pourrait être appliquée à la place à partir des ARNs d'au moins 4 plantes de chaque accession. Cette méthodologie proposée permet d'éviter la culture en serre et donc de gagner du temps et de l'espace.

Mots clés: Diagnostic, BanMMV, Séquençage à haut débit (HTS), *Musa*, plantes *in vitro*, thérapie, PCR, extraction d'ARN

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Table of Contents

List of figures

List of tables

List of abbreviations

BanMMV: banana mild mosaic virus

BBTV: banana bunchy top virus

BBrMV: banana bract mosaic virus

BLAST : Basic Local Alignment Search Tool

Bp: base pair

BSVs: banana streak viruses

cDNA: complementary DNA

CMV: cucumber mosaic virus

HTS: High Throughput Sequencing

IC: Immunocapture

ICTV: International Committee on Virus Taxonomy

ITC: International *Musa* Germplasm Transit Centre

KU Leuven: Katholieke Universiteit Leuven

NGS: Next Generation Sequencing

Nt: nucleotide

PCR: Polymerase Chain Reaction

RACE: Rapid amplification of cDNA ends

RT: Retro-transcription

UTR: untranslated region

GHU: Germplasm Health Unit

Chapter 1

Introduction

1. Banana

1.1. Introduction

Banana (*Musa* spp.) is a perennial, monocotyledonous, herbaceous plant and is one of the most important staple food crops worldwide after rice, wheat and maize (Kouakou et al., 2016). It supplies food to more than 400 million people in more than 136 countries (Ntui et al., 2020). With worldwide annual production reaching 155 million tons on 11 million hectares of land on 2018, it represents an important contribution to the economies of many countries in Asia, Africa, Latin-America and the Pacific Islands (Christelová et al., 2017; L. Tripathi et al., 2021). It has been observed that the diversity of *Musa* species is the greatest in the Southeast Asian region. Thus, it is suggested that the general centre of origin of bananas should be in this region (Teo et al., 2011). Bananas are vegetatively propagated through rhizomes and suckers, which represents a high risk of virus spread through the movement of infected planting material. It is known that several diseases, such as Panama disease or banana bunchy top disease, as well as climate change constitute a serious threat to banana production. Surprisingly, recent studies have reported that changes in weather patterns impacted differently some banana producing regions. For instance, changes in temperature and rainfall were beneficial to Africa and some countries in Latin America and the Caribbean. However, Southeast Asian countries and Brazil were negatively affected by these changes. Scientific predictions suggest that climate conditions will continue to be beneficial to African production in the future decades, which won't be the case for important producing countries as Colombia, Costa Rica or India (Bebber, 2022).

1.2. Morphology of the banana plant

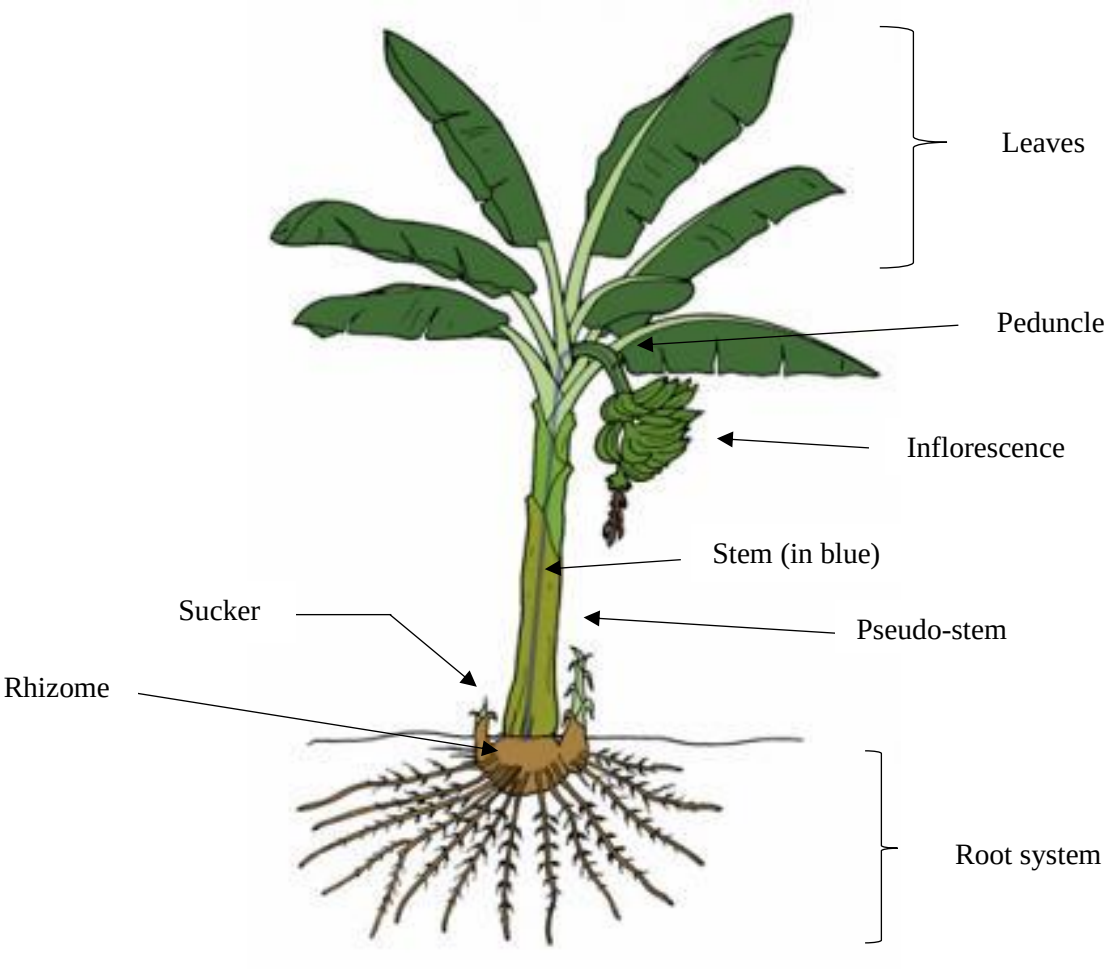

Figure 1: Morphology of the banana plant

(https://www.promusa.org/Morphology+of+banana+plant)

The banana is a tree-like giant herb due to the presence of non-woody soft stems and the death of the fruiting stem after the growing season. It belongs to *Musaceae* family. Its height varies between 1,5 and 8 m depending on the species and the variety. It is a perennial plant as it is a longer-lived species

living for three or more growing seasons (the period from when the pseudostem is removed and chopped until the development of the new plantlet from the same rhizome). In fact, the lateral buds on the rhizome give birth to suckers that develop into fruit-bearing stems (Durmic et al., 2010; Lassois et al., 2009).

The banana plant has two stems: the 'true' stem and the pseudo-stem. The 'true' stem (in blue in Figure 1) is located inside the pseudo-stem. It is composed of the rhizome (usually called corm or bulb) which grows in a horizontal underground way, the aerial stem containing the leaves, and the peduncle which is the emerging part of the stem at the top of the plant. The peduncle holds the inflorescence or the bunch which supports the fruits. The stem is white. However, the peduncle is green (Figure 2). In general, there are more than one fruiting stem simultaneously in one banana plant. The pseudo-stem is composed of overlapping leaf sheaths (Deepa et al., 2011). Figure 2 shows the insertion points of the leaves along the aerial part of the stem.

Figure 2: The banana 'true' stem after removing all leaves and leaf sheaths

(https://www.promusa.org/Morphology+of+banana+plant)

1.3. Origin and domestication of banana

The two primary diploid wild relatives of bananas and plantains are *Musa acuminata Colla* and *Musa balbisiana Colla*. They are the origin of most edible banana and plantain cultivars. Most cultivated bananas and plantains are triploids (2n =3x = 33). A few are diploid or tetraploid. They are sterile and parthenocarpic and produce large seedless fruits (Kallow et al., 2022; Panis et al., 2005). These large fruits constitute the world's most important sweet dessert crop, thus the importance of the international banana trade.

Domesticated bananas are generally triploid. These triploidizations occurred from multiple hybridizations between diploid AA cultivars (*Musa acuminata*) and BB cultivars (*Musa balbisiana*). For sweet dessert bananas, the genomes constitutions are AAA and for predominantly starchy plantains eaten after cooking, the genomes are AAB or ABB (Figure 3). Some are tetraploids with the genomes AAAA, AAAB, AABB and ABBB (De Langhe et al., 2009; Heslop-Harrison & Schwarzacher, 2007).

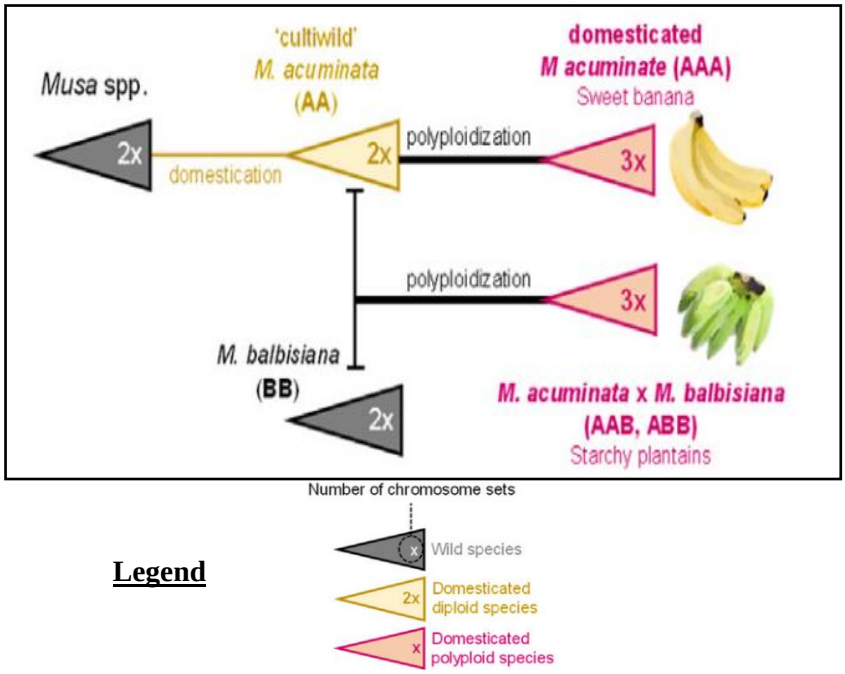

Figure 3: Proposed scenario for the polyploidization and domestication in Banana (Akagi et al., 2022)

1.4. Nutritional value and medicinal properties of banana

Banana constitutes a very good source of energy. One medium sized banana provides around 105 calories. With its energy and high level of potassium, banana is considered as the best fruit for athletes, providing them with sufficient energy for several minutes of workout and also preventing muscle spasms. This energy is provided by the carbohydrates contained in the fruit, which vary between 22 g and 27g/100g depending on the banana cultivar. After digestion process, these carbohydrates are converted into sugar. The riper the banana, the more sugar it contains (Ranjha et al., 2022).

Besides its energy and carbohydrates, banana contains a mixture of minerals (potassium, magnesium, phosphorus, calcium, manganese, and copper) and vitamins (vitamin A, vitamin B complex, vitamin C, and vitamin E).

Banana is an excellent source of potassium. This mineral is very important for the muscles and cardiovascular system. In fact, it prevents muscle contraction

and reduces the risk of stroke by helping to decrease the blood pressure. By providing around 350 mg of potassium/100g, consuming a single banana with normal size could cover about 23% of our potassium needs in one day. In addition, the amount of this mineral depends on the banana cultivar and on the soil potassium uptake and content. Potassium could reach 490 mg/100g for 'cooking bananas' or plantain bananas (Ranjha et al., 2022).

Magnesium and phosphorus are also very important minerals for the human body. A normal size banana provides respectively for magnesium and phosphorus 32 mg and 26 mg/100g, serving 8% and 4% respectively for the daily recommended intake (DRI) of magnesium and DRI of phosphorus. One median plantain banana provides 36 mg and 32 mg/100g respectively for magnesium and phosphorus (Hardisson et al., 2001; Kumar et al., 2011).

Banana is a main source of nutrients like vitamins. It contains vitamin C, vitamins B, vitamin A and vitamin E. Like minerals, the vitamins concentrations depend on the banana cultivar.

Vitamin C, known also as Ascorbic Acid, is a water-soluble vitamin. It is a very important vitamin as it is involved in several functions of the body such as the defence against infections, the iron assimilation, and the antioxidant actions. The average concentration of vitamin C is 12.7 mg/ 100 g. This vitamin represents around 8.5% of the banana pulp (Sarma et al., 2021). Table 1 shows examples of different concentrations of vitamin C depending on the banana cultivar.

Table 1 : The Vitamin C content in different banana cultivars, in an ascending order

Banana variety/ scientific name	**Vitamin C concentration**	**Reference**
Cavendish	From 2.1 to 18.7 mg/100 g	Afzal et al., 2022
Dwarf Brazilian	4.5 mg/100 g	
Williams Fruit	4.5 mg per 100 g	Ranjha et al., 2022
Musa acuminatae Colla	8.7 mg/100 g	
Lakatan/*Musa* sapientum	10 mg/100 g	
Nayak	12.7 mg/ 100 g	Afzal et al., 2022
Basak	12.7 mg/100 g	
Cooking banana/*Musa* x paradisiaca	18.4 mg/ 100 g	Ranjha et al., 2022

Vitamins B are water soluble vitamins. They play important roles in the proper functioning of the nervous system, the production of energy and the cell metabolism.

Banana is also a main source of the vitamin B complex. It contains vitamin B1 (Thiamine), vitamin B2 (Riboflavin), vitamin B3 (Niacin), vitamin B5 (Pantothenic acid), vitamin B9 (Folate), and vitamin B6 (Pyridoxine). For instance, the vitamins B content in 100 g of *Musa acuminata Colla* is as following; 31 µg of vitamin B1(representing 3% of daily requirement), 73 µg of vitamin B2 (6% of daily requirement), 660 µg of vitamin B3 (4% of the daily requirement), 330 µg of vitamin B5 (7% of the daily requirement), and finally 400 µg of vitamin B6 which importantly represents 31% of the daily requirement (Ranjha et al., 2022).

The dessert banana is also a rich source of pro-vitamin, also known as vitamin A. Pro-vitamin A carotenoids like α-carotene, β-carotene and lutein are present in a dessert banana and can be converted in the body to vitamin A. Globally, many developing countries in Asia and Africa (almost 120 countries) are victims of the deficiency of vitamin A, which a very problematic situation as it causes the death of many women and children. The average content of vitamin A in 100 g of banana is of 8.2 µg. The riper the fruit, the more carotenoids it contains. Interestingly, some bananas have orange pulp. These varieties contain high amount of vitamin A. For instance, Karat banana contains 2230 µg/ 100 g of β-carotene (Afzal et al., 2022).

On the other hand, banana is known to have potential medicinal properties. Several studies have enumerated its medicinal effects like antioxidant effect, role in digestive health, role in body muscles, role in cardiovascular diseases, role in human brain, and even benefits for smokers (Govindaraj, 2022; Muhammad Suffi et al., 2021; Panyayong & Srikaeo, 2022; Ranjha et al., 2022).

Banana is very rich in antioxidants. These antioxidants are very important for our health as they play a key role in preventing several diseases such as cancer and heart disease. In fact, they prevent oxidative damages by neutralizing free radicals (Govindaraj, 2022).

In addition, the fruit has many beneficial effects on the digestive system. It has a mild laxative property as well as the property to cure diarrhea and dysentery. Some intestine lesions and worm problems in children can be healed by eating banana. In addition, it has been shown that banana flower were very beneficial to women presenting menstrual issues. It helps them stabilize their blood flow (Kumar et al., 2011).

1.5. The international banana trade

Actually, around 150 million tonnes of bananas are produced each year across the tropics and subtropics, which puts total banana production right after tomatoes and before apples. However, in term of sweet dessert crop, banana remains the most important one. This crop is grown in many countries across the world. Figure 4 shows the most important producers of banana in 2020.

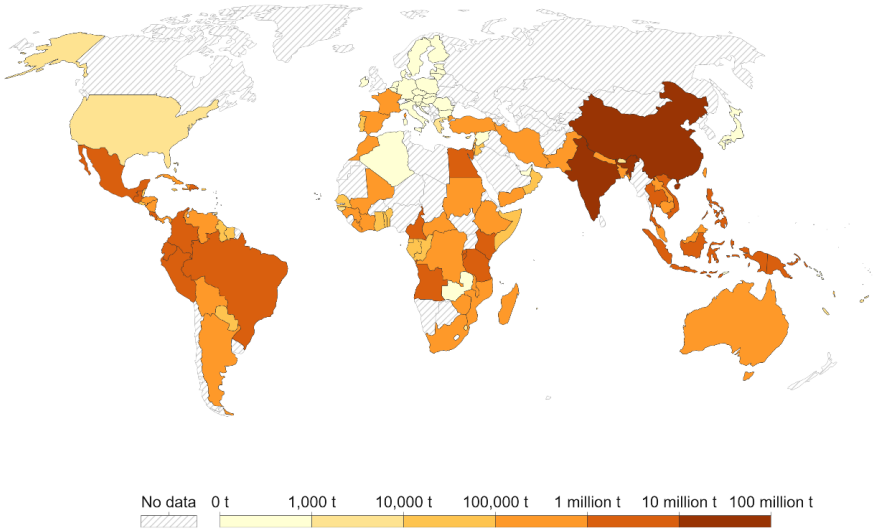

Figure 4: Banana production in 2020 (http://www.fao.org/faostat/en/#data)

As it is shown in the figure above, Asia remains the largest banana producer in the world. According to FAO data for the year 2020, India was the largest producer country in the world with a production of 31.50 million tonnes followed by China that produced 11.51 million tonnes in 2020. These two countries produce almost 41% of the world's banana production (Rincón-Catalán et al., 2022).The third position is occupied by Brazil with a production of 6.64 million tonnes followed by the United States with a production of 2,935tonnes.

The world banana exportation data revealed that the Latin America & Caribbean (LAC) region is the leading exporting region of banana in the world, followed by Asia then Africa (Figure 5).

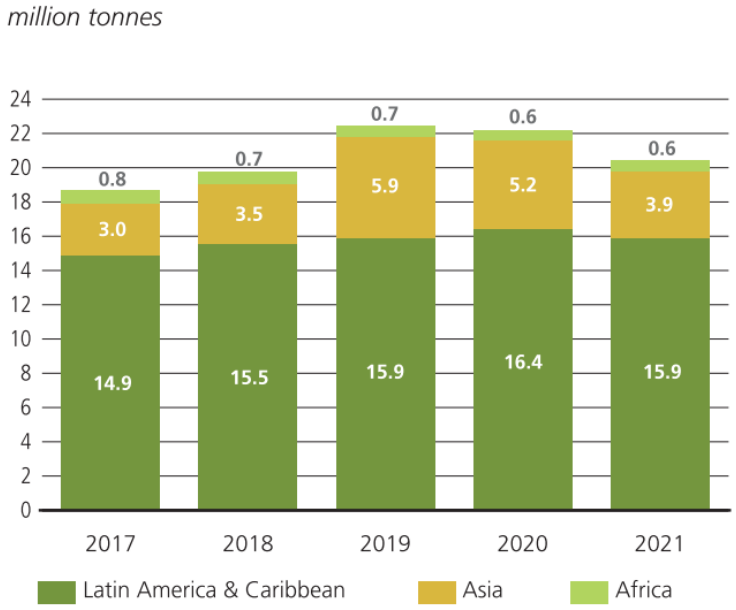

Figure 5: World banana exports by region, 2017-2021 (FAO. 2022. Banana Market Review 2021. Rome)

In LAC region, Ecuador is the largest exporter of banana globally followed by Costa Rica, Guatemala and then Colombia. However, focusing on the largest producers regardless of region, we find that Ecuador remains the largest exporter of banana in the world followed by Philippines, Costa Rica then Guatemala and Columbia (Figure 6). They export bananas mainly to the European Union and the United States of America. The banana trade is around USD 11 billion, more than any other fruit (Bebber, 2022; L. Tripathi et al., 2021).

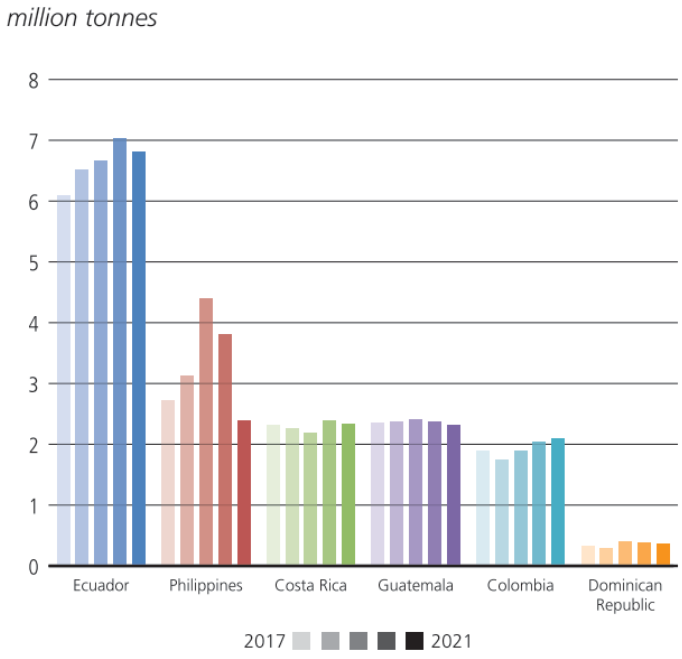

Figure 6: World banana exports by country, 2017-2021 (FAO. 2022. Banana Market Review 2021. Rome)

Even though there are many edible banana cultivars available, the international trade relies only on one cultivar which is Cavendish. An example of a Cavendish banana plantation is illustrated in Figure 7.

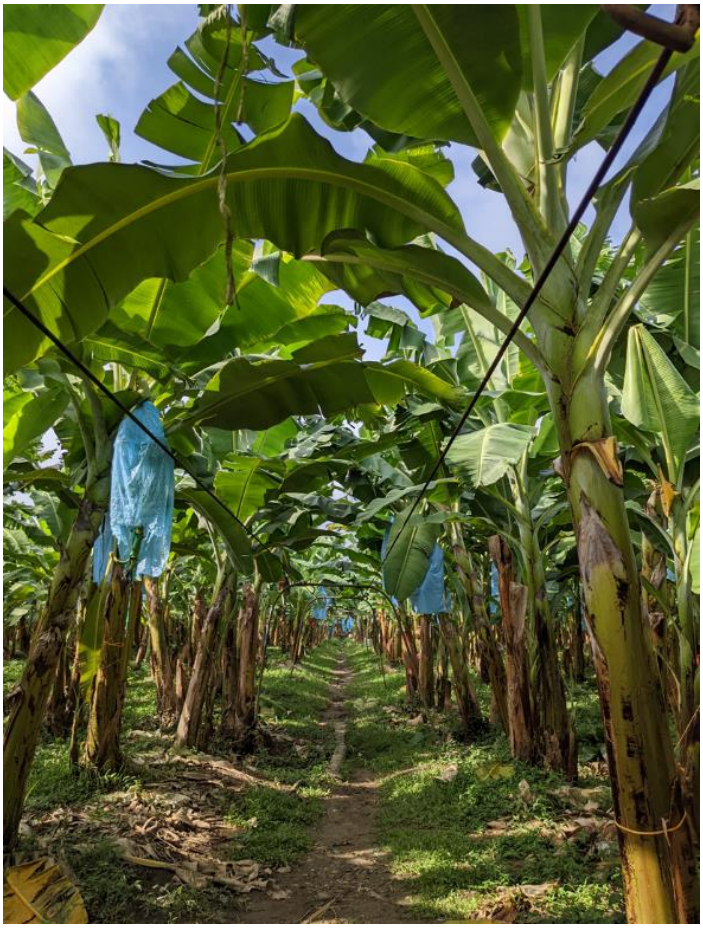

Figure 7: Cavendish banana plantation in Latin America (Bebber, 2022)

This industry choice was made for different reasons. First of all, it is easier to manage the culture of one cultivar than the culture of many cultivars. The management includes the plantation, the harvest, the transport, etc. In addition, Cavendish showed better ripening properties than the other cultivars, which makes it suitable for long distances shipping. For instance, the shipping from Latin America to Europe lasts around two weeks. During this period, the green bananas are placed in ripening rooms with ethene (also named ethylene) until they become yellow, ready for distribution (Thakur et al., 2019).

1.6. Banana valorisation and contribution in circular economy

Banana is mostly cultivated for its fruit which means that banana fields generate huge amounts of wastes and banana by-products. Hence, banana can contribute to the circular economy through the valorisation of these products and the development of value-added ones. Many parts of the banana can be reused such as leaves, stems, pith, sap, inflorescence, fibers, rhizomes and even peel. They have shown great potential value in the creation of products for food and non-food industries (Mohd Zaini et al., 2022). Figure 8 describes the utilization of banana by products from indigestible parts (pith, pseudostem, pseudostem sap and fibers) as a raw material in different industries.

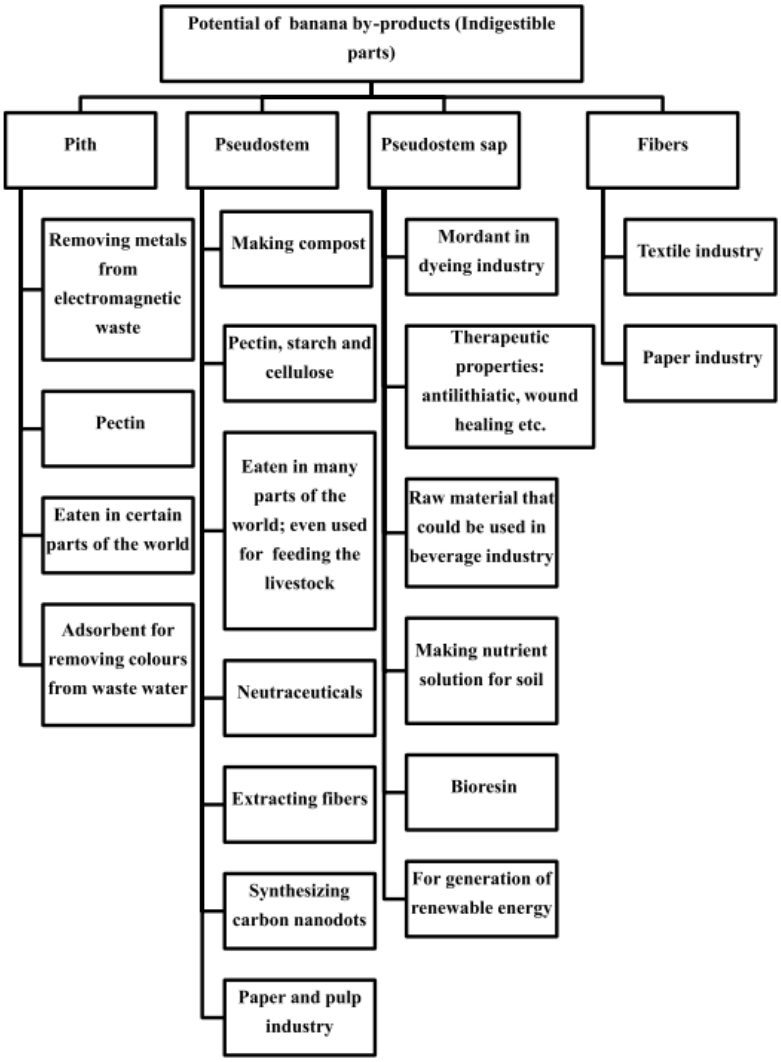

Figure 8: Use of indigestible banana by-products in different industries (Gupta et al., 2022)

Furthermore, banana leaves and stems are considered as a rich source of water-soluble and easily degraded organic matter. Therefore, they can be used as aerobic composting and soil fertilizer. In India, people usually serve meals in banana leaves (Gore & Akolekar, 2003).

Banana leaves were even used in the medical field. During the COVID-19 pandemic, in some regions in Indonesia, people have used banana leaf-filtered

cloth mask as banana leaves showed the ability to filter the bacteria. In fact, (Hakim et al., 2022b) have found out, through the bacterial filtration efficiency test, that there was significantly less bacteria in the banana leaf-filtered face masks (around $0.417x10^3$ cfu/cm2) comparing to $30x10^3$cfu/cm2 for the masks without additional leaf-filter. Thus, it was interesting for this community to increase self-defence against the virus using a biodegradable alternative (Hakim, Duhita, et al., 2022).

The weight of banana peel represents around 35% of the total weight of banana, which means that approximately 40 million tons of banana peel are produced annually. This plant resource is highly interesting as it can be used in different applications. It has a very high nutritional value due to its high content in proteins, carbohydrates and crude fiber. This unused biomass seems to be very suitable to feed animals. Moreover, in order to enhance nutritional properties of food products, some industrials tried to valorize the banana peel in food industry sector. For example, they developed a chicken sausage with a 2% banana peel powder which showed an important increase in dietary fibre and a decrease in the total fat content. The only limitations noticed were the deterioration of the sensory aspects of the products mainly the colour and texture, and the inhibition of lipid oxidation. In addition, the development of bread with 10% banana peel powder decreased the carbohydrate content by 31% which is interesting for people wishing to consume low carb bread in order to lower the level of glucose in their blood. Moreover, banana peels can also be used and valorized in food packaging, pharmacological applications, energy and fertilizer utilization, etc (Mohd Zaini et al., 2022; Putra et al., 2022).

In same line as banana peel, banana inflorescences (bracts, male flowers, rachis, and whole inflorescence) were also used in meat production due to their interesting antioxidant activity. Among these inflorescences , the male flowers showed the highest antioxidant activity due to their highest content of flavonoids and phenolic compounds. It was selected to be included in the sausage formulation as a natural antioxidant. Another interesting point is that 2% of male flowers powder didn't affect the sensory aspects of the product (Rodrigues et al., 2020).

The rhizome or corm contains is a rich source of bioactive compounds that have several functions. These components were used as an environment-friendly approach in material synthesis through the synthesis of polycrystalline Ag-NCs (Jaiswal et al., 2022).

2. Banana viruses

A plant virus is a simple structure composed of the virion and the capsid. The viral particle or virion is composed of the genetic information and the structural proteins, which form the capsid. The genetic information of viruses is very diverse. It could be composed of DNA or RNA, either single stranded or double stranded. Viruses will thus be separated according to their DNA or RNA composition. Unlike animal viruses, plant viruses generally do not possess a lipid viral envelope hence the name of naked or non-enveloped viruses, with the exception of Rhabdoviruses and Tospoviruses (Villagrana-Escareño et al., 2019).

Several viruses are infecting banana and plantain. The most prevalent and economically impactful banana viruses that constitute major constraints in banana production are *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV*), Banana mild mosaic virus* (BanMMV), and *Banana streak viruses* (BSVs).

Since cultivated varieties of bananas and plantains are seedless, they are propagated vegetatively and not sexually. This propagation method is unfortunately effective for the spread of viruses. In same line, the five most prevalent viruses mentioned above are transmitted through suckers and tissue culture (Ngatat et al., 2022).

The main methods of banana virus control include the control of banana virus vectors, if it has been identified, as well as the use of virus-free plant material. As with most viral infections, there is no curative methods in the field except the uprooting of the diseased plant to avoid secondary contamination. Plant virus diseases are difficult to manage due to their obligate intracellular properties. There are numerous strategies that aim to control the plant virus diseases. They include the risk-reducing measures, the RNA-based resistance and the CRISPR-based virus resistance. The risk-reducing measures are principally avoidance, exclusion, eradication and vector management. The

exclusion consists in the implementation of quarantine measures and certification programs in order to ensure the movement of virus-free plant material (budwood, seeds or *in vitro* plants). The avoidance consists in the elimination of other hosts potentially infected. This participates in the interruption of the disease cycle of the virus or vector (Tatineni & Hein, 2023). In the case of vegetatively propagated plants like banana, we should combine the use of clean planting material (exclusion) with cultural practices in order to limit the presence of alternative hosts for vectors and/or viruses (avoidance). In this context, it has been reported that the presence of banana bunchy top virus in the banana plantations in the Pacific and Hawaii can be controlled by the use of virus-free planting material and the disinfection of the tools used (Nyombi, 2019). On the other hand, to reduce the risk of the secondary propagation of the virus, the digging up and the destruction (the incineration) of infected plants seems to be necessary. This method is called eradication and is especially important for perennial crops. Another method is the vector management. Many insects can transmit viruses, such as aphids, thrips, mealybugs and whiteflies, etc. For instance, in the case of banana viruses, *Cucumber mosaic virus* and *Banana bunchy top virus* (BBTV) are both transmitted from plant to plant by several aphid species, although the number of vectors of BBTV is limited (in general two species; mainly *Pentalonia nigronervosa Coquerel* but also *Pentalonia caladii*) . On the other hand, the *Banana bract mosaic virus* can be transmitted by the banana black aphids, *Pentalonia nigronervosa*, after its introduction in a field by infected banana suckers,.. The vector control relies on the application of pesticides, biological control but also of the application of peptides, mineral oils and viral proteins. In this context, several authors have reported many recommendations in order to control the population of *Pentalonia nigronervosa*, the vector of banana bunchy top virus. In fact, the combination of parasitoids, predators, and entomopathogens (such as entomopathogenic fungi) can be used in the implementation of vector control programs like the biological control or the integrated pest management (Tricahyati et al., 2022). For instance, the combination of the use of natural enemies of *P. nigronervosa* with intercropping banana and pineapple (Ouma, 2009) or banana and tomato seemed to be very effective and remunerative (Lifake et al., 2018) . Other authors recommended the use of virus-free plant material and the application of pesticides to control the population of *P. nigronervosa* and reduce the incidence of the banana bunchy top disease (Kakati & Nath, 2019). Nevertheless, the use of pesticides might not be effective against this vector when it is present in young cigar leaves (Robson et al., 2007). In addition, in

the last decades, the use of CRISPR/Cas technology, which is an editing genome tool used to target the host or the virus genes, has revolutionized the development of virus resistance crops. For instance, (Tripathi et al., 2019) have shown great results of the CRISPR/Cas system in plants of banana, by comparing edited and non-edited banana plants regarding the production of episomal viral genome of banana streak virus (BSV). They found out that 75% of edited plants were without symptoms under hydric stress (Tatineni & Hein, 2023).

2.1 Banana bunchy top virus

Banana bunchy top virus (BBTV) is the causal agent of bunchy top disease of banana and plantain. BBTV belongs to the genus Babuvirus (family Nanoviridae). This small family regroups circular ssDNA plant viruses. It has two genera *Nanovirus* and *Babuvirus*. BBTV is a persistently aphid-transmitted virus (Figure 9) and is restricted to the phloem tissue (Thomas, 2015).

Figure 9: Colony of banana aphids, *Pentalonia nigronervosa* (Qazi, 2016)

This virus is characterized by two modes of transmission. It is locally transmitted by the aphid vector in a persistent circulative and non-replicative manner. In general, this transmission is carried out by *Pentalonia nigronervosa Coquerel* (Hemiptera: Sternorrhyncha: Aphididae). However, it has been shown that *Pentalonia caladii* is also a vector of BBTV. For long-

distance spread, this virus is vegetatively propagated, through infected plant material such as suckers and corms.

The vector transmission requires a latent period of 20 to 28 hours. There is no virus replication in the aphid. No mechanical transmission has been reported for this virus. It is not even transmitted by contact or agriculture tools (Bhadra & Agarwala, 2010; Qazi, 2016; Thomas, 2015).

BBTV has 18−20 nm isometric virions as shown in the following figure (Figure 10):

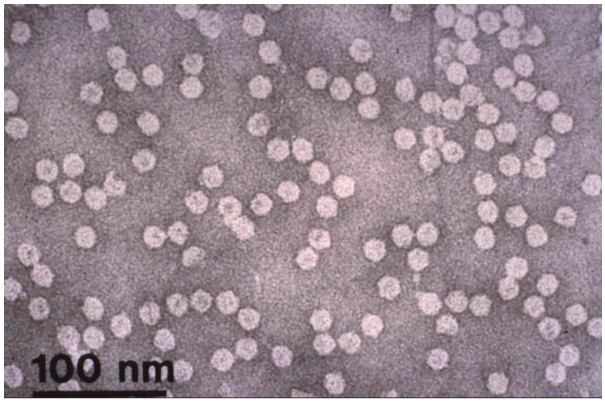

Figure 10: Isometric BBTV particles (Thomas, 2015)

Banana bunchy top disease is known to be the most economically devastating viral disease on banana and plantain. It causes significant crop losses worldwide that can reach up to 100% of yield reduction from infected plants (Bashir et al., 2022). In fact, when the infection is installed before the flowering, the virus-infected plants do not produce fruits and when it is a late infection, the plants produce deformed and inedible fruits (Ngatat et al., 2022).

In general, symptoms start to appear at the stage of two new leaves (incubation period). The infection causes strong symptoms on all leaves mainly dark green streaks on the leaves on the midrib, veins and leaf petioles (Qazi, 2016; Thomas, 2015).

All the species and cultivars from the *Musaceae* family are potential hosts for BBTV. However, attention needs to be paid for other alternate hosts that constituted inoculum sources. They include taro (*Colocasia esculenta*),

gingers such as *Alpinia zerumbet* (shell ginger) and *Hedychium coronarium* (white ginger), canna (*Canna indica*), arrowroot and anthurium (Hapsari et al., 2023).

BBTV is spread in many banana producing countries in Asia, the Oceania and Africa. The virus incidence is absent in Latin America and Carribean (Elayabalan et al., 2015; Stainton et al., 2015). Table 2 shows the distribution of BBTV in countries of the regions Asia, the Oceania and Africa.

Table 2: Distribution of BBTV in countries of the three continents: Africa, Asia and Oceania (adapted from (Thomas, 2015))

Continent	Countries	References
Africa	Angola	(Kumar et al. 2008)
	Benin	(Lokossou et al. 2012)
	Burundi	(Sebasigari and Stover 1988)
	Cameroon	(Oben et al. 2009)
	Central African Republic	(Foure and Lassoudiere, unpublished)
	Congo Republic	(Wardlaw 1961)
	Democratic Republic of Congo (formerly Zaire)	(Manser 1982) (Magee 1953)
	Egypt	(Manser 1982)
	Equatorial Guinea	(Manser 1982)
	Gabon	(Kenyon et al. 1997)
	Malawi	(Adegbola et al. 2013)
	Nigeria	(Sebasigari and Stover 1988)
	Rwanda	(Gondwe et al. 2007)
	Zambia	
Asia	Bangladesh	(Fouré and Manser 1982)
	China	(Thomas and Dietzgen 1991)
	Hong Kong	(Buddenhagen 1968)
	Indonesia	(Sulyo and Muharam 1985)
	India	(Magee 1953)
	Iran	(Bananej et al. 2007)
	Japan Ogasawara-gunto, (formerly Bonin Island) and Okinawa	(Gadd 1926) and (Kawano and Su 1993)
	Kampuchea	(Stover 1972)
	Korea	(Kiritani 1992)
	Laos	(Chittarhat et al., unpublished, 2015)
	Malaysia	(Su et al. 1993)
	Myanmar	(Furuya and Natsuaki 2006)
	Pakistan	(Soomro et al. 1992)
	Philippines	(Castillo and Martinez 1961)
	Sri Lanka	(Magee 1953)
	Taiwan	(Sun 1961)
	Thailand	(Wongsuwan and Chawpongpang 2012)
	Vietnam	(Vakili 1969)
Oceania	Australia	(Magee 1927)
	Fiji	(Magee 1927)
	Kiribati (formerly Gilbert Islands)	(hanmuganathan 1980)
	Marianas Islands (Guam; Saipan, Tinian and Rota)	(Beaver 1982; Miller et al. 2011) (Kagy et al. 2001)
	New Caledonia	(Magee 1927)
	Tonga	(Campbell 1926)
	Tuvalu (formerly Ellice Islands)	(Magee 1927; Dietzgen and Thomas 1991)
	USA (American Samoa; Hawaii)	
	Wallis Island	(Simmonds 1933)

	Western Samoa	(Magee 1927)

2.2. Banana bract mosaic virus

Banana bract mosaic virus (BBrMV) is the causal agent of bract mosaic disease which is a serious disease causing important economic losses in banana, up to 40% yield losses. This virus belongs to the genus Potyvirus of the family Potyviridae.

The primary transmission of the virus is conducted through infected suckers. Then, BBrMV is secondary spread by the banana black aphids, *Pentalonia nigronervosa* in a non-persistent manner. Moreover, it has been reported that its transmission by the aphid species *Rhopalosiphum maidis* and *Aphis gossypii* (Thomas, 2015).

BBrMV develops characteristic chlorotic spindle-shaped lesions on the leaves (parallel to the veins) and dark coloured (purplish) broad streaks and mosaic patterns on the bracts of the inflorescence in a banana plant infected with BBrMV (Madhavan et al., 2022; Siljo et al., 2012) (Figure 11).

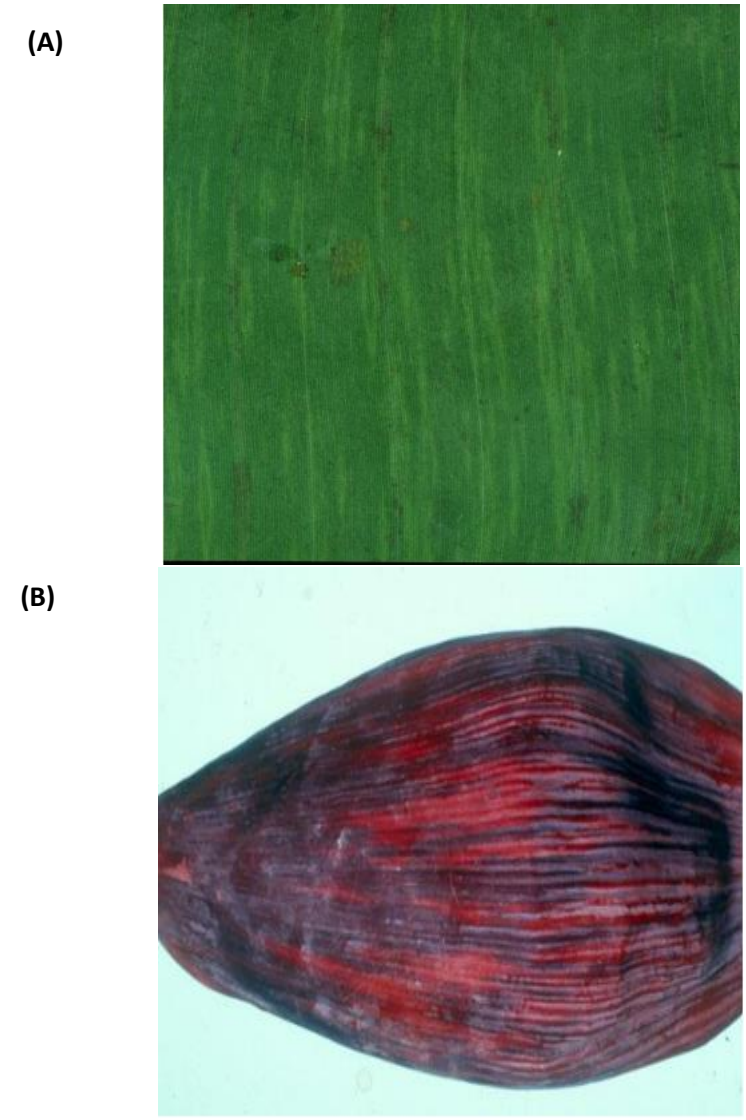

Figure 11: Symptoms of the infection of banana plant with BBrMV on (A) the leaves and (B) the bracts of the inflorescence (Thomas, 2015).

The virus has flexuous filamentous particles of about 700 to 750 × 11 nm with a single-stranded positive-sense RNA genome that consists of 9,711 nucleotides (Figure 12).

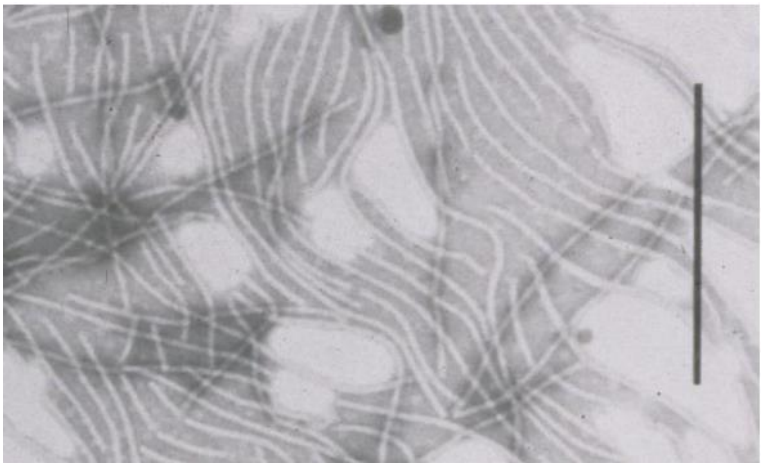

Figure 12: Purified virions of BBrMV. The scale bar represents 400 nm (Thomas, 2015)

Musa species and cultivars are the main hosts of BBrMV. Other alternate hosts of the virus were reported. They include the flowering ginger (*Alpinia purpurata*), the small cardamom (*Elettaria cardamomum*) and abaca (*Musa textilis*) (Balasubramanian & Selvarajan, 2014).

BBrMV is endemic in several banana producing countries which are the Philippines, India, Sri Lanka, Vietnam, Thailand, Western Samoa, Taiwan, and Hawaii (Thomas, 2015).

2.3. Cucumber mosaic virus

Cucmber mosaic virus (CMV) is the causal agent of banana mosaic disease. The virus belongs to the genus *Cucumovirus* of the family *Bromoviridae*.

The transmission of CMV is carried out from plant to plant using several aphid species such as *Aphis gossypi*, *Myzus persicae*, *Rhopalosiphum maidis* and *Rhopalosiphum prunifoliae*. However, *Aphis gossypii* were found to be the most common species spreading CMV, and therefore used for experiments of CMV transmissibility (Y & Wardany, 2022).

In general, symptoms include foliar mosaic, chlorotic streaking or flecking (Figure 13). In some cases, due to decreased chlorophyll infections, the chlorosis may cover the whole leaf.

Figure 13: Symptoms of the CMV infection on a banana leaf (Dheepa & Paranjothi, 2010)

CMV has icosahedral viral particles approximately 28–30 nm in diameter (Y & Wardany, 2022) (Figure 14).

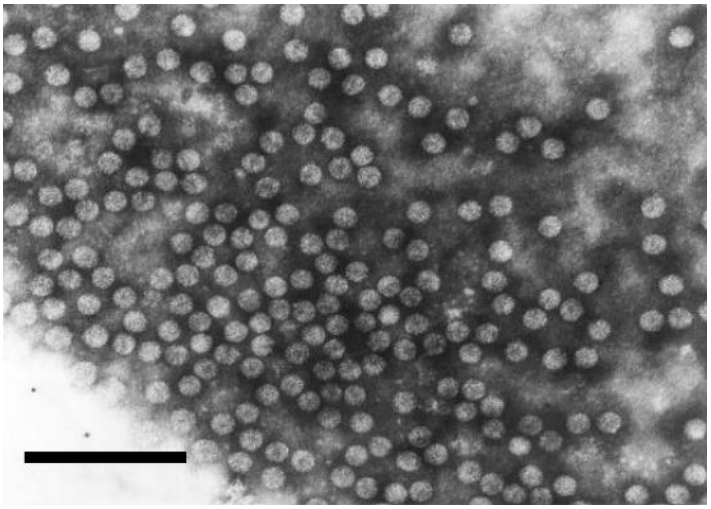

Figure 14: Virions of CMV. The scale bar represents 200 nm (Thomas, 2015)

The host range of CMV is the broadest of all plant RNA viruses. In addition to *Musa* spp., the virus infects around 1200 different plant species within more than 100 taxonomic families. The virus is commonly found in either cultivated crops such as pepper and tomato, or weeds like *Commelina* or wild populations of Arabidopsis (Montes et al., 2021).

CMV is an omnipresent virus with a very wide geographical distribution. It is found in all continents and in several countries. Nevertheless, some strains that induce serious symptoms are limited in distribution (Thomas, 2015).

2.4 Banana mild mosaic virus

2.4.1. General overview

Banana mild mosaic virus (BanMMV) is the causal agent of banana mild mosaic disease of banana and plantain. BanMMV is the first species of the proposed *Banmivirus* genus, belonging to the *Betaflexiviridae* family. It has a single stranded (ss) RNA genome. Its complete genome consists of 7,352 nucleotides, encoding five open reading frames (ORFs): one RNA dependant RNA polymerase, three triple gene block proteins and one coat protein. BanMMV has flexuous filamentous particles of about 580 nm in length (Figure 15).

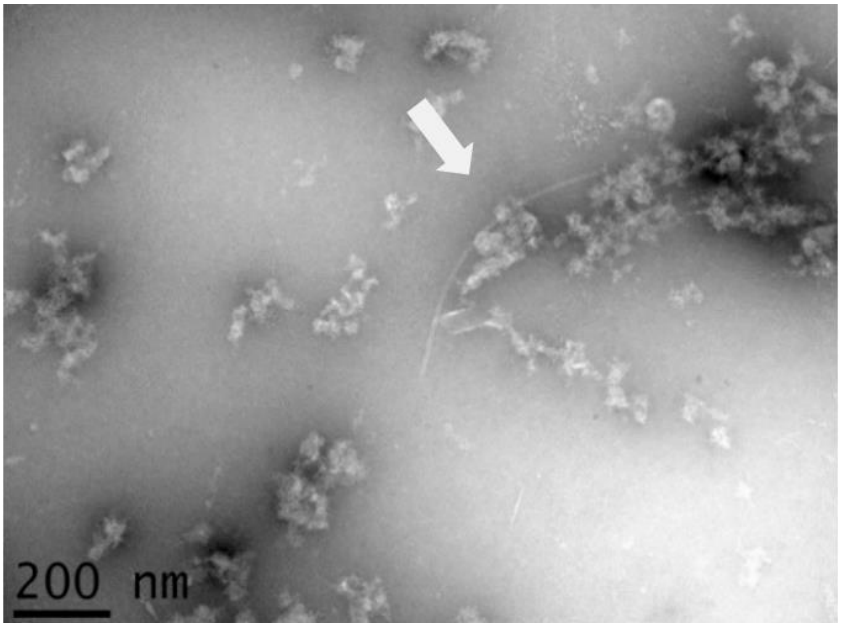

Figure 15: Flexuous filamentous particle (arrow) obtained after viral particle enrichment from leaves of one banana accession. The scale bar represents 200 nm.

BanMMV infects *Musa* spp. with a very wide geographic distribution. BanMMV is present in most, if not all, banana-producing areas of the world, in South America, Asia, Africa, Oceania and Australia. Usually, infected plants are symptomless. However, transitory symptoms, such as chlorotic streaks and mosaic, are occasionally observed on young leaves, but often disappear as the plant matures.

Musa species and cultivars are the hosts of BanMMV. The virus is found in almost all banana-producing countries (Thomas, 2015).

Previous studies have reported that some more pronounced symptoms of BanMMV infection were associated with a limited number of cultivars, including cv. 'Ducasse' (syn. 'Pisang Awak', ABB genome) (Figure 16) (Gambley & Thomas, 2001; Sharman et al., 2000).

Figure 16: Mosaic symptoms on a young leaf of banana cv. Ducasse (syn. Pisang Awak) (Thomas, 2015).

BanMMV often occurs as a mixed infection with other banana-infecting viruses such as banana streak viruses (BSV) or cucumber mosaic virus (CMV), and the mosaic symptoms of BanMMV may be masked by those of the other viruses. On the other hand, previous studies reported that, in Guadeloupe, infection by BanMMV exacerbated the symptoms of CMV causing leaf necrosis symptoms compared with mosaic symptoms from CMV infection alone (P. Y. Teycheney et al., 2005).

BanMMV is mainly transmitted through tissue culture or vegetative propagation. No natural vector has been identified and mechanical transmission has not been successful. Virus transmission attempts made from a single infection in cv. Ducasse failed with mealybugs and aphids, via soil collected from around infected plants, and by root-to-root contact. For BanMMV, it is frequent to detect highly divergent sequences simultaneously in a single plant (Pierre Yves Teycheney et al., 2005a).

2.4.2. Pest risk assessment of BanMMV

The French government has established a pest risk assessment of BanMMV in 2008 and has so prohibited the importation of vegetal materiel of banana (seeds, fruits, banana plants and their parts), although some flexibility has been noticed for the introduction of *Musa* vitroplants into the French overseas departments when the variety in question presents a real phytosanitary or phytotechnical interest in the context of local production. The country of

origin and the banana material must be exempt of BanMMV (Baker et al., 2008).

According to ANSES's expert report established in 2021, that described the obligations of the institutions producing banana vitroplants imported into French overseas departments and regions, there are strong uncertainties as to whether BanMMV fulfills the criteria of a quarantine organism due to its uncertain ability to cause damage on banana plants and to its uncertain presence in all the French overseas departments, although the evidence of BanMMV presence in Guadeloupe and Martinique (Teycheney, Laboureau, et al., 2005b). Nevertheless, the virus seems to fulfill the criteria of regulated non-quarantine pest as the risk is higher when the virus is present in the plant in a co-infection with *Cucumber mosaic virus*. For this category of organisms, their presence in banana vitroplants during the *in vitro* culture is associated with a significant risk for the banana industry, which requires the implementation of appropriate control measures. In fact, BanMMV has an important capacity to get propagated during the production of banana vitroplants, which leads to a high prevalence of contamination of vitroplants in the absence of control (especially that the virus can be asymptomatic on the mother plant), and so a high risk of introduction of the virus in a wide area. However, the probability of associations of these organisms with vitroplants during the acclimation phase seems to be low. Thus, it would be recommended to carry out visual monitoring accompanied by laboratory tests only in the event of observation of suspicious symptoms (ANSES, 2021).

2.5. Banana streak viruses

Banana streak viruses (BSVs) are the causal agents of banana streak disease. They belong to the genus *Badnavirus* of the family Caulimoviridae. BSVs exist under two different states: as an episomal form, causing infection in banana plants, or as viral DNA integrated within the nuclear genome of all diploid *Musa balbisiana* genitors. These endogenous BSV sequences are activated by biotic and abiotic stresses.

Actually, nine BSV species are recognized by the International Committee for the Taxonomy of Viruses (ICTV); Banana streak GF virus (BSGFV), Banana streak IM virus (BSIMV), Banana streak MY virus (BSMYV), Banana streak OL virus (BSOLV), Banana streak UA virus (BSUAV), Banana streak UI virus (BSUIV), Banana streak UL virus (BSULV), Banana streak UM virus (BSUMV), and finally Banana streak VN virus (BSVNV). In addition to

BSGFV, BSIMV and BSOLV for whom the endogenous viral elements (EVEs) are activated in hosts with B genome (*Musa balbisiana* and hybrids with B genome), the EVEs of BSMYV and probably those of BSVNV can also be activated. No link has been yet established between the activation of EVEs of the two viruses and their transmission via mealybugs (Iskra-Caruana et al., 2014).

Interestingly, a recent study has focused on the distribution of these nine BSV species over three clades of the *Badnavirus* genus (Figure 17).

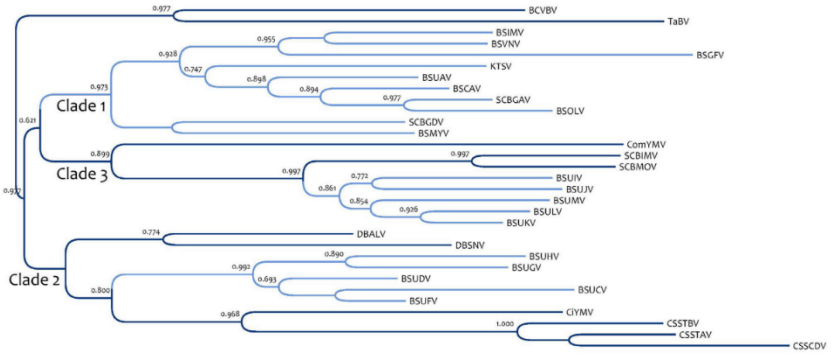

Figure 17: Phylogeny of BSV species (based on the Maximum-likelihood method with branching support >0.6) showing the structuring of species into 3 clades (Chabannes et al., 2021)

In fact, Clade 1 encompasses BSVs species detected as viral particles. This clade is composed of species integrated and activated in banana with B genome (BSIMV, BSOLV and BSGFV), other species with identified integrated sequences but with no evident ability to produce viral particles from these EVEs. Then, Clade 2 encompasses badnaviral sequences that are integrated in the banana genome. No episomal virus belongs to this clade. Finally, Clade 3 encompasses BSV species in episomal form that are not integrated into *Musa* genome. It includes species that are recognized by ICTV such as BSUIV, BSULV and BSUMV and the proposed species such as BSUJV et BSUKV (Chabannes et al., 2021).

BSVs infect all *Musa* species and cultivars. However, symptoms vary with isolates and cultivars. In most cases, the infection produces chlorotic streaks that become then darker resulting in necrotic streaks in leaves (Umber et al., 2022). Moreover, pseudostem splitting and cracks in fruit skins can be observed in the presence of the virus. BSVs are non-enveloped bacilliform

particles measuring 120−150 x 30 nm. An example of BSV viral particles is shown in Figure 18.

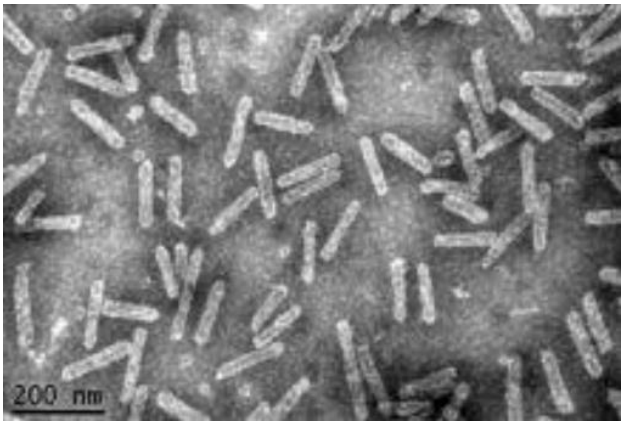

Figure 18: Viral particles of Banana streak Mysore virus (Thomas, 2015)

BSVs are spread in all continents and in several countries. Table 3 shows the affected countries per continent.

Table 3: Distribution of BSVs in the affected countries all over the world (adapted from (Thomas, 2015)

Continent	Countries	References
Africa	Benin	(Pasberg-Gauhl et al. 1996)
	Cameroon	(Gauhl et al. 1997; Lockhart 1995)
	Cape Verde	(Lockhart 1995)
	Côte d'Ivoire	(Lassoudière 1974, M-L Caruana, unpublished)
	Ghana	(Pasberg-Gauhl et al. 1996)
	Guinea Bissau	(Lockhart 1995)
	Kenya	(Lockhart 1995)
	Madagascar	(Jones and Lockhart 1993)
	Malawi	(Vuylsteke et al. 1996)
	Mauritius	(Jaufeerally-Fakim et al. 2006)
	Morocco	(Lockhart 1986)
	Nigeria	(Gauhl et al. 1999; Pasberg-Gauhl et al. 1996)
	Rwanda	(Sebasigari and Stover 1988)
	Sierra Leone	(Lockhart 1995)
	South Africa	(Jones and Lockhart 1993)
	Tanzania	(Sebasigari and Stover 1988)
	Togo	(Lockhart 1995)
	Uganda	(Dabek and Waller 1990; Geering et al. 2005; Harper et al. 2005)
	Zanzibar	(Vuylsteke et al. 1998)
Asia	China, Peoples' Republic	(Zhuang et al. 2011)
	India	(Cherian et al. 2004)
	Indonesia (New Guinea)	(Davis et al. 2000; Lockhart 1995)
	Jordan	(Lockhart 1995)
	Malaysia	(Ang and Ong 2000)
	Philippines	(Lockhart 1995)
	Sri Lanka	(Lockhart 1995)
	Taiwan	(Su et al. 1997)
	Thailand	(Aung et al. 2012; Lockhart 1995)
	Vietnam	(Lheureux et al. 2007; Lockhart 1995)
Oceania	Australia	(Thomas et al. 1994)
	New Caledonia	(Lockhart 1995)
	Papua New Guinea	(Davis et al. 2000)
	Tonga	(Thomas et al. 1994)
	Western Samoa	(Thomas et al. 1994)
Europe	Spain (Canary Islands)	(M-L Caruana, unpublished)
	Portugal (Madeira)	(Jones and Lockhart 1993)

South and Central America	Brazil	(Carnelossi et al. 2014)
	Colombia	(Reichel et al. 2003)
	Costa Rica	(Pasberg-Gauhl et al. 2000)
	Cuba	(Javer et al. 2009; Jones and Lockhart 1993)
	Dominican Republic	
	Ecuador	(P-Y Teycheney et al., unpublished)
	Grenada	(Jones and Lockhart 1993)
	Guadeloupe	(Jones and Lockhart 1993)
	Honduras	(Jones and Lockhart 1993)
	Jamaica	(Jones and Lockhart 1993)
	Martinique	(Jones and Lockhart 1993)
	Nicaragua	(Jones and Lockhart 1993)
	Peru	(Lockhart 1995)
	Trinidad	(Pasberg-Gauhl et al. 2007)
	USA (Florida, Puerto Rico, Virgin Islands)	(Jones and Lockhart 1993) (Lockhart 1995)
	Venezuela	(Garrido et al. 2005)

The main challenge in the diagnostic of BSV was to distinguish between infections from the episomal form of the virus and the one from integrated viral sequences. This was achieved through the addition of an Immunocapture step before the Polymerase Chain Reaction process in order to avoid the amplification of plant genomic DNA (Thomas, 2015). Interestingly, the CRISPR/Cas9 system, which is a recent method used as a gene-editing tool, was performed in order to inactivate the endogenous BSV by targeting the three ORFs of the virus (L. Tripathi et al., 2022).

3. Evaluation of the accuracy of a diagnostic test

The investigation of the accuracy of any diagnostic test (HTS-based protocol, PCR-based protocol, etc) requires the evaluation of its performance characteristics that are mainly sensitivity, specificity, repeatability, and reproducibility (Soltani et al., 2021). It is also important to understand other performance criteria such as predictive values and likelihood ratios (Schlattmann, 2022).

For sensitivity, we distinguish diagnostic sensitivity and analytical sensitivity. Similarly, specificity is divided into characteristics; diagnostic specificity (DSP) and analytical specificity.

Diagnostic sensitivity (DSE) is the percentage of infected samples tested positive for a test. The higher the DSE is, the lower the number of false negative results. The false negative rate (FNR) can be calculated following this formula: FNR=1-DSE. Furthermore, analytical sensitivity refers to the limit of detection of the target using diluted infected samples. It was defined by the EPPO standard PM 7/98 as ‘ the smallest amount of target that can be detected reliably’. A very high DSE value alone is not enough, it is also mandatory to have its diagnostic specificity is (Massart, Lebas, et al., 2022; Van Stralen et al., 2009).

Diagnostic specificity (DSP) is the percentage of healthy samples tested negative for a test. The higher the DSP is, the lower the number of false positive results. The false positive rate can be calculated following this formula: FPR=1-DSP. DSP should not be confused with analytical specificity. According to the EPPO guidelines, analytical specificity refers to inclusivity and exclusivity (Luigi et al., 2022).

As described in the EPPO Standard PM 7/98 (2019), the repeatability is defined as 'the level of agreement between replicates of a sample tested under the same conditions' whereas the reproducibility is 'the ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, person, equipment, location)' (Massart, Lebas, et al., 2022).

The repeatability and reproducibility are assessed through the calculation of concordant and non-concordant results between replicates, no matter the status of the samples. Thus, the repeatability is estimated by the calculation of the accordance through the evaluation of the probability of achieving the same test results for identical samples within laboratories. However, the reproducibility is estimated by the calculation of the concordance through the evaluation of the probability of achieving the same test results for identical samples between laboratories (Langton et al., 2002). It is important to note that if concordance is smaller than accordance, this means that two replicates can give the same result when they tested in the same laboratory and not different laboratories. Concordance can be affected by different factors such as the equipment, the environment, the operator, the day, etc (Massart, Lebas, et al., 2022).

4. Plant virus detection methods

The plant viral detection methods can be divided into two groups; specific and non-specific methods,. Specific or targeted methods include serological and molecular tests, thus *a priori* knowledge on the targeted virus(es) is crucial. However, in some cases, the knowledge of the targeted virus(es) is limited to the viral family or genus. Hence, the use of degenerate primers that are more universal and general than specific primers, allows the detection of several viral genera from the same family or several species from the same genus (Linhart & Shamir, 2005). On the other hand, non-specific methods, including biological indexing, electron microscopy and High Throughput Sequencing require no prior knowledge (Adams et al., 2016).

4.1. Traditional methods of plant virus detection

4.1.1. Specific methods

4.1.1.1. Biological detection

Biological indexing is an essential tool based on biological assays to detect a particular plant virus. This method consists of mechanical inoculation with sap, graft or bud in indicator plants. There are two main types of indicator plants: herbaceous and woody. The sensitive indicator hosts will subsequently develop various symptoms allowing the identification of the virus with more or less accuracy, which is defined as the proportion of true (positive and negative) test results of the tested samples. Plants can express symptoms that are similar to viral diseases due to nutritional disorders and/or harsh abiotic conditions. Thus, it is important to distinguish between nutrition or environment influence and the symptoms induced by the inoculation of a pathogenic virus through the combination of other methods to confirm the detection (Massart, Lebas, et al., 2022; Van Der Want & Dijkstra, 2006).

Biological indexing is non-destructive, convenient and simple to operate. There is also no need for expensive equipment or devices. Indeed, indicator plants provide relatively high accuracy but are excessively time-consuming as they require long time from grafting to the development of symptoms (usually

several weeks for symptom development after inoculation). Most biological assays are not specific or sensitive enough to be used on their own, in particular in the case of isolates or strains of viruses that are asymptomatic or sometimes remain latent in the planting material. Furthermore, some dedicated facilities are required to perform a biological assay, such as a temperature-controlled greenhouse, which may be labor- and space-consuming and difficult to manage especially for large scale testing (Legrand, 2015; Wang et al., 2022).

4.1.1.2. Serological detection

Serological detection approaches are based on the use of viral proteins (mainly coat protein) for virus detection. They consist of the interaction between one or many proteins in the virus particle, which constitute antigens, with antibodies selected to recognize them.

Hereunder are presented the different immunoassays used for plant virus detection; Enzyme-Linked Immunosorbent Assay (ELISA), Tissue Immunobinding Assay (TIBA) and Immunosorbent Electron Microscopy (ISEM).

4.1.1.2.1. Enzyme-Linked Immunosorbent Assay: ELISA

The use of ELISA is proved to be a very valuable tool for the detection of plant viruses. ELISA method was first used in the 1970s is based on an antigen (viral coat protein)-antibody interaction. It can use either polyclonal or monoclonal antibodies. It is so far the most widely used immunodiagnostic technique because of its several advantages (Kalimuthu et al., 2022).

4.1.1.2.2. Dot Immunobinding Assay: DIBA

Like others serodiagnostic techniques, the DIBA is based on the formation of antigen-antibody complex. It is a simple and highly reproducible dot blot assay where antigen suspensions are dotted onto nitrocellulose or nylon membranes rather than microtitre plates. Comparing to ELISA, this method is cheaper, less time consuming, shows same diagnostic sensitivity and interestingly requires small amounts of antigen (Abd El-Aziz, 2019).

4.1.1.2.3. Immunosorbent Electron Microscopy: ISEM

It is a combination serology-electron microscopy technique that has been developed as a new diagnostic tool during the late-1970s. It allows the rapid

detection and characterization of a broad range of plant viruses. Firstly, copper grids are coated with antiserum containing specific antibodies to the virus. Then, the excess of antibody is washed off, followed by the treatment of grids with infected plant sap. Thus, virus particles are selectively “trapped” and coated with the antibodies. Therefore, the grid is observed under a transmission electron microscope (Kirankumar et al., 2020).

4.1.1.3. Molecular detection

4.1.1.3.1 PCR

The Polymerase Chain Reaction (PCR) is an *in vitro* method which uses enzymes (called a polymerase) to amplify, exponentially, targeted DNA sequences (Eeles et al., 1992). It remains one of the most important scientific discoveries of the twentieth century and has been widely used for the detection and characterization of plant viruses. This method can overcome the problem associated with serological detection such as limited multiplexing options and insufficient level of analytical sensitivity. In fact, PCR showed the potential to have a greater sensitivity than ELISA (Niimi et al., 2003).

The "Polymerase Chain Reaction" or PCR consists in general on 30 to 40 cycles of 3 steps. The first step called denaturation allows the break of double-stranded DNA into single strands through heat, by disrupting hydrogen bonds between complementary bases at 94°C/95°C. The second step called annealing consists of the binding of the primers to their complementary sequences on single-stranded DNA (ssDNA) DNA. When the strands are separated, the temperature decreases between 40 and 65°C (depending on the primer sequence) which allows primers to anneal to ssDNA. The third step of the PCR technique is the extension step. As indicated by its name, this step allows the extension of DNA strands from hybridized primers. The temperature used is 72°C which is the optimal temperature for DNA polymerase activity (Zarlenga & Higgins, 2001) (Figure 19).

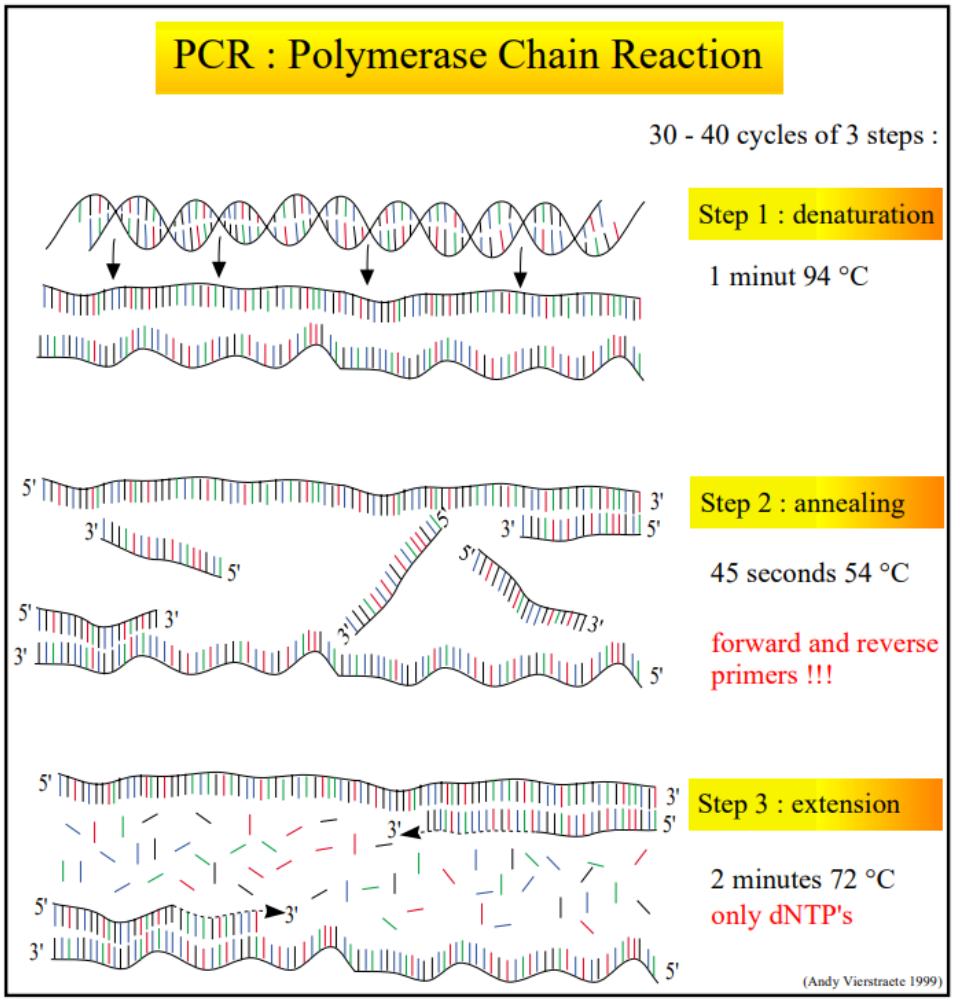

Figure 19: The three basic steps in PCR (Vierstrate,1999)

** RT-PCR

Unlike DNA viruses that can be directly detected, RNA viruses need to be reverse-transcribed to a complementary DNA before PCR. This is called Reverse Transcription (RT)-PCR. Amplification and RT can be performed in the same tube (one-step RT-PCR) or separately by transcribing first the RNA into cDNA and then using this cDNA strand as a template for amplification (two-step RT-PCR). It is methodeperformed for the detection of BBrMV, CMV, and BanMMV.

** Multiplex PCR/RT-PCR

Multiplex PCR, a variant of PCR, is the simultaneous amplification of two or more loci (James et al., 2006). For instance, (Sharman et al., 2000) have developed a multiplex, immunocapture PCR (M-IC-PCR) for the simultaneous detection of three viruses from crude sap extracts of banana and plantain. In same line, (Le Provost et al., 2006) have developed a M-IC-PCR assay for the detection of BSV. On the other hand, (Komínková and Komínek, 2020) developed and validated a multiplex RT-PCR for the simultaneous detection of two viruses and two viroids: grapevine fleck virus, grapevine Pinot gris virus, grapevine yellow speckle viroid 1, and hop stunt viroid.

** Immunocapture PCR/RT-PCR

The addition of an immunoassay to entrap virus particles before its amplification by PCR using specific oligonucleotides, allows the purification of the virions from the plant sap. This technique, valid for either RNA or DNA viruses, seems to be adequate for viruses present in low titre in the plant and/or for which certain inhibitors are present in the crude extract (Rimhanen-Finne et al., 2002).

**Real time PCR (qPCR)

Real time PCR has the advantages to be more sensitive, accurate and specific comparing to conventional PCR. It allows the quantification of the amplified nucleic acid. It is based on the use of fluorescent dyes for marking amplicons or primers and thus monitoring the amplification process of the PCR in real time (Mackay et al., 2002). For instance, (Delanoy et al., 2003) have developed a real time PCR assay in for the rapid detection of episomal BSV.

4.1.1.3.2. Isothermal amplification

Loop-Mediated Isothermal Amplification : LAMP

LAMP allows the amplification of target DNA at a constant temperature in a short span of time (within 1 hour with sensitivity similar to other reliable molecular methods). It combines high specificity, simplicity and rapidity

under isothermal conditions. Unlike PCR-based methods, the LAMP reaction does not require a thermocycler. Thus, it can be performed for either in-field or laboratory conditions. A LAMP test is an amplification assay which is performed using 4 to 6 primers (inner and outer primers) that recognize several distinct regions on the target gene. The inner primer enhances the specificity of the amplification. It consists of two types of elongation reactions occurring at the loop regions. Like PCR, only one type of enzyme is required in this reaction (Caruso et al., 2023; Notomi et al., 2015; Wilisiani et al., 2019). The LAMP assay, like PCR, has different variants such as RT-LAMP, Multiplex LAMP and Real-time LAMP. This method is used fot the detection of several plant viruses such as *banana streak virus*, *tobacco mosaic virus*, *potato mosaic virus* Y or *cauliflower mosaic virus*,etc (Garg et al., 2022) .

- Reverse transcription lamp: RT-LAMP

RT-LAMP is a one-step assay during which a complementary DNA is synthesized from RNA before its amplification at a constant temperature, and its detection. Contrary to qPCR, RT-LAMP can be used without the sample preparation which can provide results more quickly and with less costs (Schermer et al., 2020).

- Multiplex LAMP: mLAMP

Apart from its major merits, this technique has shown high specificity and inclusivity. In fact, mLAMP targets many genes in order to detect multiple pathogen sequences at the same time. It showed also great performances in distinguishing between divergent strains of the same pathogen (Garg et al., 2022).

- Real-time LAMP

Real-Time LAMP is mainly used for extensive sample analysis that requires quantitative results in short time. In this context, (Lopez-Jimena et al., 2018) have assessed the performances of this technique on the detection of the dengue virus, a human virus transmitted by mosquitoes. They found out that a 100% of reproducibility and specificity, and 95.8% of sensitivity. This method is also known with its practicality. In fact, it can be simply performed using a smartphone application and a camera for color surveillance during amplification. However, this method can show some disadvantages such as the false positives (Garg et al., 2022).

4.1.2. Non-specific methods

4.1.2.1.Visual observation of symptoms

Visual observation is based on the inspection/observation of visual symptoms of the plants in field or greenhouse and the comparison of these symptoms with a healthy plant. The general condition of the plant and several other morphological features are observed through this method, such as plant height, leaf colour and shape, the root system, etc. Some observations can provide insights about the parasitic or non-parasitic origin of the disease. Indeed, when the distribution of symptoms on the plant is irregular and when there is a progression of infection from a source with crop-specific symptoms, it is about a parasitic disease. Whereas the regular distribution of symptoms and the absence of progression of symptoms indicates that the disease is non-parasitic. Furthermore, the symptoms can be generalist or specific to the type of crop. For example, the symptoms on the cereal grass crops can be recognized through the appearance and the particular shape of the lesion. In the case of rice blast disease which is caused by the fungus *Pyricularia oryzae*, the necroses are present with darker outline. Visual observation is important but is not reliable for a definitive diagnosis, as many viral infections in plants are symptomless. Thus, the confirmation of the disease using complementary analysis is compulsory (Khakimov et al., 2022).

In the case of banana, the typical symptoms of viral diseases are the formation of chlorotic or dark coloured streaks starting from the leaf midrib to the margin. They can be interrupted with mosaic patterns. In some cases, such as the CMV infection, a leaf deformation can be occasionally observed (Thomas, 2015).

4.1.2.2. Electron microscopy

This technique is based on the observation of morphological properties of the virus particle such as the size and the shape (e.g. filamentous or rod-shaped viruses). It is used for the detection and identification of viral particles. Although this method is used reliably for the investigation of viruses ultrastructural features, it requires the preparation of tissue samples. However, such preparation is time and labor consuming (can take several days to get results). Moreover, it requires expensive and highly specialized equipment. In

addition, operators need to undergo a long period of training to correctly use this technology (Zechmann & Zellnig, 2009).

4.2. Recent and emerging methods of plant virus detection

4.2.1. High-Throughput Sequencing : HTS

High throughput sequencing (HTS), also known as deep sequencing or next generation sequencing (NGS) is a laboratory based method that has revolutionized the identification of either known or unknown viruses. This method consists of detecting nucleic acid content in biological samples, in particular plant samples, for identification of pathogens using bioinformatics. The adoption of HTS technologies for the detection of plant pathogens has been growing steadily in the last decades. They are mailny used in order to identify pathogens causing novel diseases (or diseases of unknown aetiology), to improve knowledge of genomic diversity through the sequencing of genomes from known pests, for the surveillance of pests,etc (Baranwal et al., 2021).

The HTS process can be divided into eight steps as it is shown in Figure 20.

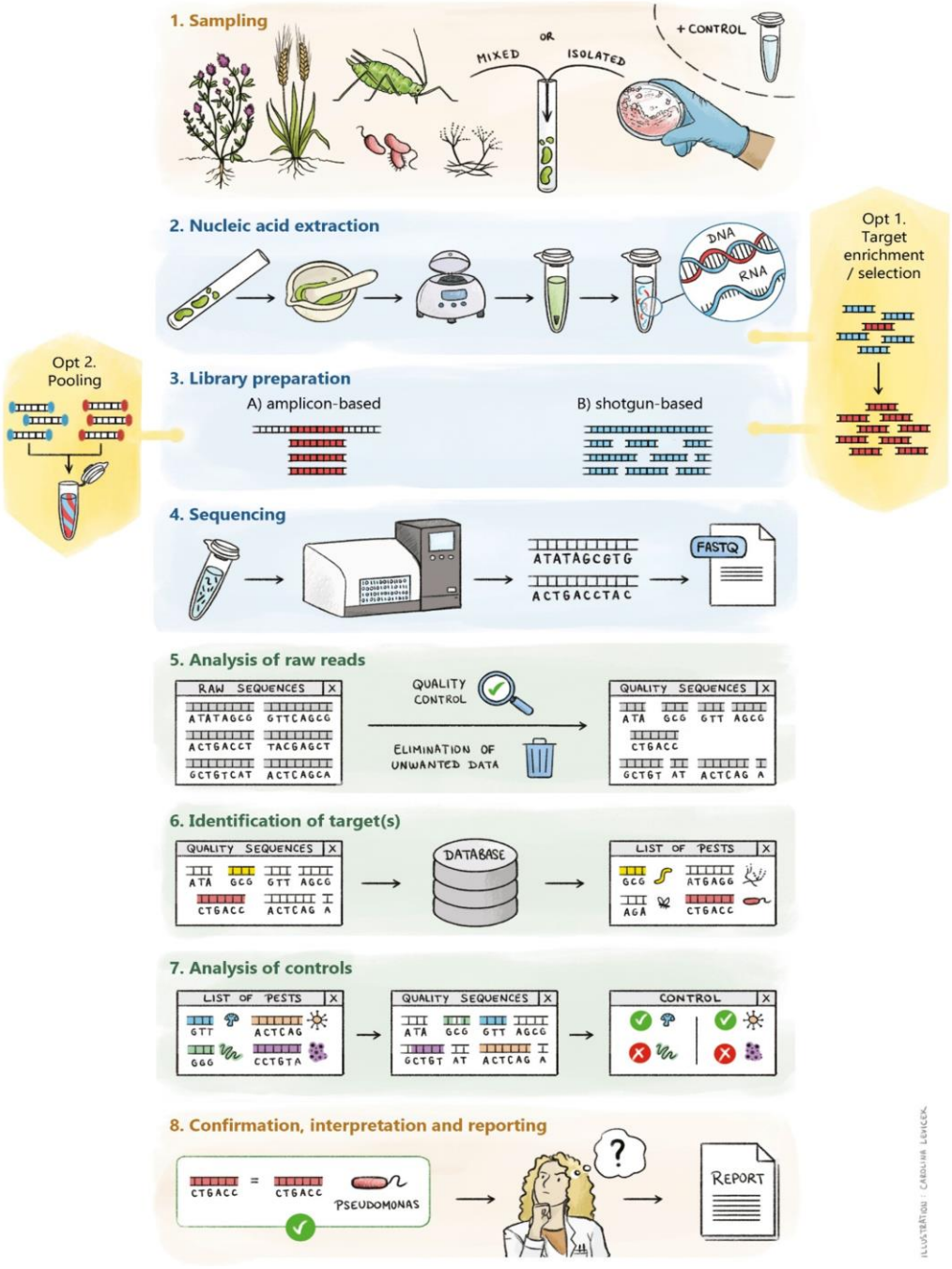

Figure 20: Schematic representation of the main steps of an HTS assay adopted in plant health diagnostics (Lebas et al., 2022)

First of all, the sampling is quite similar to any sampling for a diagnostic test. The matrix to be sampled can contain mixed or isolated organisms. It is important to sample also controls for the HTS assay. Then, the nucleic acid extraction is performed in general using a kit. The nucleic acids can be genomic DNA or RNA. The third step consists on library preparation. Two main approaches are commonly adopted for plant virus detection; amplicon sequencing or metabarcoding and shotgun sequencing. Two optional steps can be added at this stage. Firstly, the enrichment or target selection which is performed by either removing untargeted sequences (e.g. ribodepletion) or using oligonucleotides specific to the targets. Secondly, the multiplexing or the pooling of samples is an option that can be added in many library preparation protocols in order to reduce the cost per sample. The fourth step consists on the sequencing. It is important to choose the convenient sequencing platform according to the purpose of the research. Then, the bioinformatic analysis needs to be performed. It starts with the analysis of raw reads with the check of the quality control of the generated sequences and the elimination of unwanted data. Then, comes the identification of targets which aims to associate sequences with specific organisms through the comparison of the obtained sequences with sequences from database to establish the list of pathogens present in the sequenced sample. In order to make sure that the results obtained are reliable and don't include false positives and/or false negatives, the analysis of controls needs to be performed. Finally, the eighth and last step of the HTS test consists on the confirmation of the target(s) detected in the sample, the interpretation of its/their relevance and the reporting of the results (Lebas et al., 2022; Massart, Adams, et al., 2022).

These technologies showed very high sensitivity to detect viruses in plants, without *a priori* information, especially when they are present in low concentrations in the host. However, this high performance can be sometimes problematic in the case of contaminations that trigger to the detection of false positives (Rott et al., 2017).

4.2.2 Droplet Digital PCR: ddPCR

The ddPCR is a technology based on water-oil emulsion droplets. It enhances the amplification efficiency of PCR, in particular when viruses have uneven distribution in their hosts and are present in low titre (Selvarajan & Balasubramanian, 2017).

4.3. Diagnostic techniques used for banana viruses

Hereunder a summary table that shows the different diagnostic techniques according to each of the five most economically important banana viruses (Table 4).

Table 4: Diagnostic methods for each banana virus (adapted from (Thomas, 2015))

Banana virus	Diagnostic techniques used for its detection
BBTV	- ELISA (monoclonal and/or polyclonal antibodies are commercially available) - PCR assays; Real time PCR, Multiplex PCR - LAMP - Electrochemiluminescence PCR - Impedance spectroscopy
BBrMV	- ELISA (monoclonal and/or polyclonal antibodies) - PCR assays; RT-PCR, IC-RT-PCR and Multiplex-IC-PCR - LAMP
CMV	- ELISA - Multiplex PCR assays - Observation of symptoms - Electron microscopy - The use of indicator plants - Nucleic acid hybridization
BSVs	- ELISA (kits from Agdia and DSMZ companies) - ISEM - IC-PCR (addition of DNAase I treatment) - RCA - LAMP
BanMMV	- IC-RT-PCR - ISEM - Nested IC-RT-PCR

4.4 Comparison between diagnostic techniques

ELISA showed high performances as a diagnostic tool for virus certification and quarantine purposes, being robust, speed, easy to use and to implement, cost effective and adaptable for a large-scale testing of samples. But, when it is about the testing of different pathogens in one crop, nucleic acid-based

methods seemed to be more flexible and compatible. In fact, they allow several tests to be runed in parallel from the same samples (prepared from a single plant nucleic acids) or as multiplex assays (Boonham et al., 2014).The first publication of PCR methods for virus detection was in the early 1990s. They showed better specificity and sensitivity for virus detection comparing to ELISA. At the same time, this high sensitivity constituted a serious issue since it caused recurring problems with post-PCR contamination and false positive results. Therefore, the use of closed-tubes throughout real-time PCR or quantitative PCR (qPCR) helped to solve the problem of post-PCR contamination since the fluorescent signal can be detected during or at the end of amplification. This technique showed benefits of high reproducibility and accuracy. Despite the advantages of the PCR assays, these tests still require expensive reagents, laboratory equipment and highly trained professionals. They can also be time-consuming. Over the last decades, many studies have concluded that LAMP is cost-effective, rapid, and far more sensitive as well as specific in comparison PCR. They are less sensitive to inhibitors than PCR. The additional advantage is that this method can be used for the diagnosis of various diseases in particular in developing countries (as they can be used directly by farmers *in situ*). However, in contrast with PCR that requires only two primers (uniplex assay), this technique is associated with a relatively complex assay of design selecting up to six primers. Interestingly, this disadvantage can be simplified with the use of primer design softwares. An additional disadvantage of LAMP is the high possibility of contamination and the production of false positive results due to its high sensitivity comparing to PCR (Da Costa et al., 2023; Matthew et al., 2022; Tomlinson & Boonham, 2008).

Then, the application of HTS technologies has shown great advantages over other diagnostic methods. In fact, they give a complete view about the viral phytosanitary status of a plant. They can also detect all viruses, and the only limitation for that is the completeness of databases against which the sequences are compared (Maree et al., 2018).

5. Impact of climate change on plant virus diseases

The manifestations of climate change, also known as global warming, consist mainly of increasing in mean temperatures, changes in humidity conditions, mean precipitation disturbances (flooding in the tropical areas and drought in the middle latitude regions), disturbances in wind patterns, but also a rising in atmospheric carbon dioxide (CO_2) levels due to the increasing fossil fuels consumption (Trebicki et al., 2015).

Plant viruses constitute a serious constraint to productivity and yields of horticultural crops worldwide. Weather changing conditions are impacting, directly or indirectly, the plant health. In fact, the emergence of plant viral diseases and the vectors transmission are highly influenced by climate change (Pautasso et al., 2012).

Surveys have reported that higher concentrations of CO_2 might affect the physiology and the biochemistry of several plants such as wheat, barley or paddy, and increase the disease occurrence in these crops. For instance, (Trebicki et al., 2015) showed the direct link between an increased titre of *barley yellow dwarf virus* (BYDV) in wheat plants and elevated CO_2 (eCO_2) levels. Although there is no exact explanation of this phenomenon, some scientists have elaborated hypothesis about the effects of elevated CO_2 on plants. In fact, (Trębicki et al., 2016) supposed that eCO_2 conditions increase the temperature of the leaf canopy and increase relatively the carbon (C):nitrogen (N) ratios in leaves through to the higher photosynthetic and growth rates. This variability in plant growth was shown to influence the spread of contact-transmitted viruses in plants (Dash et al., 2021).

On the other hand, the gradual raise of temperatures is affecting the natural "immune" systems of the plant which are composed mainly of the PAMP-triggered immunity (PTI) and the effector/ environment-triggered immunity (ETI). In fact, these two defence systems are slowed down under high temperatures, which makes the plants stressed and vulnerable. Thus, they cannot protect themselves from viral attacks or any other pest and vector attacks. In addition, they become susceptible to emerging viral infections (Rosenzweig et al., 2007; Velásquez et al., 2018)

The most common vectors of plant viruses are Hemiptera insects like aphids, whiteflies, and hoppers. These sucking insects are responsible for the

transmission of 70% of all plant viruses. Aphids alone transmit 50% of the vector-borne plant viruses. In fact, they can achieve five extra generations in a one year with an increasing temperature of 2°C due to their short generation time. In addition, it has been reported that eCO_2 concentration play a key role in the abundance of aphids (Harrington et al., 2007; Newman et al., 2003).

An interesting example illustrated the impact of climate change on banana viruses. In this context, a survey has shown an impact of temperature on the symptom development, the transmission efficiency, the vector biology and the spread of *Banana bunchy top virus* (BBTV). The assessment of acquisition efficiency in terms of temperature and using aphids showed that the absence of virus transmission at 16°C. However, a maximum transmission was obtained at 27°C, which corresponds to the ideal temperatures for banana plant growth (Anhalt & Almeida, 2008).

Although the climate change poses a risk in increasing of banana viruses transmission and in emerging diseases, it has been recently considered as beneficing beneficial global yields for banana cultivation from 1961 to 2016. In fact, for 27 countries across the world, providing around 90% of dessert banana production in the world and covering over 80% of the total territory under cultivation, the annual yields have increased by 1.37 t ha^{-1} on average. However, for these largest banana exporters, things might change by 2050. In fact, it has been shown that global banana yields could be reduced to 0.59 t ha^{-1} by 2050 due climate change effects (Varma & Bebber, 2019).

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Chapter 2

Context and objectives of the thesis

Context

This thesis is part of the activities of the Germplasm Health Unit (GHU) which is based in the Integrated and Urban Plant Pathology Laboratory, Gembloux AgroBio Tech, University of Liège, Belgium. The GHU has two main missions; the first consists on the indexing or the determination of the viral status of *Musa* accessions. The second consists in the elimination of one or more viruses from *in vitro Musa* plants (through thermotherapy or chemotherapy). The plant material used in the GHU is mainly supplied by the Bioversity International Transit Centre (ITC). In fact, the ITC is the world's largest collection of *Musa* germplasm, founded in 1985. It is based at the Katholieke Universiteit Leuven (KUL) in Belgium. This centre holds a cryopreserved *in vitro* collection which is composed of 1,617 banana accessions from 38 countries. It has played a key role in the intercontinental exchange of banana germplasm by distributing 18,000 banana accession samples during the last 35 years in 113 countries. The ITC distributes 62% of Musa germplasm to developing countries to National Agricultural Research Systems and 38% of the germplasm to universities and institutes for advanced research in developed countries (Panis et al., 2020; Van den houwe et al., 2020).

The movement of vegetative germplasm is hampered by the presence of pests, in particular viruses. These microbes constitute a serious threat as they can be symptomless. Thus, they are easily transmitted through *in vitro* culture, especially in the case of vegetatively propagated crops like bananas. All banana viruses are not present in all banana-growing countries. That's why, it is crucial to avoid their introduction in new regions in order to prevent some eventual outbreaks. There is a requirement of guaranteeing the exchange of germplasm free from contamination through the reliable and specific detection of viruses infecting *Musa* plants. In this context, the International Plant Protection Convention (IPPC), a plant health treaty signed by over 180 countries, was developed in the 1920s in order to address pests that are both directly and indirectly harmful to plants (Schrader & Unger, 2003).

The GHU is acting in this sense by providing a comprehensive virus testing scheme for each tested accession, which allows the conservation and distribution of this accession in the collection, either for research, direct use or breeding. Indexing tests (molecular and serological) are carried out for the five most important banana viruses and are completed by electron microscopy

observation, in order to determine the viral status of the banana accession (De Clerck et al., 2017) .

However, in some cases, these tests can be obsolete in particular when the accession is infected by a new viral specie or a distant variant from known species. Thus, it is crucial to continuously optimize of the detection methods used to ensure their reliability. This has constituted the main objective of this work.

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Objectives of the thesis

The underlying objectives of this thesis were to:

i) explore *Betaflexiviridae* virus diversity within banana plants

Throughout this study, both *in vitro* and *in vivo* plants were tested for BanMMV presence using molecular tools. Firstly, 110 accessions of banana were tested regarding BanMMV in order to identify their viral status. Through this large-scale testing, two novel divergent isolates of BanMMV were identified and sequenced using High Throughput Sequencing technologies. Then, the testing of 137 *in vitro* plants triggered to the identification of a new *Betaflexiviridae* species for which the early steps of biological characterization have been already performed.

ii) improve detection of BanMMV from banana plants

The identification of two divergent isolates of BanMMV triggers to the development of a new diagnostic primer (BanMMV CP9) to improve the detection of the virus. A retrospective analysis of 110 different *Musa* accessions (from different origins and different viral statuses) was carried out in order to compare the new and the common primers. According to the results, we have recommend the use of the two primers in order to improve the inclusiveness of the protocol.

iii) compare RT-PCR and HTS technologies for the detection of BanMMV from *in vitro* banana plants

RT-PCR and HTS tests were applied on the same total RNA samples extracted from the base and leaves of in vitro banana plants. The diagnostic sensitivity was of 65% and 100% , respectively for RT-PCR and HTS. In addition to its high diagnostic sensitivity, HTS showed 100% of diagnostic specificity and allowed the identification of a new *Betaflexiviridae* species infecting *Musa* species.

Chapter 3

Identification of Divergent Isolates of Banana Mild Mosaic Virus and Development of a New Diagnostic Primer to Improve Detection

This chapter is adapted from: Hanafi M., Tahzima R., Ben Kaab S., Tamisier L., Roux N., and Massart S., (2020). Identification of divergent isolates of banana mild mosaic virus and development of a new diagnostic primer to improve detection. *Pathogens,* 1045

Abstract:

Banana mild mosaic virus (BanMMV) (*Betaflexiviridae*, Quinvirinae, unassigned species) is a filamentous virus belonging to the *Betaflexiviridae* family. It infects *Musa* spp. with a very wide geographic distribution. The genome variability of plant viruses, including the members of the *Betaflexiviridae* family, makes their molecular detection by specific primers particularly challenging.

During routine indexing of the *Musa* germplasm accessions, a discrepancy was observed between electron microscopy and immunocapture (IC) reverse transcription (RT) polymerase chain reaction (PCR) test results for one asymptomatic accession. Filamentous viral particles were observed while molecular tests failed to amplify any fragment. The accession underwent high-throughput sequencing and two complete genomes of BanMMV with 75.3% of identity were assembled. Based on these sequences and on the 54 coat protein sequences available from GenBank, a new forward primer, named BanMMV CP9, compatible with Poty1, an oligodT reverse primer already used in diagnostics, was designed. A retrospective analysis of 110 different germplasm accessions from diverse origins was conducted, comparing BanMMCP2 and BanMMV CP9 primers. Of these 110 accessions, 16 tested positive with both BanMMCP2 and BanMMV CP9, 3 were positive with only BanMMCP2 and 2 tested positive with only BanMMV CP9. Otherwise, 89 were negative with the two primers and free of flexuous virions. Sanger sequencing was performed from purified PCR products in order to confirm the amplification of the BanMMV sequence for the five accessions with contrasting results. It is highly recommended to use the two primers successively to improve the inclusiveness of the protocol.

Keywords: high throughput sequencing (HTS); diagnostic; BanMMV; *Betaflexiviridae*; primers; novel isolates; plant virus; total RNA

1. Introduction

Banana (*Musa* spp.) is a perennial herbaceous plant and is among the most important staple food crops worldwide. It is also considered to be a substantial source of income for producers [1].

With worldwide annual production reaching 144 million tons, it represents an important contribution to the economies of many countries in Asia, Africa, Latin-America and the Pacific Islands [2]. Bananas are vegetatively propagated, which involves a high risk of virus spread through the movement of infected planting material [3].

There is a requirement for virus-tested planting material for guaranteeing the exchange of germplasm and for disease control [4]. In this context, the International Plant Protection Convention (IPPC), a plant health treaty signed by over 180 countries, was developed in the 1920s in order to address organisms that are both directly and indirectly harmful to plants [5]. The reliable detection of plant pathogens is therefore a crucial step in the proper management of many diseases, and to avoid their geographical extension due to exchanges of planting material.

However, some problematic situations make the detection of viruses in banana more difficult and therefore time-consuming. For instance, viruses that may be symptomless or at a very low titer in in vitro plants or young plantlets pose a special threat to the germplasm distribution [6]. Alternatively, the presence of endogenous infective *Banana streak viruses* (eBSV) in almost all B genome-containing *Musa* cultivars makes the detection process trickier, since it requires the distinction between episomal and endogenous viral sequences [7]. Furthermore, viruses that have high genome variability, such as Banana mild mosaic virus (BanMMV), make molecular detection particularly challenging. The latter virus displays an important degree of genetic diversity [3,8].

For example, divergent isolates of grapevine virus T, another member of the *Betaflexiviridae* family, presented nucleotide mismatches at the primer-binding site, which could hamper their detection [9,10]. Therefore, some BanMMV isolates could be missed by very specific diagnostic primers, resulting in false-negative results.

BanMMV is an unassigned member in the *Betaflexiviridae* family [11]. Its complete genome consists of 7352 nucleotides, encoding five open reading

frames (ORFs) [4]. BanMMV is present in most, if not all, banana-producing areas of the world, in South America, Asia, Africa, Oceania and Australia [8,12]. Usually, infected plants are symptomless. However, transitory symptoms, such as chlorotic streaks and mosaic, are occasionally observed on young leaves, but often disappear as the plant matures [8]. Gambley and Thomas (2001) and Sharman et al. (2001) have reported that some more pronounced symptoms of BanMMV infection were associated with a limited number of cultivars, including cv. 'Ducasse' (syn. 'Pisang Awak', ABB genome). BanMMV often occurs as a mixed infection with other banana-infecting viruses such as banana streak viruses (BSV) and cucumber mosaic virus (CMV), and the mosaic symptoms of BanMMV may be masked by those of the other viruses [1,13,14].

On the other hand, previous studies reported that, in Guadeloupe, infection by BanMMV exacerbated the symptoms of CMV causing leaf necrosis symptoms compared with mosaic symptoms from CMV infection alone [8].

BanMMV is only known to infect species from the *Musa* genus. BanMMV is mainly transmitted through tissue culture or vegetative propagation. No natural vector has been identified and mechanical transmission has not been successful. Virus transmission attempts made from a single infection in cv. Ducasse failed with mealybugs and aphids, via soil collected from around infected plants, and by root-to-root contact [8].

Several diagnostic protocols have been published for BanMMV. Monoclonal and polyclonal antibodies developed against BanMMV [8,13] can be used for the detection of the virus by ELISA.

Monoclonal antisera were unsuitable for the routine indexing of BanMMV since they were greatly specific to the viral strain that was used to produce them, while BanMMV has shown a very high level of genome variability [11,13]. However, polyclonal antisera were suitable and successfully used for immunosorbent electron microscopy (ISEM) and in immunocapture (IC) reverse transcription (RT) polymerase chain reaction (PCR) [13]. A nested IC-RT-PCR assay has been described [13] for BanMMV detection, targeting the RNA-dependent RNA polymerase (RdRp) region. It is based on polyvalent degenerate primers (PDO) developed by the authors of Ref. [15]. Downstream (toward the 3' end) primers targeting the coat protein (CP) gene have been also developed to recognize BanMMV isolates [3,8].

One approach to minimize this issue is the inclusion of degenerated bases within the oligonucleotide primers. However, a primer with a high level of degeneracy might lead to a loss of primer template-specificity [16,17].

High-throughput sequencing (HTS), also called next-generation sequencing (NGS) or deep sequencing, has proposed a solution to these issues and thus revolutionized plant virus diagnostics. The availability of these powerful tools offers new opportunities and possibilities in routine diagnostics as they enable the simultaneous sequencing and detection of multiple viruses in a sample, regardless of their genome sequence and, therefore, without any a priori knowledge [18]. Currently, they are accelerating the identification of viruses associated or not with a disease of unknown etiology [19–21].

This allows unbiased and hypothesis-free testing in particular for symptomless plant material [22]. This study aimed to describe the identification and genome sequencing of two isolates of BanMMV, and, based on the virus sequences available in GenBank, to design and test a new diagnostic primer for a routine indexing use. A retrospective analysis of 110 accessions from the international banana germplasm collection was conducted using this newly designed primer to compare its performance with that of an existing diagnostic primer.

2. Materials and Methods

2.1. Plant material

Banana accession ITC0763 from the International *Musa* Germplasm Transit Centre (ITC) (Bioversity International) presented discrepancies between diagnostic techniques used for indexing when assessed under post entry quarantine in Australia [23]. No viral symptoms were observed for the accession.

To investigate this, three deflasked in vitro plantlets were grown in 25 cm diameter pots in Gembloux, Belgium, initially in a humid chamber (90% relative humidity) at a temperature of 23 ± 2°C and a 16 h photoperiod, and then after three weeks, in a heated insect-proof greenhouse with the same conditions of temperature and photoperiod.

After 6 months of acclimatization, 1/3 of the youngest leaves were sampled from each plant using a disposable sterile scalpel blade. In the laboratory, 8 discs/leaf samples were subsampled from both laminar and midrib using a

water-cleaned then bleached 4 mm leaf punch. These 24 samples were directly processed (but they may also be stored at −80 ◦C).

One hundred and ten additional banana accessions from different countries and continents were tested in order to validate the new diagnostic primer from this study. They were all grown and sampled as ITC0763. Details are listed in Supplementary File S1. Healthy accessions were also treated as these accessions.

2.2. RNA Extraction

Total RNA was extracted from leaf subsamples (100 mg in total) using RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified RNA concentration was quantified by spectrophotometry, and quality was evaluated using the Agilent 2100 Bioanalyzer, Belgium (2100 expert software, ver B.02.07.SI532).

2.3. Crude Extracts Preparation

Crude extracts were prepared by grinding the leaf subsamples using a TissueLyser (QIAGEN, Hilden, Germany) in 1 mL of a CMV milk extraction buffer called common extraction buffer (CEB) (0.05 M citrate, pH 8.0, containing 0.5 mM EDTA, 2% PVP, 0.05% Tween-20 and 0.5% monothiogylcerol) [24] and then clarifying the sap extract by centrifuging at 10,000× g for 5 min. The supernatant was aliquoted into 500 µL thin-walled polypropylene tubes and stored at −80 ◦C.

2.4. Routine Diagnostic Assays

Routine germplasm screening molecular diagnostic tests were carried out on the crude extracts following the procedure described by [23]. Then, an IC-RT assay was conducted to produce the complementary DNAs, on which the PCR test was carried out. All the details about the molecular diagnostic test used in this study are shown in Supplementary File S2.

2.5. Immunosorbent Electron Microscopy (ISEM) of Viral Minipreps

Partially purified and concentrated viral minipreps were prepared as described previously [23]. 'Necoloidine' solution (Stanvis)-coated copper grids were

coated with a mixture of BSV, BanMMV and BBrMV antibodies before being floated over a droplet of viral miniprep. The prepared grids were stained with 1% ammonium molydbate and viewed using a JEM-1400 TEM (JEOL Ltd., Tokyo, Japan).

The micrographs were taken with an ORIUS SC1000 CCD camera (Gatan Inc., Pleasanton, CA, USA). The detailed protocol is described in Supplementary File S3.

2.6. Library Preparation and High-Throughput Sequencing

The sequencing library was prepared using the Ribo-Zero™ Plant Leaf Kit (Illumina Inc., San Diego, CA, USA) for ribodepletion (ribosomal RNA depletion) followed by the TruSeq Stranded Total RNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) using the standard protocol as previously described for another virus of Beteflexiviridae [25]. The sample was sequenced on the Illumina Nextseq 500 platform with paired sequencing reads of 2 × 151 nt at the GIGA facilities of Liège University (Liège, Belgium).

2.7. Bioinformatics Analysis

The Geneious software v10.2.6 (Biomatters Ltd., Auckland, New Zealand) [26] was used for sequence analysis. The RNA-seq reads were paired, merged using BBMerge from the BBtools suite and the duplicates eliminated. They were then de novo assembled into contigs for genome reconstruction, using the SPAdes software embedded in Geneious with default parameters and a k-mer of 55 [27].

De novo contigs were annotated using the tBLASTx module with the refseq database of viral nucleotides sequences downloaded from GenBank on 15 November, 2019. All the non-redundant reads (merged and unmerged) from the sample were mapped to selected contigs using the Geneious mapper with the following parameters: minimum mapping quality 30, word length 17, maximum mismatches per read 2%, do not allow gaps, minimum overlap identity 98%, index word length 9, maximum ambiguity 16, search more thoroughly for poor matching reads set to yes [28]. Whole genome alignments between our contigs and the refseq sequence of other *Betaflexiviridae* species were done using multiple sequence comparison by log-expectation (MUSCLE) alignment embedded in Geneious with a maximum number of 8

iterations. SNP calling was performed using Geneious software v10.2.6 with default parameters (Minimum frequency (%) = 0.25, Minimum coverage = 1) in order to determine in which positions there are polymorphisms. To remove possible sequencing artifacts, only the SNPs that appeared at a frequency higher than 1% were retained for further analyses [29].

2.8. Phylogenetic and Sequence Analysis

The RdRp and CP genomic regions of the sequenced genomes in this study were retrieved using the ORF finder online tool from GenBank (https://www.ncbi.nlm.nih.gov/orffinder/). In addition to these sequences, the complete genomes of the BanMMV and RdRp nucleotide sequences were aligned and used for phylogenetic analyses with the MEGA software package version 7.0. In addition to these sequences, full nucleotide genomes of BanMMV and other *Betaflexiviridae* members, RdRp aa sequences of BanMMV and CP nucleotide sequences of the virus were separately aligned using MUSCLE implemented in MEGA, then used for phylogenetic analyses with the MEGA software package version 7.0. [30]. The phylogenetic relationships were inferred using UPGMA embedded in the same version of the MEGA software. The stability of the topology was evaluated using bootstrap (1000 replications) [30,31]. Accession numbers and origins (when available) of virus sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) have been integrated in the phylogenetic trees.

2.9. Primer Design

Two hundred and ten nucleotide sequences of BanMMV were publicly available in the Genbank database on 16 March 2020. Among them, 54 sequences correspond to the CP sequences of the virus (40 partial and 14 complete sequences), and 154 correspond to partial RdRp sequences. A single complete genome of BanMMV was available before the identification of the two novel genomes from this study.

The new forward primer (BanMMV CP9) was designed via the Geneious v10.2.6. software based on the Muscle Alignment of the 54 CP region sequences, the two novel isolates from this study and the single complete BanMMV genome (GenBank accession NC_002729).

The candidate primer was evaluated for its Tm and self- or heterodimer formation using the IDT OligoAnalyzer 3.1 software

(https://eu.idtdna.com/calc/analyzer). A list of all the published primers that could detect BanMMV sequences is represented in Supplementary File S4a. The inclusivity of all the published primers, and of the new one, was assessed in silico to test if they are able or not to detect the novel genomes. The analysis for matches was carried out using Geneious 10.2.6 (Biomatters Ltd., Auckland, New Zealand) [32].

2.10. Gradient PCR

In order to optimize the annealing temperature of the newly designed forward primer, eight different temperatures ranging from 47°C to 59°C were tested, during the gradient PCR process, on healthy and infected banana accessions.

3. Results and Discussion

3.1. Immunosorbent Electron Microscopy (ISEM) Analysis

Like PCR tests and other methods in plant virus diagnostics, electron microscopy is a specific approach that is based on the use of antibodies as well as knowledge of the target. It can help in the detection of novel viruses. Flexuous rod-shaped virions were detected in a partial purification of banana accession ITC0763 (Figure 21), while negative results were obtained by targeted IC-RT-PCR used to detect filamentous viruses of banana (BanMMV and banana bract mosaic virus (BBrMV)).

The particles were approximately 580 nm in size, suggestive of members of the family *Betaflexiviridae*.

Like all virions of the latter family, these particles showed a diameter ranging from 10 to 15 nm [33].

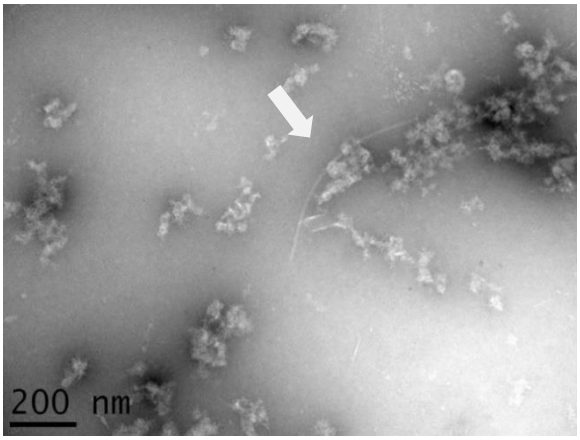


Figure 21: Flexuous filamentous particle (arrow) obtained after viral particle enrichment from leaves of banana accession ITC0763. The scale bar represents 200 nm.

3.2. High-Throughput Sequencing and Bioinformatics Analysis

The HTS yielded a total number of 8,683,460 sequence reads ranging from 50 to 151 nucleotides.

After removing duplicates and de novo assembly, a total of 101,160 contigs was obtained, summing 33,003,893 nucleotides. The maximum, minimum and mean lengths were 16,883 bp, 86 bp and 386 bp, respectively. Two contigs, contig1 and contig 2, each approximately 5 kb in length, had high homology (E-value of 0) to the reference Banana mild mosaic virus (BanMMV) sequence (GenBank accession NC_002729). The contigs were further extended by iterative mapping to lengths of 7336 and 7311 nt, respectively. The genome coverage of both isolates reached almost 100%.

The two new nucleotide sequences were deposited on GenBank. They are hereafter called MT872724 and MT872725 with their GenBank accessions numbers, respectively, for contig 1 and contig 2. They shared a pairwise identity of 75.3% at the nucleotide level, and had pairwise identities of 75.2% and 75.4%, respectively, with the BanMMV reference genome (GenBank accession NC_002729). They had pairwise identities of 76.5% and 75.9%, respectively, with the translated amino acid sequence of the BanMMV reference genome (GenBank accession NC_002729). MT872724 and MT872725 shared 80% nt identity (91.1% aa identity) between their

respective CPs. They shared 76% nt identity (84.3% aa identity) between their respective RdRps. All percentages of identity between BanMMV (GenBank accession NC_002729) and the two isolates regarding CP and polymerase genes, either at the nt or the aa level, are detailed in Table 5.

Table 5: Percentage of identity between BanMMV reference genome and the two novel isolates regarding CP and polymerase genes, at both the nucleotide (A) and amino acid (B) levels.

		(A) Nucleotide Level				**(B) Amino Acid Level**			
		MT872724		**MT872725**		**MT872724**		**MT872725**	
		CP	**RdRp**	**CP**	**RdRp**	**CP**	**RdRp**	**CP**	**RdRp**
BanMMV NC_002729	CP	77.6 %	n.a.	76.8 %	n.a.	88 %	n.a.	87.8 %	n.a.
	RdRpp	n.a.	75.4 %	n.a.	75.4 %	n.a.	81%	n.a.	81.6 %

With n.a. refers to non-available.

Throughout the *Betaflexiviridae* family, isolates of different species should have less than about 72% nt identity (or 80% aa identity) between their respective CP and polymerase genes [34]. Therefore, MT872724 and MT872725 can be considered as different isolates of BanMMV.

3.3. Phylogenetic Analysis

The whole genome sequences of MT872724 and MT872725 (7336 nt and 7311 nt, respectively) were compared and aligned together with the single complete genome of BanMMV (GenBank accession NC_002729; 7352 nt), and with other members from the different genera of the *Betaflexiviridae* family.

The phylogenetic tree reconstructed from the full genome sequences (Figure 22) showed the clustering of the new genomes into a group along with BanMMV, confirming their taxonomical placement as novel isolates of this unassigned member of the *Betaflexiviridae* family. They are also close to the other genera of the same family, but the closest one seems to be the Foveavirus, as it is the genus of Peach chlorotic mottle virus.

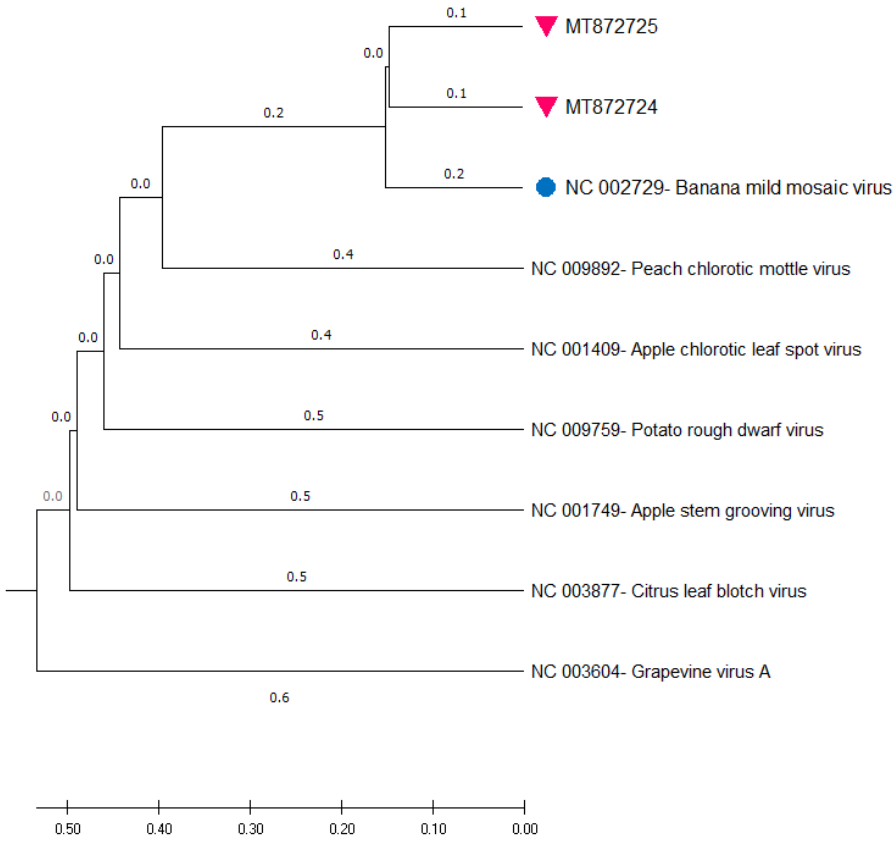


Figure 22: Unweighted Pair Group Method with Arithmic mean (UPGMA) phylogenetic tree inferred from full genomes of nt sequences of BanMMV, isolates from this study, and members from different genera of the Betaflexiviridae family The cyan dot denotes the single complete genome of the virus (GenBank accession NC_002729). The purple triangles denote the BanMMV isolates characterized during this study. Branches were bootstrapped with 1000 replications. The scale bar indicates the number of substitutions per site.

The phylogenetic tree reconstructed from CP nucleotide sequences (Supplementary File S5) had a similar topology to the one inferred from full genomes, and also showed that the new genomes clustered together into a group with the single BanMMV genome (GenBank accession NC_002729).

Further analysis was carried out in order to assess how the new genomes of BanMMV related to the currently known viral diversity. Phylogenetic analysis

inferred from RdRp nucleotide sequences showed that several monophyletic groups were generated, which confirms that BanMMV is a genetically diverse virus species (Figure 23). Similarly, Meena et al. [35] reported the extreme variability of the RdRp genomic region of flexiviruses. In the case of the novel genomes characterized during this investigation, each genome clustered in a different group.

The co-existence of two distinct and divergent isolates of BanMMV in the same plant can be explained by the possibility of the horizontal transfer of the virus between plants. Similarly, a previous analysis supported the existence of the horizontal spread of BanMMV between plants [8]. In fact, Caruana and Galzi [36] showed that virus-free planting material became infected with CMV and BanMMV once planted in the field. In the same line, Teycheney et al. [3] reported that identical RdRp sequences were obtained from two independent pairs of plants, and there was a strong dispersion gradient of the virus between plants. No insight into the mechanism underlying the plant to plant transfer has yet been provided. Mechanisms are still unknown. Subsequently, further studies need to be done [3,4,8].

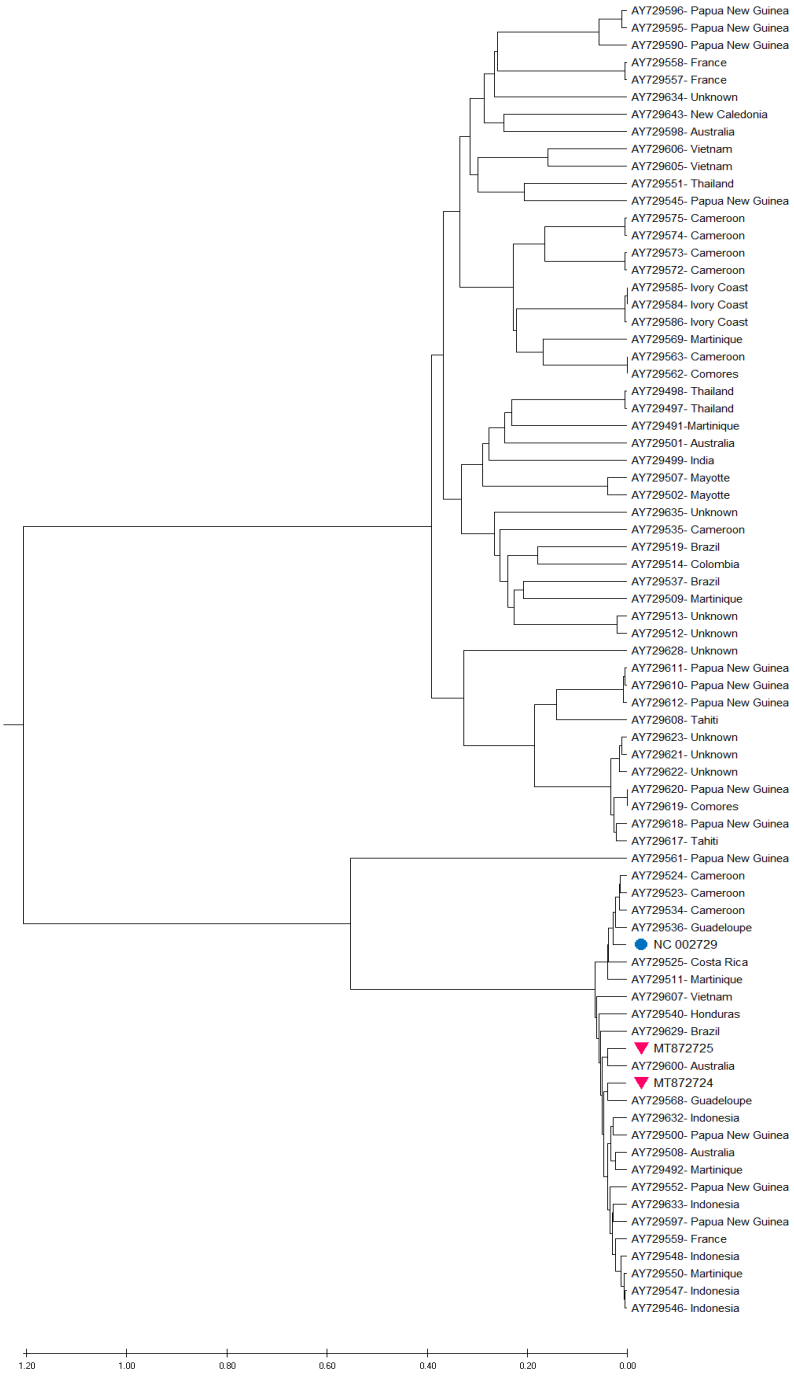

AY729596- Papua New Guinea
AY729595- Papua New Guinea
AY729590- Papua New Guinea
AY729558- France
AY729557- France
AY729634- Unknown
AY729643- New Caledonia
AY729598- Australia
AY729606- Vietnam
AY729605- Vietnam
AY729551- Thailand
AY729545- Papua New Guinea
AY729575- Cameroon
AY729574- Cameroon
AY729573- Cameroon
AY729572- Cameroon
AY729585- Ivory Coast
AY729584- Ivory Coast
AY729586- Ivory Coast
AY729569- Martinique
AY729563- Cameroon
AY729562- Comores
AY729498- Thailand
AY729497- Thailand
AY729491-Martinique
AY729501- Australia
AY729499- India
AY729507- Mayotte
AY729502- Mayotte
AY729635- Unknown
AY729535- Cameroon
AY729519- Brazil
AY729514- Colombia
AY729537- Brazil
AY729509- Martinique
AY729513- Unknown
AY729512- Unknown
AY729628- Unknown
AY729611- Papua New Guinea
AY729610- Papua New Guinea
AY729612- Papua New Guinea
AY729608- Tahiti
AY729623- Unknown
AY729621- Unknown
AY729622- Unknown
AY729620- Papua New Guinea
AY729619- Comores
AY729618- Papua New Guinea
AY729617- Tahiti
AY729561- Papua New Guinea
AY729524- Cameroon
AY729523- Cameroon
AY729534- Cameroon
AY729536- Guadeloupe
NC 002729
AY729525- Costa Rica
AY729511- Martinique
AY729607- Vietnam
AY729540- Honduras
AY729629- Brazil
MT872725
AY729600- Australia
MT872724
AY729568- Guadeloupe
AY729632- Indonesia
AY729500- Papua New Guinea
AY729508- Australia
AY729492- Martinique
AY729552- Papua New Guinea
AY729633- Indonesia
AY729597- Papua New Guinea
AY729559- France
AY729548- Indonesia
AY729550- Martinique
AY729547- Indonesia
AY729546- Indonesia
1.20
1.00
0.80
0.60
0.40
0.20
0.00

Figure 23: UPGMA phylogenetic tree inferred from the RdRp aa sequences of BanMMV and isolates from this study. The cyan dot denotes the single complete genome of the virus (GenBank accession NC_002729). The purple triangles denote the BanMMV isolates characterized during this study. Branches were bootstrapped with 1000 replications. The scale bar indicates the number of substitutions per site.

The percentage identities of RdRp and CP nucleic acid and the translated amino acid sequences confirmed that both contigs are independent full genomes of BanMMV [33].

3.4. Genetic Diversity of the Two Identified Isolates

The analysis of minor SNPs in the two novel BanMMV genomes resulted in 31 SNPs for MT872724 (of which 2 are on the non-coding region) and 14 SNPs for MT872725 (Table 6). Details about base change, variant frequency and SNP type are shown in Supplementary File S6.

Table 6: Distribution of single nucleotide polymorphisms (SNPs) with a frequency higher than 1% in the five ORFs of BanMMV populations.

A-	**First Novel BanMMV Genome MT872724**					
BanMMV New Genome 1	**Total SNPs**	**RdRp**	**TGB2**	**TGB3**	**TGB4**	**CP**
MT872724	31	20	2	1	2	3
Length (nt)	-	5328	675	339	213	678
B-	**Second Novel BanMMV Genome MT872725**					
BanMMV New Genome 2	**Total SNPs**	**RdRp**	**TGB2**	**TGB3**	**TGB4**	**CP**
MT872725	14	9	1	3	0	1
Length (nt)	-	5307	675	339	213	717

The number of SNPs in the RdRp varied from 9 to 20 between the two BanMMV viral populations from this study; there were one to two SNPs in TGB2, one to three SNPs in TGB3, and one to three SNPs in the CP region. No SNP was detected in the TGB4 region for MT872725, whereas two SNPs were located in the same region for MT872724.

The percentages of SNPs in the five regions are 4.9, 4.6, 3.3, 4.5 and 5.5%, respectively for RdRp, TGB2, TGB3, TGB4 and CP. The variant frequency

was slightly higher in the CP coding region. This is in line with a previous study [3], which showed that little selection pressure is applied on BanMMV coding genomic sequences above that necessary for the conservation of the encoded proteins.

Around 80% of SNPs for MT872724 and the majority of SNPs for MT872725 were present at a frequency of less than or equal to 10%, supposing that large numbers of low frequency variants are present in sequence clouds of BanMMV genotypes [37]. These minor SNPs support the fact that the quasispecies is a well-connected group of variants, and not a collection of random mutants. In the same line, [38] such a cloud of variants was proposed to be genetically linked through mutation, to interact cooperatively on a functional level, and to collectively contribute to the characteristics of the population. For the same isolate, 5 out the 31 SNPs had a high frequency greater than 20%, suggesting that they might be part of mutant networks [37]. No SNPs located at the same nucleotide position have been identified in both quasispecies.

3.5. Nucleotide Sequence Analysis and Primer Design

The 210 available nucleotide sequences of the BanMMV isolates were downloaded from NCBI and aligned together with complete genomes of MT872724 and MT872725. Around half of the bases covered by several sequences presented a polymorphism, which makes primer design more complicated. In this context, a previous study described that fairly high nucleotide sequence variation in conserved regions of the polymerase and coat protein has been reported between isolates, which is frequently observed for this family of viruses [39]. In the same vein, Teycheney et al. [3] supposed through an observation of similar levels of amino acid conservation between pairs of isolates that there were wide variations in their nucleotide sequences. Likewise, high variabilities were observed for other members of the *Betaflexiviridae* family, which suggests that this property is shared by a large number of members of this family. The alignment results of all BanMMV published primers with the two novel genomes are shown with details in Supplementary File S4b. In fact, the two following primer pairs, BanCP1 and BanCP2 [3] and BanMMVCPFP and BanMMVCPRP [40], were unable (based on in silico tests) to bind with MT872724 and MT872725. Thus, no molecular detection could be achieved with these primers.

For PDO-F2i and PDO-R1i [15], there was binding with both isolates. However, one mismatch in the 3' end was detected for each primer, in both cases. A molecular test with the primer pair was not done since the nested PCR method generates highly concentrated amplicons and a high risk of contamination [41]. This might be a serious constraint, especially for routine diagnostic centers.

In addition to these three primer pairs, there are three other forward primers that could bind (with Poty1) to BanMMV isolates on the CP region. Both molecular and in silico tests are available for these primers (Supplementary File S4b). The three pairs are BanMMCP2 and Poty1, BanMMV CP8 and Poty1, and BanMMV CP9 and Poty1.In the case of BanMMCP2, three mismatches were present in the 10 bases of the 3' end (including one mismatch in the 5 bases of the 3' end) for MT872724, and two mismatches were detected in the 10 bases of the 3' end (no mismatch detected in the 5 bases of the 3' end) for MT872725. Five mismatches were detected in the 25 bases of the primer for both genomes.

This could explain the absence of molecular detection with the primers used for the routine indexing of BanMMV. In the case of BanMMV CP8, although there are no reported mismatches in the 10 bases of the 3' end of BanMMV CP8, as they are covered by the degenerate bases (but three mismatches the whole primer length), the primer failed to give amplicons with ITC0763. This might be explained by the presence of many degenerate bases (at least four degenerate bases) in the 3' end of the primer.

In fact, it has been reported that primers with high levels of degeneracy could decrease the specific detection of the virus [42].

As such, there is a need to develop a polyvalent and degenerated primer with inosine bases to detect a broad range of BanMMV isolates. In this context, a previous survey reported the advantages of the use of Inosine-containing primers [43]. In fact, this kind of primer is considered very useful in detecting more diverse populations in the environment. The use of inosine enhances primer inclusiveness while retaining exclusiveness. In the same line as the current survey, inosine is the best base to use in particular sites with three or four base ambiguities [43,44].

A new primer, called BanMMV CP9, was designed to amplify, with Poty-1, a fragment of BanMMV coat protein. In silico evaluation has been carried out in order to highlight potential mismatches for the 54 CP sequences of

BanMMV available on GenBank, the single complete genome of the virus and the new isolates from this study. Data are shown in Supplementary File S7. According to these results, BanMMV CP9 could be able to detect all these isolates since it presents only one mismatch at the fourth (JX183725.1) or fifth base (AY730743.1) in the 3' end of the primer.

3.6. Primer Validation

Based on the results of gradient PCR using the new primer, 51.6°C was considered as the optimal annealing temperature (Ta) to proceed with the PCR assay with BanMMV CP9. The concentration of 20 µM for this primer has been kept since other concentrations tested did not improve the results.

As expected, the newly developed primer was able to detect BanMMV from our sample of interest (Supplementary File S8), and from other samples tested with this primer.

Further analysis was carried out in order to confirm that the obtained amplicons came from the amplification of BanMMV sequences, and not from other origins. As is shown in Table 7, BanMMV genomes detected by RT-PCR using BanMMV CP9, then sanger-sequenced, exhibited considerable homology to multiple BanMMV sequences. This homology varied from 69% to 89% with BanMMV CP sequences of different isolates.

Table 7: BlastN results of sequenced samples tested with BanMMV CP9.

Sample	BlastN Results		
	% nt Identity	**Sequence ID**	**«Organism»**
ITC 1022	84%	AY730737.1	BanMMV isolate CP2.3 CP gene, partial cds
ITC 1709	89%	AY730742.1	BanMMV isolate CP4.2 CP gene, partial cds
ITC 1746	84%	KT780866.1	BanMMV isolate TN1 CP gene, complete cds
ITC 1518	85%	AY730732.1	BanMMV isolate CP18.1 CP gene, partial cds
ITC 1628	87%	AY730744.1	BanMMV isolate CP6.1 CP gene, partial cds

ITC 1651	83%	AY730748.1	BanMMV isolate CP77.2 CP gene, partial cds
ITC1681	87%	AY730743.1	BanMMV isolate CP4.3 CP gene, partial cds
ITC 1733	81%	AY730754.1	BanMMV isolate CP82.2 CP gene, partial cds
ITC 1740	83%	AY730740.1	BanMMV isolate CP3.3 CP gene, partial cds
ITC 1794	77%	AY366187.1	BanMMV isolate F8 CP gene, complete cds
ITC 1929	69%	EF143978.1	BanMMV isolate Q6.2T CP gene, complete cds

3.7. Laboratory Validation of the New Primer in Banana Germplasm Collection

In order to validate the primer, a retrospective analysis of 110 accessions from different geographical origins and with different viral statuses was conducted, comparing BanMMCP2 and BanMMV CP9 forward primers as an IC-RT-PCR test with the Poty1 reverse primer. These PCR tests were carried out on the complementary DNAs (cDNAs) prepared with Poty-1 from 110 accessions. Detailed results are shown in Supplementary File S1. Of these 110 accessions, 105 (95.45%) showed a concordance in the results with both primers (16 positive accessions and 89 negative accessions), while five discrepancies were noticed. Previously undetected infections of two accessions were identified only by BanMMV CP9. Three infections were missed by the same primer but detected by BanMMCP2. The five detections were confirmed by sequencing of the PCR product.

The three BanMMV-infected accessions that were not recognized by BanMMV CP9 have different origins—ITC1709 originates from Cameroon, ITC1894 from China and, unfortunately, the origin of ITC1686 is unknown. This downplays the hypothesis that the undetected isolates correspond exclusively to a certain geographic origin. This loss of inclusiveness could be explained by the decrease in its robustness due to the presence of either three degenerate bases or two inosine bases. In the same context, Ref. [45] confirmed that universal primers containing inosine do not necessarily amplify all heterologous or divergent sequences of different species of viruses.

In a preliminary analysis, the analytical sensitivity of the new diagnostic primer (BanMMV CP9) was similar to, and even better than, that of the CP2 primer on three accessions. The results were identical for two accessions, but, for one accession, CP9 was still able to detect one isolate of the virus with a 100× diluted sample, while BanMMV CP2 failed. Details are shown in Supplementary File S9.

4. Conclusions

Viral diseases, among which is BanMMV, are one of the main constraints for *Musa* germplasm movement and vegetative propagation. Sensitive and specific diagnostic tools are needed to help in their control. It is nevertheless highly challenging to develop inclusive primers that could detect all BanMMV isolates, which is supported by several studies reporting the very high level of molecular variability of this virus and other species from the *Betaflexiviridae* family [13]. So far, six primer pairs have been designed and published to detect this virus, but in silico analyses, completed by laboratory tests for some primer pairs, suggested that all the BanMMV isolates could not be detected by a single test because of the presence of several mismatches, particularly at the 3' end of primers.

In the current study, two divergent isolates of this virus were fully sequenced, adding two full genomes of BanMMV to the unique genome in GenBank. A new degenerate, inosine-containing diagnostic primer was designed to improve the inclusiveness of the detection protocol, which failed to detect these novel isolates. In fact, this kind of primer could help to overcome the high genetic diversity of the virus, even if a lower sensitivity could sometimes result from the degeneracy and inosine contents of the primers [9,13,14]. The new primer detected BanMMV infection in two samples that tested negative with the existing primer, but failed to detect three infections among the 110 tested accessions.

Therefore, as the very high genetic diversity of BanMMV is a strong challenge for the development of a single protocol with appropriate inclusiveness, our recommended approach would be to continue to use one of these primers for routine indexing and to confirm negative results with the other primer.

This two-step strategy might be preferable over the use of nested PCR, which would require two PCR reactions for all samples, with a high risk of contamination.

On the other hand, this can be time-consuming, and unknown divergent isolates might exist.

As such, HTS technologies that analyze the plant virome present in a sample with minimal bias and independently of the genome sequence of the target can be a good alternative for routine indexing in the future.

Supplementary File S1

Table 8: Banana accessions and their viral status using two different primers BanMMCP2 and BanMMV CP9

ITC number	Origin	Presence or absence of the virus			
		BanMMCP2	Band Intensity	BanMMV CP9	Band Intensity
103	Unknown	0		0	
136	Unknown	0		0	
194	Unknown	0		0	
324	Unknown	0		0	
489	Unknown	0		0	
516	Unknown	0		0	
759	Unknown	0		0	
763	Unknown	0		1	Very high
881 (VT-0881.1)	Papua New Guinea	0		0	
1022	Papua New Guinea	0		1	Very high
1049	Unknown	0		0	
1073	Unknown	0		0	
1493	Congo (Kinshasa)	1	High	1	High
1494	Congo (Kinshasa)	0		0	
1496	Congo (Kinshasa)	0		0	
1505	Congo (Kinshasa)	0		0	
1506 (VT-1606.1)	Congo (Kinshasa)	0		0	
1518	China	1	Very low	1	Very high
1530	Malaysia	0		0	
1533	Thailand	0		0	

1543	Tanzania	0		0	
1560	Tanzania	0		0	
1562	Tanzania	0		0	
1589	Unknown	0		0	
1600	Cuba	0		0	
1602	Cuba	0		0	
1604	Uganda	0		0	
1606	India	0		0	
1609	India	0		0	
1612	India	0		0	
1621	Unknown	0		0	
1628	Vietnam	1	Very high	1	High
1632	India	0		0	
1633	India	0		0	
1643	India	0		0	
1644	Vietnam	0		0	
1651	India	1	High	1	Very high
1652	India	0		0	
1681	Vietnam	1	Very high	1	Low
1686	Unknown	1	Low	0	
1697	Indonesia	0		0	
1709	Cameroon	1	High	0	
1720	India	0		0	
1722	India	0		0	
1729	India	0		0	
1733	Myanmar	1	Very high	1	Very high
1740	India	1	Low	1	High
1746	India	0		1	Very high
1786	Philippines	0		0	
1793	Uganda	0		0	
1794	Uganda	1	Very high	1	Very high
1795	Uganda	0		0	
1796	Uganda	0		0	

1797	Uganda	0		0	
1798	Uganda	0		0	
1802	Uganda	0		0	
1803	Uganda	0		0	
1805	Uganda	0		0	
1806	Uganda	0		0	
1807	Uganda	0		0	
1809	Nigeria	0		0	
1810	Nigeria	0		0	
1811	Nigeria	0		0	
1812	Nigeria	0		0	
1849	Uganda	0		0	
1859	Congo (Kinshasa)	1	High	1	Very low
1860	Indonesia	0		0	
1861	Congo (Kinshasa)	1	Very high	1	Low
1862	Congo (Kinshasa)	0		0	
1863	Congo (Kinshasa)	1	Very low	1	High
1864	Indonesia	0		0	
1865	Indonesia	0		0	
1866	Indonesia	0		0	
1867	Congo (Kinshasa)	1	High	1	High
1868	Indonesia	0		0	
1880	Indonesia	0		0	
1881	Papua New Guinea	0		0	
1886	Unknown	0		0	
1887	Malaysia	0		0	
1888	China	0		0	
1890	Unknown	0		0	
1891	Unknown	0		0	

1892	Unknown	0		0	
1894	China	1	Low	0	
1895	Japan	0		0	
1897	Papua New Guinea	0		0	
1899	Papua New Guinea	0		0	
1900	Papua New Guinea	0		0	
1902	Papua New Guinea	0		0	
1903	Papua New Guinea	0		0	
1904	Papua New Guinea	0		0	
1905	Papua New Guinea	0		0	
1906	Papua New Guinea	0		0	
1907	Papua New Guinea	0		0	
1909	Papua New Guinea	0		0	
1910	Papua New Guinea	0		0	
1911	Unknown	0		0	
1912	Papua New Guinea	0		0	

1919	Papua New Guinea	0		0	
1923	Papua New Guinea	1	Low	1	Low
1926	Papua New Guinea	0		0	
1928	Papua New Guinea	0		0	
1929	Papua New Guinea	1	Very high	1	Very high
1930	Papua New Guinea	0		0	
1932	Papua New Guinea	0		0	
1933	Papua New Guinea	0		0	
1934	Papua New Guinea	0		0	
1935	Papua New Guinea	0		0	
1936	Papua New Guinea	0		0	
1938	Papua New Guinea	1	Very high	1	Very high
1940	Papua New Guinea	1	Very high	1	Very high

Supplementary File S2

Molecular diagnostic test used to detect BanMMV in this study: IC- RT-PCR

Twenty-five microlitres of an antibody mixture containing 5 µg/ml of BanMMV IgG (J. Thomas, unpublished) in sterile carbonate coating buffer was added to each thin-walled PCR tube and incubated for 2 h at 37°C before being washed three times for three min with PBS-T (0.05 M phosphate buffer pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 0.05% Tween20). Twenty-five microlitres of the clarified leaf extract (crude extract) was then added to the washed, coated tube and incubated at room temperature for 4°C overnight. Tubes were washed three times for three min at room temperature with PBS-T and rinsed once with sterile distilled water.

After the water was removed from the immunocapture tubes, reverse transcription was carried out in order to generate cDNA for the virus with RNA genomes. Diluted primers (750 nM Poty1 and 750 nM CMV3′; 12.5 µl total) were added to the immunocapture tube, which was incubated at 80ºC for 10 min, then rapidly chilled on ice. The following reagents were added (final concentration in 7.5 µl): 1× first strand buffer (Invitrogen, USA), 10 mM DTT, 500 nM dNTPs, 10 U RNaseOUT (Invitrogen), 50 U Superscript III reverse transcriptase (Invitrogen), and the tubes incubated at 50 ºC for 45 min, then 70°C for 15 min.

Two microliters of the cDNA mixture was added to 23 µl of the BanMMV PCR mastermix, prepared as follows: 1× MangoTaq coloured PCR buffer (Bioline, Australia), 2 mM $MgCl_2$, 200 nM dNTPs, 400 nM Poty1 primer, 800 nM BanMMCP2 primer, 2.5 U MangoTaq DNA polymerase (Bioline, Australia). The thermal cycling conditions were: 94°C for 1 min, followed by 35 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 20 s, then 72°C for 3 min. The expected product size was ~280 bp.

In all cases, the PCR products were separated by electrophoresis in a 1% agarose gel in 0.5× TBE, and stained with GelRed (Biotium).

Supplementary File S3

Immunosorbent Electron Microscopy (ISEM) of Viral Minipreps:

Three to five clonal plants of each germplasm line were grown out from tissue culture under glasshouse conditions. Plants were sampled at three and six months after deflasking. Samples at each time point were pooled for the indexing process.

For the viral miniprep, the 2 g subsample of leaf lamina and midrib tissue was ground in 6 mL of extraction buffer (0.2 M potassium phosphate buffer (pH 7.0), 15 mM EDTA, 2% polyvinylpyrrolidone, 2% polyethylene glycol and 0.4% Na_2SO_3) using a mortar and pestle, and the slurry filtered through cheesecloth. The filtrate was clarified by centrifugation at 12,000 *g* for 15 min at 5°C and the supernatant recovered. Triton X-100 (120 µL of 33% v/v solution) was added and the mix was vortexed for 1 min. A cushion of 1 mL of 30% sucrose in 0.2M potassium phosphate buffer at pH 7.0 was under laid and the tube ultracentrifuged at 163,000 *g* for 30 min at 5°C. This pellet was resuspended in 100 µL of 10 mM potassium phosphate buffer, pH 7.0 and transferred to a microcentrifuge tube. Thirty µL of chloroform was added to the tube and the mixture vortexed for 30 s prior to centrifugation for 5 min at 12,000 *g*. The aqueous supernatant was stored at -70°C.

ISEM was conducted as follows. Nitrocellulose- or Nicolodion-coated, carbon-stabilised copper electron microscope grids were placed film side down on 10 µl droplets of antibody mixture containing 5 µg mL^{-1} each of SCBV IgGs (Agdia, USA), BSMYV IgG (J. Vo and A. Geering, unpublished), BBrMV IgG (Agdia, USA) and BanMMV IgG (J. Thomas, unpublished), and 0.2 µg mL^{-1} (a 1/200 dilution) of CMV IgG (Sediag, France).

Grids were incubated in a humid chamber for 30–60 minutes at room temperature, then washed with 20 drops of a 10 mM sodium phosphate buffer, pH 7.0 and dried with filter paper. The grids were placed film side down on a 10 µL drop of the viral miniprep and incubated in a humid chamber at 4°C overnight. The grids were then washed again with 20 drops of 10 mM sodium phosphate buffer, pH 7.0 and dried. The grids were negatively contrasted with five drops of 1% ammonium molybdate, pH 6.8–7.0 and touch dried with filter paper. Grids were observed for 10 minutes with an electron microscope

(Hitachi H-7000 or JEOL-TEM-1400). Micrographs were captured on film or using an Orius digital camera (Gatan Inc, USA).

Supplementary File S4

Table 9: List of the published BanMMV primers

Target virus	Primer name	Sequence (5'-3')	Reference	Direction	Location
BanMMV	BanCP1	GGATCCCGGGTTTTTTTTTTTTTTT	Teycheney et al., 2005b	Forward	CP and the adjacent 3' NCR
	BanCP2	TATGCNTTYGAYTTCTTRGAYG		Reverse	
	Poty1	GGATCCCGGGTTTTTTTTTTTTTTTV	De Clerck et al., 2017	Reverse	3' terminal
	BanMMCP2	TGCCAACTGAYGARGAGCTRAATGC		Forward	CP
	BanMMV CP8	TGCCAACTGAYGARGARYTRAAHGC	JE Thomas, unpublished	Forward	
	BanMMV CP9	TCTGATKCIGCIATHATSCC	This study	Forward	
	PDO-F2i	GCYAARGCiGGiCARACiYTKGCiTG	Foissac et al., 2005	Forward	RdRp
	PDO-R1i	TCHCCWGTRAAiCKSATiAiiGC		Reverse	
	BanMMVCPFP	ATGGCAACDGGGGAAAAGAAGG	Selvarajan et al., 2016	Forward	CP
	BanMMVCPRP	TTAATTATTCAATTTGAGGCTCA		Reverse	

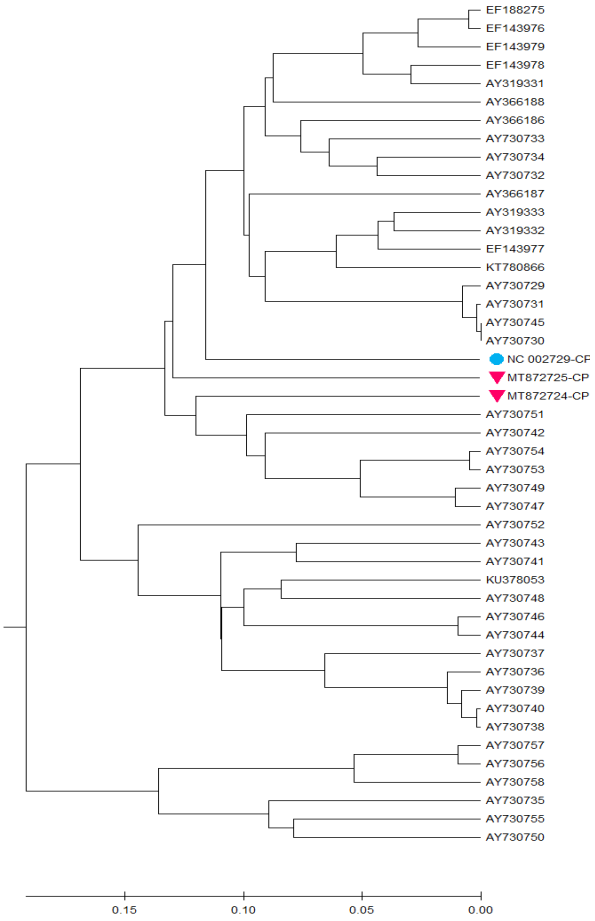
Table 10: *In silico* analysis of matches

Target sequence	Primer pair		Moleculer test	In silico test	Number of mismatches	
MT872724	BanMMCP2 (with Poty1)		No detection	Binding	5 mismatches	
	BanMMV CP8 (with Poty1)		No detection	Binding	3 mismatches	
	BanMMV CP9 (with Poty1)		Detection	Binding	0	
	BanCP1	BanCP2	not possible	No binding	-	-
	BanMMVCPFP	BanMMVCPRP	not possible	No binding	-	-

	PDO-F2i	PDO-R1i	was not done	Binding	1 mismatch in 3'	1 mismatch in 3'
MT872725	BanMMCP2 (with Poty1)		No detection	Binding	5 mismatches	
	BanMMV CP8 (with Poty1)		No detection	Binding	3 mismatches	
	BanMMV CP9 (with Poty1)		Detection	Binding	0	
	BanCP1	BanCP2	not possible	No binding	-	-
	BanMMVCPFP	BanMMVCPRP	not possible	Binding only for BanMMVCPRP => No binding for the primer pair	-	3 mismatches in 5'
	PDO-F2i	PDO-R1i	was not done	Binding	1 mismatch in 3'	1 mismatch in 3'

Supplementary File S5

Phylogenetic analysis-CP



Supplementary Figure 24. UPGMA phylogenetic trees inferred from CP nucleotide sequences of BanMMV and isolates from this study**.** The cyan dot denotes the single complete genome of the virus (GenBank accession NC_002729). The purple triangles denote the BanMMV isolates characterized during this study.

Supplementary File S6 – SNPs

Table 11: Distribution of SNPs on MT872724

Isolate	Position des SNPs	Change	%SNP	Region	Region delimitation	Coverage
Isolate1	421	T→C	6.1	RdRp	64 →5391	197
	438	G→A	7	RdRp	64 →5391	214
	738	T→C	1.5	RdRp	64 →5391	337
	1,448	G→T	8.2	RdRp	64 →5391	268
	1,692	T→C	4.9	RdRp	64 →5391	184
	1,697	T→A	2.7	RdRp	64 →5391	184
	1,715	T→C	2.3	RdRp	64 →5391	173
	1,821	G→A	4.1	RdRp	64 →5391	123
	1,881	G→A	22.7	RdRp	64 →5391	132
	2,628	C→T	1.6	RdRp	64 →5391	183
	2,985	C→T	3.2	RdRp	64 →5391	190
	3,027	T→C	2.6	RdRp	64 →5391	190
	3,031	A→G	2.6	RdRp	64 →5391	193
	3,058	T→C	2.3	RdRp	64 →5391	177
	3,119	G→A	2.3	RdRp	64 →5391	175
	3,587	G→A	4.6	RdRp	64 →5391	130
	3,843	G→A	17.8	RdRp	64 →5391	152
	3,852	G→A	3.5	RdRp	64 →5391	144
	5,211	C→T	9.1	RdRp	64 →5391	66
	5,289	A→G	9.1	RdRp	64 →5391	55
	5,446	C→T	7.7	TGB protein 2	5354→ 6028	91
	5,567	G→A	4.3	TGB protein 2	5354→ 6028	93
	5,944	T→C	3.3	TGB protein 2	5354→ 6028	123
	6,110	T→A	4.6	TGB protein 3	6028→ 6366	152
	6,427	T→C	9.1	TGB protein 4	6281→ 6493	55
	6,488	C→A	8.9	TGB protein 4	6281→ 6493	56
	6,812	A→G	8.7	CP	6564→ 7241	46

	7,239	T→G	94.1	CP	6564→ 7241	17
	7,241	A→T	94.1	CP	6564→ 7241	17
	7,247	T→G	92.9	NCR	NCR	14
	7,255	G→T	66.7	NCR	NCR	3

Table 12: Distribution of SNPs on MT872725

Isolate	Position des SNPs	Change	%SNP	Region	Region delimitation	Coverage
Isolate2	1808	G→A	1.5	RdRp	50 →5356	409
	2138	C→T	1.7	RdRp	50 →5356	291
	2564	A→G	2	RdRp	50 →5356	295
	2603	G→A	1.7	RdRp	50 →5356	294
	2955	A→G	1.2	RdRp	50 →5356	322
	3811	G→A	3.6	RdRp	50 →5356	197
	4023	T→C	2.5	RdRp	50 →5356	239
	5113	G→A	2.6	RdRp	50 →5356	227
	5170	C→T	5.5	RdRp	50 →5356	201
	5827	C→T	3	TGB2	5319→5993	232
	6062	G→T	2	TGB3	5993→6331	254
	6192	G→A	2.7	TGB3	5993→6331	149
	6330	T→C	4	TGB3	5993→6331	200
	6879	A→G	5.2	CP	6528→7244	115

Supplementary File S7

Mismatches with CP9

Table 13: Mismatches with CP9

Primer	T	C	T	G	A	T	K	C	I	G	C	I	A	T	H	A	T	S	C	C
AY730758.1																				
AY730756.1																				
AY730755.1			A																	
AY730754.1																				
AY730753.1																				
AY730751.1																				
AY730750.1																				
AY730749.1																				
AY730748.1																				
AY730747.1																				
AY730746.1			A																	
AY730745.1			A																	
AY730744.1			A																	
AY730743.1	C															G				
AY730742.1						C														
AY730741.1			A																	
AY730740.1																				
AY730739.1																				
AY730738.1																				
AY730737.1																				
AY730736.1																				
AY730735.1																				
AY730734.1			A																	
AY730733.1			A																	
AY730732.1			A																	
AY730731.1			A																	
AY730730.1			A																	
AY730729.1			A																	
FJ179164.1																				
JX014304.1			A			C														
AY366188.1			A																	
AY366187.1			A										G							
AY366186.1			A			C														
AY319333.2			A																	
AY319332.1			A																	
AY319331.1			A																	
EF188275.1			A																	
EF143979.1			A																	
EF143978.1			A																	
EF143977.1			A																	
EF143976.1			A																	
KU378053.1																				
KT780866.1			A																	
AY730757.1																				
AY730752.1			A																	
JX183730.1		T		T										A						
JX183729.1			A																	
JX183728.1		T				G														
JX183727.1																				
JX183726.1																				
JX183725.1			A														A			
JX183724.1			A			G														
JX183723.1			A			C														
JX183722.1			A																	
NC_002729.1			A			C														
MT872724						C														
MT872725																				

Supplementary File S8

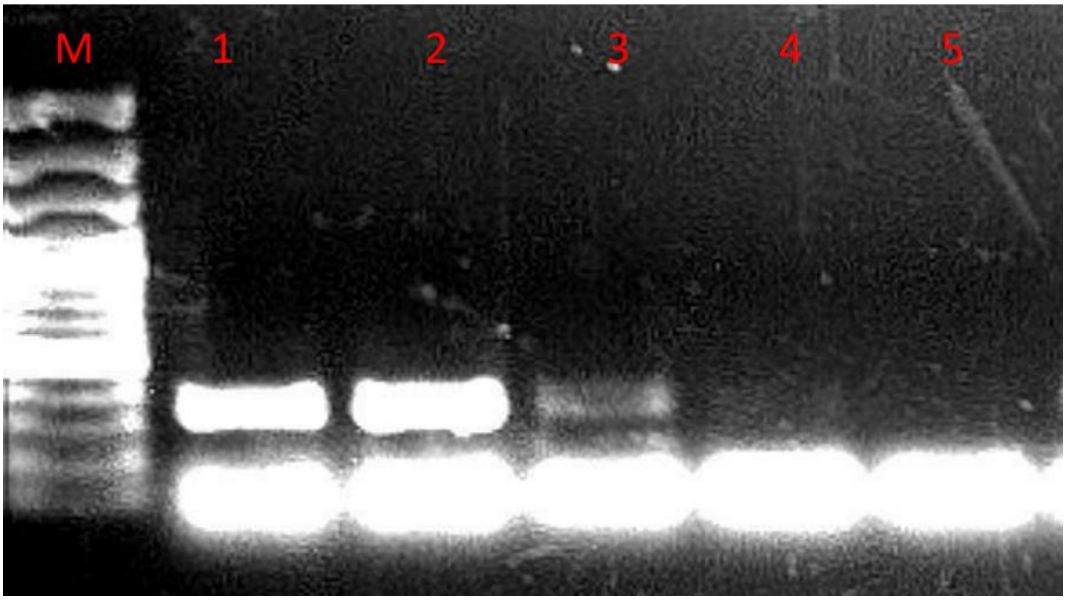


Figure 25: RT-PCR with BanMMV CP9 RT-PCR using BanMMV CP9 (and Poty1). M- Marker (100bp) ; Lanes 1 to 5 : ITC0763, ITC0763 (repetition), ITC1160, ITC1859 and healthy banana (negative control).

Supplementary File S9

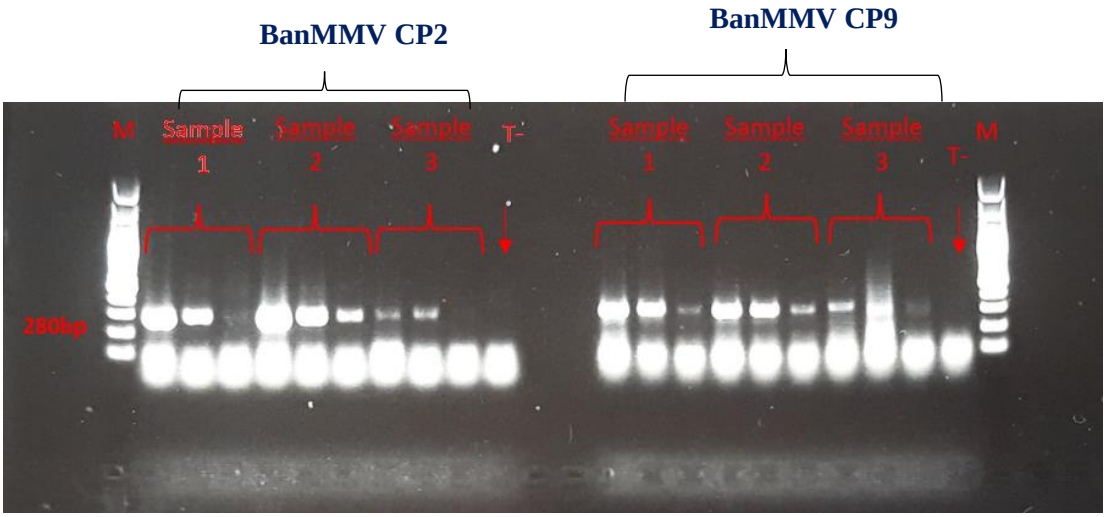


Figure 26: Gel electrophoresis of the amplification of BanMMV with two different primers

With M : 100bp molecular weight marker and T- refers to negative control (healthy banana).

Sample1: ITC1859, Sample2: ITC1861, Sample3: ITC0412

The samples were tested in three contexts: sample not diluted, sample diluted 10x, and sample diluted 100x

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Chapter 4

Detection of Banana Mild Mosaic Virus in *Musa In Vitro* plants : High-Throughput Sequencing Presents Higher Diagnostic Sensitivity Than (IC)-RT-PCR and Identifies a New *Betaflexiviridae* Species

This chapter is adapted from: Hanafi M., Rong W., Tamisier L., Berhal C., Roux N., and Massart S., (2022). Detection of Banana Mild Mosaic Virus in *Musa* In Vitro Plants: High-Throughput Sequencing Presents Higher Diagnostic Sensitivity Than (IC)-RT-PCR and Identifies a New *Betaflexiviridae* Species. *Plants*

Abstract:

The banana mild mosaic virus (BanMMV) (*Betaflexiviridae*, Quinvirinae, unassigned species) is a filamentous virus that infects *Musa* spp. and has a very wide geographic distribution. The current BanMMV indexing process for an accession requires the testing of at least four plants cultivated in a greenhouse for at least 6 months and causes a significant delay for the distribution of the germplasms. We evaluated the sensitivity of different protocols for BanMMV detection from in vitro plants to accelerate the testing process. We first used corm tissues from 137 in vitro plants and obtained a diagnostic sensitivity (DSE) of only 61% when testing four in vitro plants per accession. After thermotherapy was carried out to eliminate BanMMV infection, the meristem was recovered and further grown in vitro. So, the same protocol was evaluated in parallel on the corm tissue surrounding the meristem, as a rapid screening to evaluate virus therapy success, and compared to the results obtained following the standard protocol. The obtained results showed 28% false negatives when conducting testing from corm tissues, making this protocol unsuitable in routine processes. RT-PCR and high-throughput sequencing (HTS) tests were applied on tis-sues from the base (n = 39) and the leaves (n = 36). For RT-PCR, the average DSE per sample reached 65% from either the base or leaves. HTS was applied on 36 samples and yielded 100% diagnostic specificity (DSP) and 100% DSE, whatever the sampled tissue, allowing the identification of a new *Betaflexiviridae* species infecting *Musa*. These results suggest that a reliable diagnostic of BanMMV from in vitro plants using RT-PCR or HTS technologies might represent an efficient alternative to testing after greenhouse cultivation.

Keywords: high-throughput sequencing; (IC)-RT-PCR; diagnostic performance; BanMMV; in vitro plants; meristem culture; RNA extraction.

1. Introduction

Plant pests are seriously threatening food security worldwide. The damages caused by plant pathogens could reach 40% of food production all over the world [1]. The risk of epidemics is elevating due to climate change and increasing commercial trade [2]. Thus, there is a requirement for virus-tested planting material for guaranteeing the exchange of germplasms and for disease control [3,4]. In this context, the International Plant Protection Convention (IPPC), a plant health treaty signed by over 180 countries, was developed in the 1920s in order to address organisms that are both directly and indirectly harmful to plants. The reliable detection of plant pathogens is therefore a crucial step in the proper management of many diseases and to avoid their geographical extension due to exchanges of planting material [5].

The banana (*Musa* spp.) is one of the most important staple food crops, supplying food to more than 400 million people in more than 136 countries. Its global production is approximately 153 million tons annually [6]. However, pests and diseases greatly con-tribute to the decline in banana yields. They represent significant threats to banana production and have the potential to devastate entire plantations [1,7]. Among these pests, viruses constitute an important concern to banana and plantain production, as they directly affect the yield and quality. They also remain a serious constraint to the inter-national exchange of *Musa* germplasms. About 20 virus species belonging to five families have been reported to infect bananas and plantains worldwide [8]. Several species of Banana streak viruses (BSVs, genus Badnavirus, family *Caulimoviridae*), the banana bunchy top virus (BBTV, genus Babuvirus, family *Nanoviridae*), and the banana bract mosaic virus (BBrMV, genus Potyvirus, family *Potyviridae*) have caused documented epidemics [8,9], while *Musa* plants can be infected by other viruses such as banana mild mosaic virus (BanMMV) and banana virus X (BVX), both unassigned members in the family *Betaflexiviridae*, or cucumber mosaic virus (CMV, genus Cucumovirus, family Bromoviridae) [8].

Among these banana viruses, BanMMV and BSVs are the most prevalent viruses in germplasm accessions collected from Asia, Africa, Oceania, and America [10]. BanMMV has flexuous filamentous particles of about 580 nm in length, with a coat protein of ca. 26.8 kDa and a single-stranded positive RNA genome [9,11]. The infection often results in symptomless plants of *Musa* spp., and the virus has a worldwide distribution, which can explain its

high prevalence [9,10]. The impact of BanMMV seems somewhat mild on banana crop, although mixed infections (mostly with BSV and BBrMV) can lead to severe leaf necrosis [9,11,12]. The virus displays a very high molecular diversity [13]. Its presence in the banana collection must be avoided to prevent the emergence of more virulent strains and to reduce the risk of unpredictable variations in symptoms, infectivity, accumulation, and/or vector transmissibility due to co-infection with other banana viruses [10].

Currently, the indexing protocols require a growth of banana plants for 6 months in a greenhouse to minimize the risk of false-negative results for the diagnostic test. Leaf samples, both limb and midrib from the three youngest leaves, are collected from four individual plants of each accession and then bulked together for testing. PCR and electron microscopy tests are conducted from these samples at two stages: 3 and 6 months of growth in a greenhouse. Thus, reliable testing requires a significant amount of resources and time.

Therefore, another detection protocol called "pre-indexing" has been proposed to determine the health status of banana accessions after 3 months in the greenhouse. It consists of preliminary and quick (RT)-PCR tests from only a single plant. A positive plant will be directly processed for virus therapy, while a negative plant will be fully indexed. The pre-indexing protocol saves a significant amount of time, as 68% of the received plants in the international germplasm collection are infected by a virus and they can be sanitized directly without the need of complete indexing [10]. In this context, BanMMV testing directly from in vitro plants, as carried out for other viruses on several crops such as cassava [14], yam [15], potato [16], and sweet potato [17], holds interesting potential to save time and resources. In addition, virus testing by high-throughput sequencing technologies (HTS) represents a promising alternative to PCR-based tests in plant virus diagnostics, with potentially an improved inclusivity for divergent isolates and species and a similar analytical sensitivity [18].

Comparison of HTS performance with RT-PCR holds great interest for improving virus diagnostics [19]. On the other hand, the sanitation process of banana accession includes chemo-therapy, thermotherapy, and meristem culture [20]. It is very long and can last from 12 months to several years. One bottleneck is the ability to rapidly and reliably test the presence of the infecting virus BanMMV, as it is currently carried out after in vitro plant recovery and

growth and an additional period of at least 3 months in the greenhouse. Evaluating the success of therapy at the earliest stages is particularly important to save resources and quickly eliminate plants that are not sanitized. An interesting possibility is to test the BanMMV presence in the tissue surrounding the meristem that is sampled after thermotherapy. A quick decision of keeping the plant or not could be made at this stage if such a test performs well.

In this study, we evaluated the possibility of using in vitro plantlets to speed up diagnostic tests and alleviate testing and labor costs. Our goals were to: (i) study the reliability of the BanMMV testing (IC-RT-PCR) carried out on corm tissues (material surrounding the meristem) of in vitro plants while preserving the meristem; (ii) com-pare the performance of a BanMMV diagnostic test (IC-RT-PCR) performed after thermotherapy, either from corm tissue from in vitro plantlets or from the same plants grown 3 months in a greenhouse; (iii) evaluate and compare the performance of a BanMMV diagnostic test (RT-PCR) performed on RNAs extracted from either the base (area regrouping the corm and me-ristem) or leaves of in vitro plants; and (iv) evaluate the performance of HTS technologies applied on the base or leaves from in vitro plants and to compare it to RT-PCR.

2. Materials and Methods

2.1. Plant Material and Tissue Culture Conditions

Banana plantlets were multiplied and maintained in sterile conditions on semi-solid growth medium corresponding to powdered Murashige and Skoog (1962) (MS) macronutrients, MS micronutrients, and vitamins (Duchefa Farma, Haarlem, The Netherlands) with 3% sucrose solidified using 3 g/L Gelrite™ (Duchefa Farma, Haarlem, The Netherlands) [20]. Media were supplemented with 10mg/L of ascorbic acid (vitamin C), 0.19 µg/mL of indole-3-acetic acid (IAA) and 2.52 µg/mL of 6-benzylaminopurine (BAP). For all the media, pH was adjusted to 6.12- 6.14 using NaOH or HCl prior to autoclaving at 110 °C for 20 min.

Plant material originated from the International *Musa* Germplasm Transit Centre (ITC) managed by the Alliance of Bioversity International and CIAT

and hosted at KU Leuven in Belgium and were conserved under in vitro conditions. In this study, 137 plants from 19 BanMMV-infected accessions were used for in vitro testing (only IC-RT-PCR) of the plant tissue surrounding the meristem that went to meristem therapy. Moreover, the tissue surrounding the meristem was sampled for 41 plants, from 21 accessions, after thermotherapy when the meristem is further grown in vitro. Three banana accessions (9 plants) known to be healthy were also used as negative controls during tests. A detailed list of this plant material is provided in Supplementary File S1. All information about accessions was found in *Musa* Germplasm Information System (MGIS) database [21].

In addition, seven banana accessions (23 plants) known to be infected with BanMMV were used for testing (RT-PCR and HTS) of either r basal or leaf tissues. Four accessions (16 plants) that tested negative for BanMMV infection were also used as negative controls during these tests. Two out of these four accessions were used for HTS. Details are provided in Supplementary File S1.

2.2. Sanitation Process

Twenty-one banana accessions infected with BanMMV (and not available for distribution) and three healthy accessions (ITC0245, ITC0654, and ITC1120) were received from the ITC collection of Bioversity International. Only infected plants were submitted to a cycle of sanitation by thermotherapy with meristem culture (Figure 27).

During the thermotherapy process, infected plants were placed in a temperature-controlled chamber (TCC) with a progressively rising temperature program for one month. This program started with a temperature of 28 °C. It increased by 3 °C per day, for 5 days, until reaching 40 °C. Then, temperature stabilized at 40 °C for 25 days.

After the thermotherapy, the apical meristem tip (1 mm diameter) was dissected following the protocol described by Lassois et al. [20] and transferred monthly for three months to a new glass tube containing sterilized fresh growth medium until plantlets had two or three leaves and some roots. This process corresponded to the in vitro stage. Later, plantlets were acclimated for three months in the greenhouse, at a temperature of 23 ± 2 °C

and a 16 h photoperiod. This period of acclimation was the greenhouse stage (Figure 27).

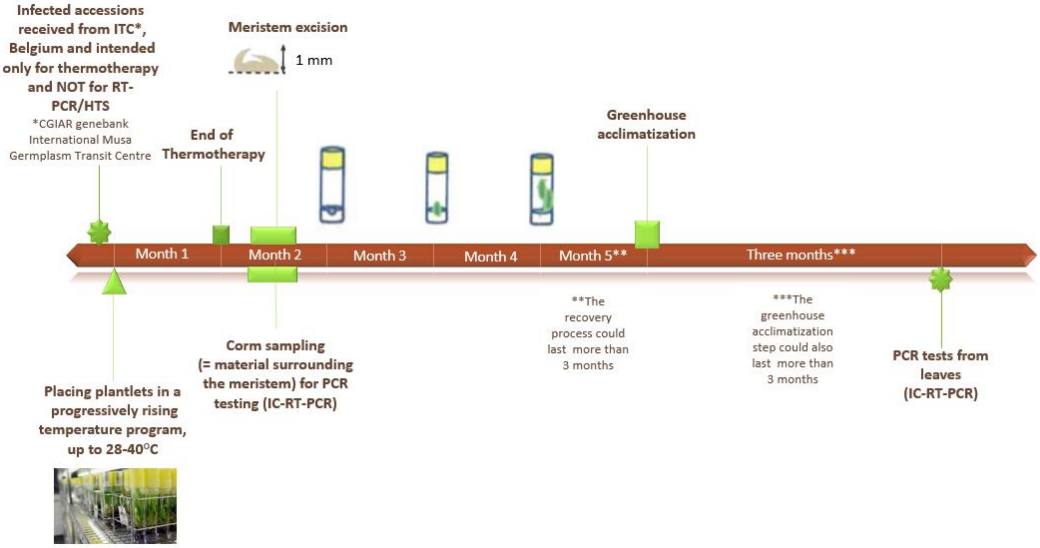

Figure 27: Flowchart of the sanitation process used in this work, describing the different steps involved in the work carried out and the tests performed throughout the experiment.

2.3. Sampling of In Vitro Plants

2.3.1. Meristem Sampling

The meristem sampling and in vitro culture were performed in aseptic conditions. Meristem excision was conducted following the protocol described by Lassois et al. [20]. Firstly, successive leaves, overlapping the meristem, were carefully removed by cutting with a scalpel through the circular insertion of each one. Then, a binocular microscope was used for a precise excision of the small explant obtained. The apical meristem tip (1 mm diameter) was isolated with the second sterile scalpel and transferred to a new tube with 10 mL of regeneration medium.

2.3.2. Corm Sampling

In vitro tissues surrounding the meristem, the zone henceforth called corm, were sampled at the same time as meristem sampling. In total, 100 mg of the corm was kept for testing while isolating the meristem and preserving it for the in vitro plant regeneration.

2.3.3. The Base Sampling

For some plants, corm tissues and meristem area, the zone henceforth called the base, were sampled and a weight of 100 mg from these tissues was used for total RNA extraction. There was no preservation of the meristem through this sampling. Thus, the plant was killed.

2.3.4. Leaf Sampling

Leaf sampling from in vitro plants was carried out by punching one or two times the three youngest leaves of the plant. A weight of 100 mg from these tissues was used for total RNA extraction. In some cases, the whole in vitro leaf was sampled if the weight was under 100 mg.

2.3.5. Sampling of Plants in Greenhouse

When the acclimatization period was over, 1/3 of the youngest leaves were sampled from each plant using a disposable sterile scalpel blade. In the laboratory, 8 discs/leaf samples were subsampled from both laminar and midrib using a water-cleaned, bleached, then water-rinsed 4 mm leaf punch. These samples were directly processed or stored at −80 °C.

2.4. RNA Extraction

Total RNA was extracted from in vitro tissues (100 mg in total) of either the base or leaves (separately) using RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified RNA concentration was quantified by spectrophotometry, and quality was evaluated using the Bioanalyzer 2100 expert soft-ware, version B.02.07.SI532 (Agilent technologies, CA, USA). RNA extracts from each in vitro plant were used for both targeted molecular test (RT-PCR) or high-throughput sequencing.

In addition, two alien external controls were used. They corresponded to plant samples infected at high concentration by a plant virus, called the alien plant virus, which cannot infect banana. They were processed in parallel to the samples from sampling to bioinformatic analysis. The presence of sequences from alien plant viruses in the banana samples can give a useful indication of the cross-contamination level be-tween samples. Therefore, total RNA was also extracted from greenhouse leaf samples (100 mg in total) of one wheat infected by barley yellow dwarf virus (BYDV) as well as a tomato plant infected by Pepino mosaic virus (PepMV). Our two alien viruses were therefore BYDV and PepMV.

2.5. Molecular Assays

2.5.1. Targeted Molecular Diagnostic on In Vitro Plants

An IC-RT-PCR test was carried out from crude extracts of corm tissues following the protocol described by De Clerck et al. [10]. Details are included in Supplementary File S2.

For the base and leaf samples, an RT-PCR test was conducted from RNA extracts following the same protocol but without IC.

For IC-RT-PCR and for RT-PCR, a non-template control corresponding to molecular-grade water was used.

2.5.2. Targeted Molecular Diagnostic on Greenhouse Plants

An IC-RT-PCR test was conducted on crude extracts of leaf samples from plants grown in greenhouse for at least three months. The protocol was the same as the one used from in vitro tissues. Details are included in Supplementary File S2.

PCR products were separated by electrophoresis in a 1% agarose gel in 0.5× TBE and stained with GelRed (Biotium). The electrophoresis of PCR products was carried out in the same way as for the IC-RT-PCR test from in vitro plants. Different healthy accessions were used as negative controls for both tests.

2.6. Library Preparation and High-Throughput Sequencing

The sequencing libraries were prepared using the Ribo-Zero™ Plant Leaf Kit (Il-lumina Inc., San Diego, CA, USA) for ribodepletion (ribosomal RNA depletion) followed by the TruSeq Stranded Total RNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) using the standard protocol as previously described [22]. The samples were sequenced on the Illumina Nextseq 500 platform with paired sequencing reads of 2 × 151 nt at the GIGA facilities of Liège University (Liège, city, Belgium).

2.7. Statistical Analyses

The statistical analyses were performed using R software (http://www.r-project.org/ , accessed on 1 April 2021). The sensibility was measured for all the possible combinations of 1 to 4 plants using the comb function.

The performance criteria of the diagnostic tests were analysed through the calculation of the diagnostic sensitivity (DSE) and the diagnostic specificity (DSP) of tests, taking into account the status of the accession (healthy or infected) and as follows:

$$diagnostic\ sensitivity\ (in\ \%) = \left(\frac{True\ positives}{True\ positivies + False\ negatives}\right) \times 100$$

$$diagnostic\ specificity\ (in\ \%) = \left(\frac{True\ negatives}{True\ negatives + False\ positives}\right) \times 100$$

2.8. Bioinformatic analysis

The obtained sequence reads from HTS were subjected to demultiplexing and removal of Illumina adapter sequences before all reads were paired, quality filtered, and trimmed by BBDuk. In this study, reads shorter than 35 bp and Phred score less than 25 on both ends of reads were trimmed. Later, the

trimmed reads were further merged by BBMerge, and "normal" rate was used. All the paired, trimmed, and merged reads were mapped to the custom-built database with all the reference sequences of BanMMV, one reference genome sequence for barley yellow dwarf virus (BYDV, GenBank Accession No. KU170668), and one reference genome sequence for Pepino Mosaic virus (PepMV, GenBank Accession No. FJ457096). Two hundred and ten nucleotide sequences of BanMMV were downloaded from NCBI on 12 December 2020. Among them, 54 sequences corresponded to the CP sequences of the virus (40 partial and 14 complete sequences) and 154 corresponded to partial RdRp sequences. A single complete genome of BanMMV (GenBank accession NC_002729) was available. GenBank accessions numbers and names of these sequences are listed in Supplementary File S3.

The mapper "Geneious", which is a fast mapping method with high sensitivity, was selected. In order to improve the results by aligning reads to each other in addition to the reference sequence, the fine tuning for mapping was set to "Iterate 2 times". In order to save calculation time and improve mapping efficiency, "20% mismatches" tolerance and customer sensitivity were selected for the sensitivity option for BanMMV, PepMV, and BYDV mapping, respectively. The "map multiple best matches" option was set to "Randomly", under which the reads will be mapped randomly to one of the best hits presenting equal scoring. A sample was considered positive by HTS if more than 10 reads were mapped to one of the BanMMV downloaded sequences [23].

De novo assembly into contigs was carried out to reconstruct the genome sequence of a potential new viral species, using the SPAdes software embedded in Geneious with default parameters and a k-mer of 55 [22]. The full genome sequence was aligned and used for phylogenetic analyses with the MEGA software package version 7.0. The phylogenetic relationships were inferred using neighbor-joining method embedded in the same version of the MEGA software. The stability of the topology was evaluated using bootstrap (1000 replications) [22]. Accession numbers of virus sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/ , accessed on 1 October 2021) have been integrated in the phylogenetic trees.

2.9. Validation of detection of the new species

To confirm the sequence of the new species, its RdRp and CP genes were amplified by RT-PCR and sequenced. Firstly, nine primer pairs were designed using Geneious software (v11.0.4). Seven pairs were used to amplify the RdRp of the new sequence, and two pairs were used to amplify the CP of the same sequence. Primers sequences and corresponding PCR programs are detailed in Supplementary File S4. The Tm and dimer formation of the selected primers were also checked using Geneious. Then, Sanger sequencing (Macrogen Europe BV, Amsterdam, The Netherlands) was performed from purified PCR products.

3. Results

3.1. Evaluation of Diagnostic Sensitivity of BanMMV Detection from Corm Tissues of Banana In Vitro Plants by IC-RT-PCR

3.1.1. Results on In Vitro Plants

Table 14: BanMMV detection by IC-RT-PCR from corm tissue of infected in vitro plants (19 accessions).

Accession code	Number of tested plants	Plants with true positive testing	Plants with false negative testing	Diagnostic sensitivity
ITC0099	10	7	3	70%
ITC0240	7	3	4	≈ 43%
ITC0519	5	2	3	40%
ITC1171	5	1	4	20%
ITC1434	10	4	6	40%
ITC1460	10	2	8	20%
ITC1541	7	1	6	≈ 14%

ITC1564	7	2	5	≈ 29%
ITC1664	10	8	2	80%
ITC1681	6	5	1	≈ 83%
ITC1691	5	0	5	0%
ITC1758	10	10	0	100%
ITC1767	5	0	5	0%
ITC1768	5	0	5	0%
ITC1776	11	5	6	≈ 45%
ITC1792	9	2	7	≈ 22%
ITC1808	5	0	5	0%
ITC1832	5	0	5	0%
ITC1833	5	0	5	0%
Total	**137**	**52**	**85**	**38 %**

The healthy accessions used as negative controls during this experiment were: ITC0450, ITC1304, and ITC1586, and negative results were always obtained.

According to these results (Table 14), 52 out of 137 plants tested positive. Thus, 38% of overall diagnostic sensitivity has been recorded. The percentage of detection per accession varied from 0 to 100%, depending on the accession. Among the 19 accessions tested, six showed 0% of virus detection, and only one showed 100% of detection. Some results of this testing are shown in Supplementary File S5.

The six BanMMV-infected accessions, for which 0% of virus detection was recorded, had different geographical origins. Details are provided in Supplementary File S1. This downplays the hypothesis that the undetected isolates correspond exclusively to a certain geographic origin. Unfortunately, it was not possible to check if there was an impact of genotypes on the absence of detection, since the genotype of three of these accessions is still unknown.

Nine plants from three accessions (ITC0450, ITC1304, and ITC1586) known to be healthy tested negative. Accessions details are provided in Supplementary File S1. They were used as negative controls for this test. Thus, 100% of the diagnostic specificity was obtained.

In addition, the results of up to four plants per accession were combined in order to follow the recommendations of the technical guidelines [24] for indexing from banana leaves, which recommend the testing of four individual plants per accession. The results are presented in Figure 28.

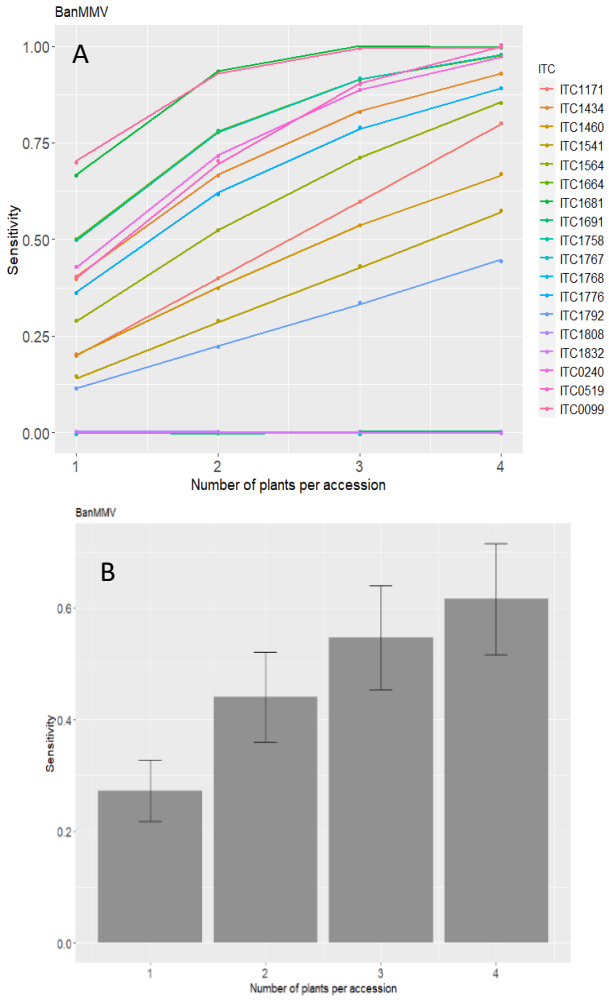

Figure 28: (A) Percentage of sensitivity of diagnostic of BanMMV from *in vitro* plants using 1, 2, 3, and 4 plants per combination for each accession. (B) Comparison of average sensitivity between combinations regardless of accessions; refers to standard deviation

Figure 28 illustrates that the diagnostic sensibility increased when the number of plants per combination increased, considering the virus detected when at least one plant is positive for the combination. Overall, average diagnostic sensitivity per accession varied from 45 to 100% when taken into account all the combinations of four plants/accession (except two accessions that remained at 0% and which corresponded to false-negative results),

considering a positive if at least one plant among the four tested positive. Figure 28(B) shows that the average diagnostic sensitivity varied from 27 to 61%, respectively, for combinations with one to four plants. The simulated diagnostic

sensitivity increased with the number of plants tested in the combinations. The highest sensitivity, being 61%, was reached with combinations of four plants for each accession of the six accessions that would have at least one positive combination with four plants. It has been also shown that for two out of the 19 accessions (ITC1171 and ITC1691), 100% of the detection was achieved with combinations of three plants/accession.

3.1.2. Comparison of Virus Detection after Thermotherapy from Corm Tissues of In Vitro Plants and from Leaves of Greenhouse Plants Using an IC-RT-PCR Assay

According to Table 15, BanMMV was detected from either corm tissues (in vitro tissues) or leaves of greenhouse plants, even after heat treatment, meristem culture, and a greenhouse acclimatization step. A success rate of 73% (per plant) for banana sanitation has been recorded in this study, confirming that thermotherapy in combination with meristem culture does not have a 100% efficacy for eradicating the virus.

Table 15: Comparison of BanMMV detection between in vitro corm tissues and greenhouse leaf samples of the same 41 banana plants (21 accessions) using IC-RT-PCR.

Accession Code	Tested Plant	Corm Results	Leaf Results (in Greenhouse)
ITC0099	1	-	-
ITC0240	1	-	-
ITC0321	1	-	-
ITC0519	1	-	-
	2	-	-
ITC1380	1	+	-
	2	+	-
	3	+	-
ITC1434	1	+	-
	2	-	-
ITC1460	1	+	-
	2	-	-
ITC1564	1	-	-
	2	+	-
	3	+	-
ITC1664	1	+	+
	2	+	-
	3	+	+
ITC1681	1	+	+
	2	-	+

	3	-	+
ITC1734	1	-	-
	2	-	-
ITC1748	1	-	-
	2	-	-
	3	-	+
ITC1752	1	-	+
ITC1758	1	-	+
ITC1767	1	-	-
ITC1768	1	+	-
	2	-	-
	3	-	-
ITC1776	1	-	+
ITC1792	1	+	-
	2	+	-
	3	+	-
ITC1808	1	+	+
	2	-	-
ITC1831	1	-	+
	2	-	-
ITC1857	1	-	-

"+" refers to a positive result; "-" refers to a negative result; the healthy accessions used as negative controls through this experiment were ITC0245, ITC0654, and ITC1120, and they tested negative.

Tests from tissue surrounding the meristem, were carried out directly after one month of heat treatment and the meristem excision. The percentage of in vitro plantlets that tested negative was 61% (25 plantlets out of 41 tested). We must take into account also that the false-negative rate when testing such tissue from in vitro plant is quite important. On the other hand, leaf tests from fully developed plants were carried out after thermotherapy, meristem culture, and a greenhouse acclimatization of these plants for at least three months. Thirty of the 41 tested plantlets tested negative, showing a banana sanitation rate of 73%.

As a result, we noticed that 22 out of 41 tested plants (53,7%) presented similar test results between in vitro and greenhouse samplings, corresponding to 18 negative and four positive results, whereas discrepancies were observed for 19 out of 41 tested plants (46,3%). For seven plants, the corm testing was negative, and the greenhouse testing was positive, while the opposite results were observed for 12 plants.

3.2. Diagnostic Sensitivity of RT-PCR from Corms and Leaves of In Vitro Plants

3.2.1. PCR Results of Infected and Healthy Banana Accessions

In vitro tissues from either the basal section or leaves were sampled. RT-PCR was carried out on the purified total RNA extracted from these samples. The testing was conducted on seven BanMMV-infected banana accessions (23 plants) and four healthy accessions known to be BanMMV-virus-free (16 plants). Results are detailed in Table 16.

Table 16: Testing of BanMMV infection from either basal section or leaves of infected in vitro plants. DSE = diagnostic sensitivity.

Accession Code	Nb. Of Tested Plants	Status	The Base of the Plant			Leaves of the Same Plant		
			Plants Tested +	Plants Tested -	DSE	Plants Tested +	Plants Tested -	DSE
ITC0476	**2**	Infected	2	0	100%	2	0	100%
ITC0528	4	Infected *	0	4	0%	0	4	0%
ITC1129	3	Infected	3	0	100%	3	0	100%

ITC1677	3	Infected	1	2	33%	2	1	67%
ITC1700	4	Infected	4	0	100%	4	0	100%
ITC1705	3	Infected	2	1	67%	0	3	0%
ITC1706	4	Infected	3	1	75%	4	0	100%
ITC0245	4	Healthy	0	4	n.a.	0	4	n.a.
ITC0654	4	Healthy	0	4	n.a.	0	4	n.a.
ITC1120	4	Healthy	0	4	n.a.	0	4	n.a.
ITC1586	4	Healthy	0	4	n.a.	0	4	n.a.

*: The infection status of this accession was determined by electron microscopy and the observation of filamentous particles; "+" means positive; " –" means negative; n.a. refers to not applicable.

Out of these 23 plants, fifteen tested positive for BanMMV presence when sampling in vitro tissues from either the base part or the leaves, reporting a diagnostic sensitivity of 65% from these two sections. The percentage of detection per accession varied from 0% to 100%. Among the seven infected accessions, three showed 100% of virus detection from both tissues. One accession (ITC0528) showed no virus detection from any sample, but the virus presence was based on the observation of a flexuous virus by electron microscopy. A lack of inclusivity of the tested primers could be the origin of the negative result and was further investigated (all the healthy accessions tested negative, reporting a diagnostic specificity of 100% through this molecular test).

Then, the results on individual plants were combined with up to four plants per accession (Figure 29). The diagnostic sensitivity increased when the number of plants per combination increased, regardless of the sampled tissue. Interestingly, Figure 29B showed that a plateau was reached with combinations of two plants/accession, showing a sensitivity of 100% through the test conducted from leaf tissues. This means that, for the tested accessions, 100% diagnostic sensitivity is reached when considering individual RT-PCR tests of at least two plants per accession.

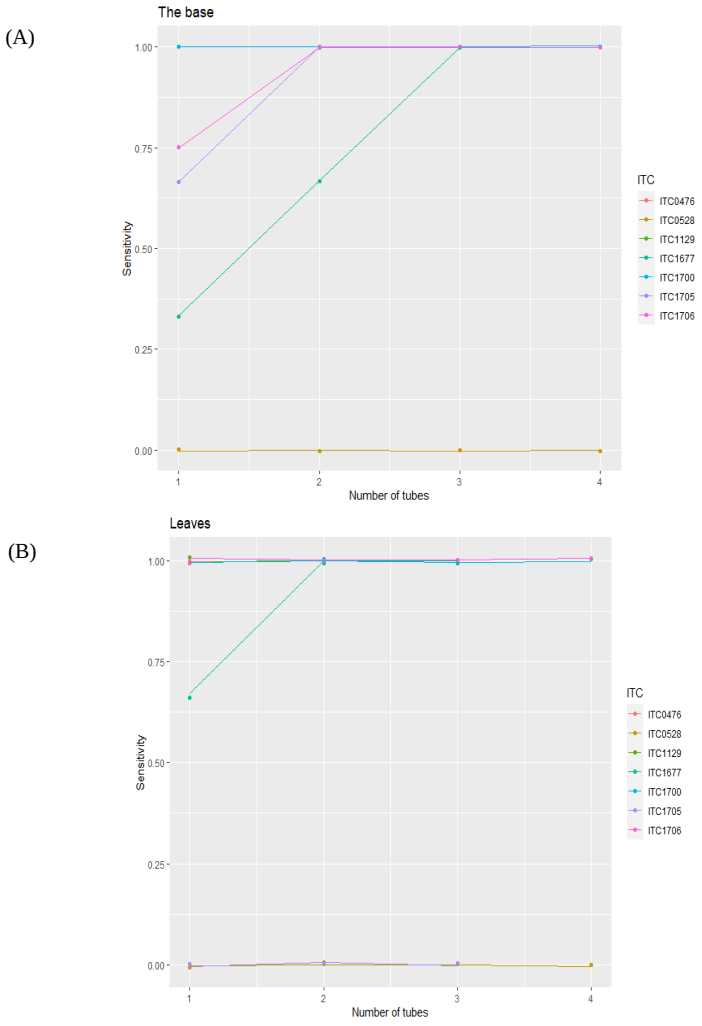

Figure 29: Diagnostic sensitivity of the test detecting BanMMV from two different in vitro tissues using combinations of plants per accession : (A) from the base (corm + meristem); (B) from leaves.

3.2.2. Detection of BanMMV by High-Throughput Sequencing Test on In Vitro Plants

To determine whether HTS could consistently detect BanMMV from in vitro plants, we selected nine banana accessions previously tested by RT-PCR and corresponding to seven infected and two healthy ones. To mimic the indexing protocol relying on the analysis of four plants pooled for each accession [10] and to take into account its higher cost, HTS was only applied on mixes of four plants per accession from either the base or leaves. Results are detailed in Table 17.

Table 17: HTS results of BanMMV detection from RNA extracts of the base or the leaves of in vitro plants. The number of reads per sample ranged between 6,499,298 and 10,357,840

Accession	Tissue	Individual	RT-PCR Result	Number of Reads Mapped to BanMMV Sequences (with 20% of Mismatches)	1st Alien Control (BYDV)	2nd Alien Control (PepMV)	Total Number of Reads
ITC0476	Base	Mix	+	5135	0	2	9,007,880
	Leaves	Mix	+	1822	0	3	6,499,298
ITC0528	Base	Pl(1)	-	49	0	2	9,211,966
		Pl(2)	-	67	1	0	10,346,472
		Pl(3)	-	46	0	0	8,606,200
		Mix	-	311	0	0	10,357,840
	Leaves	Pl(1)	-	162	0	2	9,419,300
		Pl(2)	-	193	0	1	8,544,882
		Pl(3)	-	171	0	1	7,473,540
		Mix	-	715	0	3	9,139,050
ITC1129	Base	Mix	+	667	0	2	9,791,204
	Leaves	Mix	+	683	0	3	8,717,902
ITC1677	Base	Mix	+	282	0	2	9,671,458
	Leaves	Mix	+	109	1	18	8,755,638
ITC1700	Base	Mix	+	2260	0	1	9,777,910
	Leaves	Mix	+	3499	0	0	8,245,604
ITC1705	Base	Mix	+	477	0	0	8,759,412
	Leaves	Mix	+	384	1	4	8,711,232
ITC1706	Base	Mix	+	1620	0	5	9,822,322

	Leaves	Mix	+	625	0	15	6,335,434
ITC1586 *	Base	Mix	-	0	0	0	8,414,752
ITC0654 *	Leaves	Mix	-	0	0	0	8,443,436
BYDV-infected alien	Leaves	Pl(1)	-	0	49,563	2	10,623,038
		Pl(2)	-	0	41,868	60	10,151,628
		Pl(3)	-	0	25,845	2	9,080,280
PepMV-infected alien	Leaves	Pl(1)	-	0	2	53,331	10,563,714
		Pl(2)	-	0	0	54,681	7,808,992
		Pl(3)	-	0	0	73,675	10,736,536

* ITC1586 and ITC0654 are healthy accessions used as negative controls; Pl refers to plant; Mix corresponds to a mix of four RNA extracts, each coming from one plant of the accession; "+" refers to a positive result; "-" refers to a negative result.

To check for cross-sample contamination, 10,623,038 reads, 10,151,628 reads, and 9,080,280 reads were generated from the first external alien control (corresponding to wheat sample infected by BYDV), among which 49,563 reads, 41,868 reads, and 25,845 reads corresponded to BYDV. In addition, 10,563,714 reads, 7,808,992 reads, and 10,736,536 reads were generated from the second external alien control (corresponding to tomato samples infected by PepMV), among which 53,331 reads, 54,681 reads, and 73,675 reads corresponded to PepMV. A very low PepMV contamination was observed through this test, with a maximum contamination ratio of 18 on banana samples (for ITC1677) and 60 for the BYDV alien control (on a maximum of 73,675 reads), corresponding to a contamination ratio of 1:1227. These results underlined the interest of adding an alien control to monitor the cross-contamination between samples.

In addition, no BanMMV contamination was observed in the two negative controls nor in the alien controls. The risk of detecting cross-sample contamination was indeed low for BanMMV, since all BanMMV-infected samples presented a very low proportion of BanMMV sequences (a maximum of 5135 reads on nine million reads for sample ITC0476 and a minimum of 46 for ITC0528-Pl(3)). This also underlined the importance of using positive controls at a low concentration in routine detection by HTS tests. Supplementary data from the sequencing are presented in Supplementary File S6. BanMMV reads were detected in all the infected samples. The DSE and DSP of HTS on pooled plant tissues were both at 100% from either the base or the leaves.

In same line with the RT-PCR results, the average DSE from the base was the same as the one from the leaves of the same in vitro plant. This sensitivity was similar when comparing HTS to RT-PCR for the same samples.

A BanMMV infection was also detected by HTS on the accession ITC0528, although the accession tested negative by RT-PCR. There were four mismatches between the BanMMCP2 primer sequence and the sequence of the isolate, explaining the false-negative result obtained by RT-PCR (unpresented results).

3.2.3. Identification of a New *Betaflexiviridae* Species Infecting *Musa* from ITC0528

A divergent *Betaflexiviridae* isolate with a reconstructed genome of 7364 bp was obtained by analysing the sequencing data from the corms and leaves of ITC0528, an accession belonging to *Musa* ornata Roxb. species. The pairwise alignment showed that the new genome sequence shared a pairwise identity of 62.7% at the nucleotide level with the BanMMV reference genome (GenBank accession NC_002729). Sequences from the new virus were not detected in the other accessions sequenced during this study nor in the more than 40 other accessions of *Musa* sp. sequenced at high throughput (Rong Wei, personal communication). Through the targeted RT-PCR and Sanger sequencing of amplicons, RdRp and CP sequences were confirmed. They presented 99.3% and 99.7% of identity, respectively, with the RdRp and CP consensus sequences generated by HTS.

The conserved domain assessment of the new species revealed the presence of all the plant viral domains of the *Betaflexiviridae* family (Table 18).

Table 18: List of conserved domains of the new species predicted by NCBI Conserved Domain Search.

Name	Accession	Description	Interval	E-value
Vmethyltransf	pfam01660	Viral methyltransferase	181–1125	2.18×10^{-65}
RdRP_2 super family	cl03049	RNA dependent RNA polymerase	4096–5229	1.86×10^{-37}
Viral_helicase1 super family	cl26263	Viral (Superfamily 1) RNA helicase	2911–3630	3.54×10^{-09}
Peptidase_C23 super family	cl05111	Carlavirus endopeptidase	2398–2655	1.28×10^{-03}

Viral_helicase1	pfam01443	Viral (Superfamily 1) RNA helicase	5372–5956	7.06×10^{-36}
Plant_vir_prot	pfam01307	Plant viral movement protein	5945–6238	2.25×10^{-23}
Flexi_CP super family	cl02836	Viral coat protein	6690–7094	6.96×10^{-41}
7kD_coat	pfam02495	7kD viral coat protein	6216–6380	1.26×10^{-04}

Two non-coding regions were found at the genome ends, 5' UTR and 3' UTR of 51 and 126 nt, respectively. The comparison of these values with members of the genus Foveavirus, for instance, belonging to the *Betaflexiviridae* family (5'UTR of 33–72nt and 3'UTR of 176–312nt) or with the BanMMV reference genome (GenBank accession NC_002729) (69nt 5′ UTR and 77nt 3′UTR and), showed that the genome reported lacks less than 50 nucleotides at its 3' end and a maximum of 200 nt at its 5′ end [25].

Further annotation revealed that the new contig has the typical genome organization of *Betaflexiviridae* members (Table 19). The genome organization and ORF sizes are similar to BanMMV but with a protein identity of a maximum of 65% for RdRp.

Table 19: BLASTP results of the five ORFs of the new *Betaflexiviridae* species.

	Interval	**Maximum Protein Identity (Protein-Protein BLAST)**	**Organism**	**Accession**
ORF1	52 -> 5268	64.6%	RNA-dependant RNA polymerase (Banana mild mosaic virus)	QVD99720.1
ORF2	5261 -> 5935	46%	Triple gene block protein 2 (Banana mild mosaic virus)	QVD99726.1
ORF3	5936 -> 6274	58%	Triple gene block protein 3 (Banana mild mosaic virus)	QVD99727.1
ORF4	6192 -> 6398	64%	Triple gene block protein 4 (Banana mild mosaic virus)	QVD99723.1
ORF5	6471 -> 7238	60.8%	Coat protein (Banana mild mosaic virus)	ACN91624.1

At the nucleotide level, the highest percentages of identity between the sequence of the new species and a BanMMV sequence were 58% (with FJ179164.1) for complete CP (n = 14) and 64% (NC_002729) for complete RdRp (n = 3). At the protein level, the highest percentages of identity between the sequence of the new species and a BanMMV sequence were 57% for CP (with FJ179163.1, AY319333, and AY319332) and 65% for RdRp (NC_002729). Throughout the *Betaflexiviridae* family, isolates of different species should have less than about 72% nt identity (or 80% aa identity) between their respective CP and polymerase genes [25]. In addition, the new genome has the conserved domains and the five ORFs of *Betaflexiviridae* members.

A phylogenetic tree including the sequenced genome sequence of the new isolate and the full genomes of some viruses from different genera of the *Betaflexiviridae* family (Figure 30) showed the clustering of the new species into a group with BanMMV, confirming its taxonomical position within this family of viruses and close to BanMMV, potentially belonging to the Banmivirus genus. Therefore, the sequenced isolate could be considered as a putative new species infecting *Musa* ornata, tentatively named *Musa* ornata associated Banmivirus (MoaBV).

This putative new species might be the second species of the proposed Banmivirus genus (with BanMMV currently as the unique species) within the *Betaflexiviridae* family.

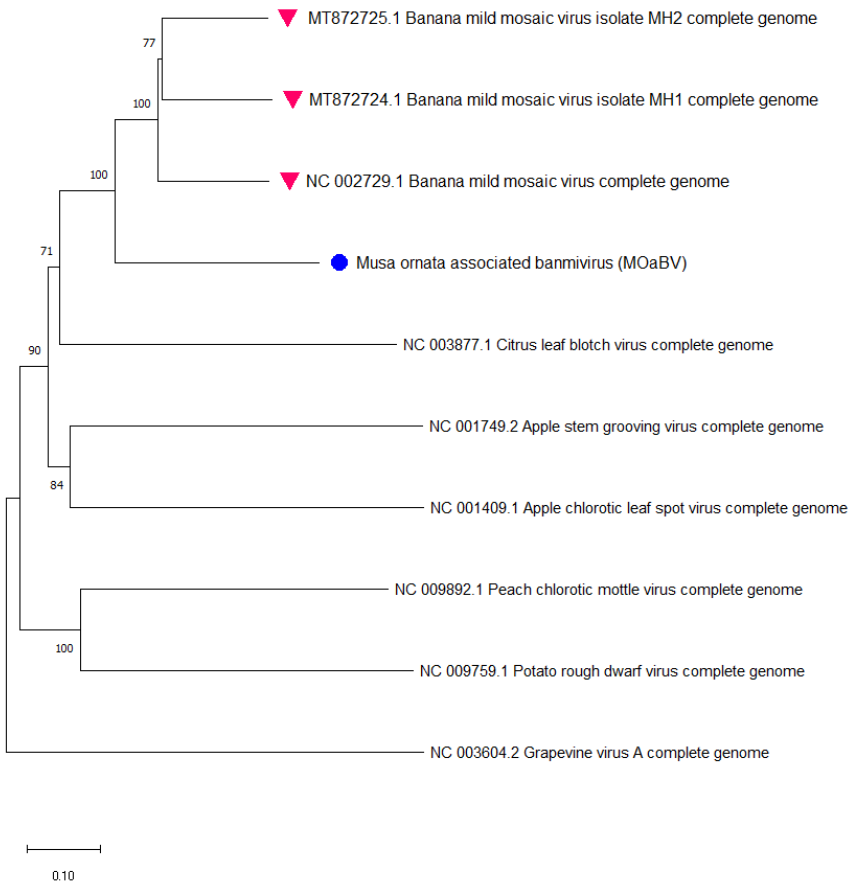

Figure 30: Neighbor-joining method phylogenetic tree inferred from full genomes of nt sequences of the new species from this study, complete genomes of BanMMV, and members from different genera of the *Betaflexiviridae* family. The purple triangle denotes the 3 existing complete genomes of BanMMV. The cyan dot denotes the new species identified throughout this work. Branches were bootstrapped with 1000 replications. The scale bar indicates the number of substitutions per site.

4. Discussion

In this publication, several detection tests have been applied on *Musa* in vitro plants. The DSE of IC-RT-PCR and RT-PCR applied on individual plants were 38% and 65%, respectively. For the same virus, De Clerck et al. [10] observed only 20% diagnostic sensitivity using a one-step RT-PCR from crude extracts on a single leaf per plant. Our results improved the DSE but also underlined the challenge to reliably detect BanMMV from in vitro plants.

The false-negative results can arise for different reasons. First, the uneven distribution of viruses in plantlets. For instance, Helliot et al. [26] have reported the heterogeneous distribution of CMV and BSV viral particles in the meristematic cells of *Musa* plantlets. In a similar context, Spiegel et al. [27] have described the uneven distribution of the tobacco streak virus (TSV) in the shoot and root systems of infected strawberry plantlets and that up to 30% of the progeny plants tested negative by ELISA. Plotnikov et al. [28] have shown that there is a correlation between the viral load of the cucumber green mottle mosaic virus (CGMMV) and the part of the cucumber plant ($r = 0,99$). Moreover, the same authors have found that high values of CGMMV concentration in the cucumber leaves of greenhouse plants were observed on the middle (46%) and lower (36%) leaves. Jones et al. [29] also highlighted heterogeneity in virus composition and concentration in different tissues of the plant. This heterogeneity is taken into account for the BanMMV testing of plants in a greenhouse, as it is recommended to sample the three youngest leaves for each plant to carry out a complete indexing of an accession [24]. The better results obtained from the base of the plant compared to the corm can be explained by the heterogeneous distribution of the virus in the plants. Indeed, even if some plant RNA viruses are known to infect meristematic cells [30,31], many viruses do not invade or are at very low concentrations in the meristem and surrounding young tissue, causing false-negative results from corm sampling.

Another hypothesis that can explain this phenomenon is the fact that viral titers can be very low, e.g., under the limit of detection, in in vitro plant tissues. Similarly, Umber et al. [15] have outlined the low viral titers in yam in vitro plants, possibly below detection thresholds. Moreover, Azad et al. [32] have suggested that the absence of vascular elements in meristem cells in potatoes

might be the reason for the low virus concentration in the meristem. This low concentration could also explain the observed results during post-therapy testing that are discussed hereunder.

An effect of the treatment applied on the in vitro plant was observed on the ability to detect BanMMV. Indeed, the detection by IC-RT-PCR of BanMMV from corm tissue surrounding the meristem was still more challenging when sampling it after thermo-therapy. Although the same results between in vitro corm and greenhouse leaf testing were obtained for 54% of the plants, the DSE dropped to 36% (four in vitro plants detected positive on the 11 plants tested positive in the greenhouse). A very low and heterogenous concentration of BanMMV in these tissues could explain the observed results. In addition, corm tissue surrounding the meristem from 12 plants tested positive while the plant tested negative in the greenhouse. These 12 positive results might be explained by the fact that the virus was present in the plant tissue surrounding the meristem but absent in the meristem, which is the objective of the thermotherapy (meristem-free explants). Thermotherapy can reduce virus movement towards the meri-stem [31]. In fact, the exposure of infected plants to high temperature could eliminate synthesis of both coat protein and movement proteins. This would likely restrict the cell-to-cell movement of a pre-existing virus [33]. It also allows the reduction of the viral replication rate in vascularized tissues [15]. However, several studies have highlighted the limited efficiency of the use of thermotherapy alone [15,31]. In addition, Umber et al. [15] have reported that meristem excision favors the regeneration of healthy plantlets from virus-free totipotent cells. Furthermore, it has been described that testing the virus status after greenhouse acclimation remains important, as the virus may be suppressed and not completely eliminated in some treatments at the tissue culture stage [31]. The sampling of this tissue for BanMMV detection is therefore not recommended from an in vitro plant. The sampled tissue influenced the performance of BanMMV testing by RT-PCR from RNA extracts of in vitro plants. The amplification from the base (including the meristem) or the three youngest leaves of individual plants presented a similar DSE of 67%. Nevertheless, for one accession (ITC1705), no BanMMV detection was recorded from leaves, whereas 67% of DSE was obtained from the base. This might be explained by the low and/or heterogenous viral titer in leaf tissues of this accession that could possibly be below detection thresholds [15]. Thus, a slightly better sensitivity might probably be obtained from the base compared to the leaves for some accessions. Nevertheless, sampling from the base causes the death of the plant,

as these tissues contain the meristem. Thus, it is not possible to regenerate the plant after the experiment. This can be problematic in the case of precious plants or can delay the testing until after in vitro multiplication.

Whatever the sampled tissue and the detection test applied, testing several plants individually for each accession and considering the accession as infected if a single plant is positive improved the DSE of the test. This decision reduced the false-positive rate to zero for the healthy accessions. This observation confirmed for in vitro plants the recommendations of the guidelines [24] for virus indexing on greenhouse *Musa* plants. These guidelines recommend the testing of four individual plants per accession, as the distribution of BanMMV can be heterogeneous between the plants. Therefore, any BanMMV testing from in vitro plants should also include at least four plants, even if for some accessions 100% DSE was obtained by RT-PCR considering only two in vitro plants.

The proportion of false negative per individual plant was also variable between accessions. Although a DSE of 100% was reached when testing the base of four individual plants, these results should be further confirmed on a larger number of accessions.

The HTS test was applied on a pool of four plants per accession and the sampling of either three leaves per plant or the complete base to address the virus heterogeneity in and between the plants. Pooling samples also lowered the cost of the HTS technologies, which is still much higher than RT-PCR. In addition, the very high analytical sensitivity of HTS represented an asset for detecting viruses present in very low concentrations [34,35].

HTS technologies showed excellent performances from pools of four plants, achieving 100% of DES from the base or the leaves of the accessions, although with a very low proportion of BanMMV sequencing reads for some accessions. The DSP of HTS was 100%, as it was for the PCR-based technique. In addition, the very high inclusivity of HTS was again demonstrated with the ability to identify a putative new species of *Betaflexiviridae* and an isolate of BanMMV presenting mismatches in the primer sequence. Ultimately, thanks to their untargeted nature, HTS technologies could also be used in the future to detect other viruses infecting the in vitro plants of a *Musa* accession. In addition, the use of an alien control would strengthen the reliability of the results by monitoring the contamination burden.

5. Conclusions

In summary, for detecting BanMMV from in vitro tissues, two tests can be recommended. First, HTS technologies could be applied on the RNA extracted from pooled leaves or bases from at least four plants per accession. Alternatively, if HTS technologies are not available or too expensive, RT-PCR could be applied on the total RNAs extracted from the base or, if the plant cannot be destroyed, from three leaves of at least four in-dividual in vitro plants (four biological replicates) per accession. These encouraging preliminary results warrant further application and evaluation of BanMMV detection on a larger panel of accessions and the extension of the proposed methodology to other viruses infecting *Musa* plants for which greenhouse cultivation is also mandatory.

Supplementary File S1

Table 20: List of the banana accessions used in this study

Accession code	Accession name	DOI	Genome	SubGroup	Geographical origin	Infected by BanMMV	IC-RT-PCR on	Thermotherapy	IC-RT-PCR on	IC-RT-PCR from greenhous	RNA extraction and RT-PCR	HTS	Remark
ITC0099	Ngougou	10.18730/9JAP0	AAB	Plantain	Unknown	x	x	x	x	x			
ITC0240	Oyoko Akpan	10.18730/9JKHR	AAB	Plantain	Unknown	x	x	x	x	x			
ITC0321	Dwarf French Plantain	10.18730/9JRCZ	AAB	Plantain	Unknown	x		x	x	x			
ITC0476	Pisang Relong	10.18730/9K0T5	AAB	Pisang Nangka	Malaysia	x					x	x	
ITC0519	Obubit Ntanga green mutant	10.18730/9K3GH	AAB	Plantain	Unknown	x	x	x	x	x			
ITC0528	Kluai Bou	10.18730/9K3VW	ornata	subsp. ornata	Unknown	x					x	x	Filamentous particles
ITC1171	Mai'a popo'ulu lahilahi	10.18730/9M8CD	AAB	Maia Maoli/ Popoulu	Unknown	x	x						
ITC1129	Big Ebanga	10.18730/9M5V6	AAB	Plantain	Unknown	x					x	x	
ITC1380	Chuoi Com	10.18730/9MPBG	AAB	Mysore	Vietnam	x		x	x	x			
ITC1434	BITA 5	10.18730/9MT1Q	Unknown	Unknown	Uganda	x	x	x	x	x			

ITC1460	Ijihu Inkundu	10.18730/9MVW8	AA	Unknown	Tanzania	x	x	x	x	x			
ITC1541	Kambani (unknown)	10.18730/9N1SC	AB	Ney Poovan	Tanzania	x	x						
ITC1564	Mlali	10.18730/9N3GY	AAA	Unknown	Tanzania	x	x	x	x	x			
ITC1664	Ebang Violet	10.18730/9NAQ2	AAB	Plantain	Unknown	x	x	x	x	x			
ITC1677	74-79	10.18730/9NBP~	AAB	Plantain	Unknown	x					x	x	
ITC1681	Chuoi hot	10.18730/9NBY4	balbisiana	Unknown	Vietnam	x	x	x	x	x			
ITC1691	Chuoi cau rung	10.18730/9NCMT	Unknown	Unknown	Unknown	x	x						
ITC1700	Kepok Kuning	10.18730/9ND89	ABB	Saba	Indonesia	x					x	x	
ITC1705	Mantreken	10.18730/9NDRS	AAB	Unknown	Indonesia	x					x	x	
ITC1706	Pagat	10.18730/9NDST	Unknown	Unknown	Unknown	x					x	x	
ITC1734	Hnget Pyaw Chin	10.18730/9NFTH	AAB	Mysore	Myanmar	x		x	x	x			
ITC1748	Boddida Bukkisa	10.18730/9NGQ9	ABB	Pisang Awak	India	x		x	x	x			
ITC1752	Poovilla Chundan	10.18730/9NH1K	AB	Kunnan	India	x		x	x	x			
ITC1758	Thu Ngel Sar	10.18730/9NHF~	Unknown	Unknown	Myanmar	x	x	x	x	x			
ITC1767	Phee Hsone	10.18730/9NJ2F	Unknown	Unknown	Myanmar	x	x	x	x	x			
ITC1768	Gubao	10.18730/9NJ4H	ABB	Unknown	Philippines	x	x	x	x	x			
ITC1776	Pusit	10.18730/9NJM~	AA	Unknown	Unknown	x	x	x	x	x			

ITC1792	NARITA 10	10.18730/9NM03	AAA	Unknown	Uganda	x	x	x	x	x			
ITC1808	25291-1A	10.18730/9NNHF	Unknown	Unknown	Nigeria	x	x	x	x	x			
ITC1831	Phee Kyan Sein	10.18730/9NQ6Z	Unknown	Unknown	Myanmar	X		X	X	X			
ITC1832	Wet Ma Lut	10.18730/9NQ7*	AAA	Cavendish	Myanmar	x	x						
ITC1833	Shwe Ni	10.18730/9NQ9$	AAA	Red	Myanmar	x	x						
ITC1857	Libanga Likale	10.18730/9NRXC	AAB	subgr. Plantain	Congo (Kinshasa)	x		x	x	x			
ITC0245	Safet Velchi	10.18730/9JM13	AB	subgr. Ney Poovan	Unknown			x	x	x	x		
ITC0450	Pisang Palembang	10.18730/9JZAZ	AAB	subgr. Pisang	Malaysia		x						
ITC1304	Kluai	10.18730/9MH70	ABB	Unknown	Unknown		x						
ITC1586	Grande Naine	10.18730/9N504	AAA	Cavendish	Unknown		x	x	x	x	x	x	
ITC0654	Petite Naine	10.18730/9KC5=	AAA	Cavendish	Unknown			x	x	x	x	x	
ITC1120	Tani	10.18730/9M56P	balbisiana	Unknown	Unknown			x	x	x	x		

Supplementary File S2

BanMMV detection protocol

Immunocapture

Twenty-five microlitres of an antibody mixture containing 5 µg/ml of BanMMV IgG (J. Thomas, unpublished) in sterile carbonate coating buffer was added to each thin-walled PCR tube and incubated for 2 h at 37°C before being washed three times for three min with PBS-T (0.05 M phosphate buffer pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 0.05% Tween20). Twenty-five microlitres of the clarified leaf extract was then added to the washed, coated tube and incubated at room temperature for 4°C overnight. Tubes were washed three times for three min at room temperature with PBS-T and rinsed once with sterile distilled water.

RT or cDNA synthesis

After the water was removed from the immunocapture tubes, reverse transcription was carried out in order to generate cDNA for the virus with RNA genomes. Diluted primer (750 nM Poty1; 12.5 µl total) was added to the immunocapture tube, which was incubated at 80ºC for 10 min, then rapidly chilled on ice. The following reagents were added (final concentration in 7.5 µl): 1× first strand buffer (Invitrogen, USA), 10 mM DTT, 500 nM dNTPs, 10 U RNaseOUT (Invitrogen), 50 U Superscript III reverse transcriptase (Invitrogen), and the tubes incubated at 50 ºC for 45 min, then 70°C for 15 min.

PCR

Two microliters of the cDNA mixture was added to 23 µl of the BanMMV PCR mastermix, prepared as follows: 1× MangoTaq coloured PCR buffer (Bioline, Australia), 2 mM MgCl2, 200 nM dNTPs, 400 nM Poty1 primer (reverse primer), 800 nM BanMMCP2 primer (forward primer) [1], 2.5 U MangoTaq DNA polymerase (Bioline, Australia). The thermal cycling conditions were: 94°C for 1 min, followed by 35 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 20 s, then 72°C for 3 min. The expected product size

was ~280 bp. In all cases, the PCR products were separated by electrophoresis in a 1% agarose gel in 0.5× TBE, and stained with GelRed (Biotium).

Reference

1. Hanafi, M.; Tahzima, R.; Kaab, S. Ben; Tamisier, L.; Roux, N.; Massart, S. Identification of Divergent Isolates of Banana Mild Mosaic Virus and Development of a New Diagnostic Primer to Improve Detection. 2020.

Supplementary File S3:

Sequences List

Table 21: List of all the BanMMV sequences published in GenBank

Sequence	GenBank accession number	Genomic regions
Banana mild mosaic virus, complete genome	NC_002729.1	complete genome
Banana mild mosaic virus clone 84_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729643.1	RdRp
Banana mild mosaic virus clone 84_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729642.1	RdRp
Banana mild mosaic virus clone 83_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729641.1	RdRp
Banana mild mosaic virus clone 83_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729640.1	RdRp
Banana mild mosaic virus clone 83_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729639.1	RdRp
Banana mild mosaic virus clone 82_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729638.1	RdRp
Banana mild mosaic virus clone 82_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729637.1	RdRp
Banana mild mosaic virus clone 82_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729636.1	RdRp
Banana mild mosaic virus clone 78_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729635.1	RdRp
Banana mild mosaic virus clone 78_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729634.1	RdRp
Banana mild mosaic virus clone 77_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729633.1	RdRp
Banana mild mosaic virus clone 77_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729632.1	RdRp
Banana mild mosaic virus clone 77_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729631.1	RdRp
Banana mild mosaic virus clone 93_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729630.1	RdRp
Banana mild mosaic virus clone 113_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729629.1	RdRp
Banana mild mosaic virus clone 112_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729628.1	RdRp

Banana mild mosaic virus clone 106_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729627.1	RdRp
Banana mild mosaic virus clone 7_4 nonfunctional RNA-dependent RNA polymerase (RdRp) gene, partial sequence	AY729626.1	RdRp
Banana mild mosaic virus clone 7_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729625.1	RdRp
Banana mild mosaic virus clone 23_1 nonfunctional RNA-dependent RNA polymerase (RdRp) gene, partial sequence	AY729624.1	RdRp
Banana mild mosaic virus clone 23_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729623.1	RdRp
Banana mild mosaic virus clone 23_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729622.1	RdRp
Banana mild mosaic virus clone 23_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729621.1	RdRp
Banana mild mosaic virus clone 43_7 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729620.1	RdRp
Banana mild mosaic virus clone 1_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729619.1	RdRp
Banana mild mosaic virus clone 21_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729618.1	RdRp
Banana mild mosaic virus clone 8_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729617.1	RdRp
Banana mild mosaic virus clone 22_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729616.1	RdRp
Banana mild mosaic virus clone 8_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729615.1	RdRp
Banana mild mosaic virus clone 22_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729614.1	RdRp
Banana mild mosaic virus clone 22_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729613.1	RdRp
Banana mild mosaic virus clone 25_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729612.1	RdRp
Banana mild mosaic virus clone 25_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729611.1	RdRp
Banana mild mosaic virus clone 25_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729610.1	RdRp
Banana mild mosaic virus clone 1_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729609.1	RdRp
Banana mild mosaic virus clone 8_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729608.1	RdRp
Banana mild mosaic virus clone 86_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729607.1	RdRp

Banana mild mosaic virus clone 89_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729606.1	RdRp
Banana mild mosaic virus clone 85_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729605.1	RdRp
Banana mild mosaic virus clone 6_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729604.1	RdRp
Banana mild mosaic virus clone 6_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729603.1	RdRp
Banana mild mosaic virus clone 6_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729602.1	RdRp
Banana mild mosaic virus clone 31_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729601.1	RdRp
Banana mild mosaic virus clone 21_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729599.1	RdRp
Banana mild mosaic virus clone 21_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729599.1	RdRp
Banana mild mosaic virus clone 99_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729598.1	RdRp
Banana mild mosaic virus clone 19_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729597.1	RdRp
Banana mild mosaic virus clone 13_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729596.1	RdRp
Banana mild mosaic virus clone 35_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729594.1	RdRp
Banana mild mosaic virus clone 35_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729594.1	RdRp
Banana mild mosaic virus clone 34_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729593.1	RdRp
Banana mild mosaic virus clone 30_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729592.1	RdRp
Banana mild mosaic virus clone 13_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729591.1	RdRp
Banana mild mosaic virus clone 13_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729590.1	RdRp
Banana mild mosaic virus clone 2_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729589.1	RdRp
Banana mild mosaic virus clone 110_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729588.1	RdRp
Banana mild mosaic virus clone 44_8 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729587.1	RdRp
Banana mild mosaic virus clone 44_7 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729586.1	RdRp
Banana mild mosaic virus clone 44_9 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729585.1	RdRp

Banana mild mosaic virus clone 44_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729584.1	RdRp
Banana mild mosaic virus clone 44_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729583.1	RdRp
Banana mild mosaic virus clone 44_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729582.1	RdRp
Banana mild mosaic virus clone 38_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729581.1	RdRp
Banana mild mosaic virus clone 15_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729580.1	RdRp
Banana mild mosaic virus clone 12_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729579.1	RdRp
Banana mild mosaic virus clone 12_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729578.1	RdRp
Banana mild mosaic virus clone 12_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729577.1	RdRp
Banana mild mosaic virus clone 17_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729576.1	RdRp
Banana mild mosaic virus clone 17_3 nonfunctional RNA-dependent RNA polymerase (RdRp) gene, partial sequence	AY729575.1	RdRp
Banana mild mosaic virus clone 17_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729574.1	RdRp
Banana mild mosaic virus clone 10_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729573.1	RdRp
Banana mild mosaic virus clone 10_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729572.1	RdRp
Banana mild mosaic virus clone 10_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729571.1	RdRp
Banana mild mosaic virus clone 11_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729570.1	RdRp
Banana mild mosaic virus clone 11_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729569.1	RdRp
Banana mild mosaic virus clone 32_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729568.1	RdRp
Banana mild mosaic virus clone 30_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729567.1	RdRp
Banana mild mosaic virus clone 103_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729566.1	RdRp
Banana mild mosaic virus clone 9_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729565.1	RdRp
Banana mild mosaic virus clone 40_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729564.1	RdRp
Banana mild mosaic virus clone 9_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729563.1	RdRp

Banana mild mosaic virus clone 1_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729562.1	RdRp
Banana mild mosaic virus clone 7_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729561.1	RdRp
Banana mild mosaic virus clone 9_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729560.1	RdRp
Banana mild mosaic virus clone 98_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729559.1	RdRp
Banana mild mosaic virus clone 108_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729558.1	RdRp
Banana mild mosaic virus clone 109_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729557.1	RdRp
Banana mild mosaic virus clone 20_7 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729556.1	RdRp
Banana mild mosaic virus clone 20_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729555.1	RdRp
Banana mild mosaic virus clone 19_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729554.1	RdRp
Banana mild mosaic virus clone 20_8 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729553.1	RdRp
Banana mild mosaic virus clone 19_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729552.1	RdRp
Banana mild mosaic virus clone 4_1_16 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729552.1	RdRp
Banana mild mosaic virus clone 2_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729549.1	RdRp
Banana mild mosaic virus clone 2_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729549.1	RdRp
Banana mild mosaic virus clone 5_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729548.1	RdRp
Banana mild mosaic virus clone 5_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729547.1	RdRp
Banana mild mosaic virus clone 5_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729546.1	RdRp
Banana mild mosaic virus clone 21_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729545.1	RdRp
Banana mild mosaic virus clone 21_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729544.1	RdRp
Banana mild mosaic virus clone 3_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729543.1	RdRp
Banana mild mosaic virus clone 3_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729542.1	RdRp
Banana mild mosaic virus clone 102_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729541.1	RdRp

Banana mild mosaic virus clone 104_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729540.1	RdRp
Banana mild mosaic virus clone 90_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729539.1	RdRp
Banana mild mosaic virus clone 35_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729538.1	RdRp
Banana mild mosaic virus clone 16_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729537.1	RdRp
Banana mild mosaic virus clone 96_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729536.1	RdRp
Banana mild mosaic virus clone 92_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729535.1	RdRp
Banana mild mosaic virus clone 97_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729534.1	RdRp
Banana mild mosaic virus clone 18_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729533.1	RdRp
Banana mild mosaic virus clone 18_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729532.1	RdRp
Banana mild mosaic virus clone 18_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729531.1	RdRp
Banana mild mosaic virus clone 105_1 nonfunctional RNA-dependent RNA polymerase (RdRp) gene, partial sequence	AY729530.1	RdRp
Banana mild mosaic virus clone 88_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729529.1	RdRp
Banana mild mosaic virus clone 87_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729528.1	RdRp
Banana mild mosaic virus clone 15_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729527.1	RdRp
Banana mild mosaic virus clone 15_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729526.1	RdRp
Banana mild mosaic virus clone 15_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729525.1	RdRp
Banana mild mosaic virus clone 14_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729524.1	RdRp
Banana mild mosaic virus clone 14_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729523.1	RdRp
Banana mild mosaic virus clone 14_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729522.1	RdRp
Banana mild mosaic virus clone 14_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729521.1	RdRp
Banana mild mosaic virus clone 91_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729520.1	RdRp
Banana mild mosaic virus clone 16_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729519.1	RdRp

Banana mild mosaic virus clone 16_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729518.1	RdRp
Banana mild mosaic virus clone 88_7 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729517.1	RdRp
Banana mild mosaic virus clone 88_6 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729516.1	RdRp
Banana mild mosaic virus clone 87_5 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729515.1	RdRp
Banana mild mosaic virus clone 87_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729514.1	RdRp
Banana mild mosaic virus clone 34_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729513.1	RdRp
Banana mild mosaic virus clone 30_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729512.1	RdRp
Banana mild mosaic virus clone 22_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729511.1	RdRp
Banana mild mosaic virus clone 11_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729510.1	RdRp
Banana mild mosaic virus clone 11_4 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729509.1	RdRp
Banana mild mosaic virus clone 107_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729508.1	RdRp
Banana mild mosaic virus clone 101_8 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729507.1	RdRp
Banana mild mosaic virus clone 101_9 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729506.1	RdRp
Banana mild mosaic virus clone 101_10 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729505.1	RdRp
Banana mild mosaic virus clone 100_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729504.1	RdRp
Banana mild mosaic virus clone 100_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729503.1	RdRp
Banana mild mosaic virus clone 100_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729502.1	RdRp
Banana mild mosaic virus clone 111_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729501.1	RdRp
Banana mild mosaic virus clone 7_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729500.1	RdRp
Banana mild mosaic virus clone 94_1 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729499.1	RdRp
Banana mild mosaic virus clone 4_1_12 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729498.1	RdRp
Banana mild mosaic virus clone 4_1_7 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729497.1	RdRp

Banana mild mosaic virus clone 4_1_13 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729496.1	RdRp
Banana mild mosaic virus clone 4_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729495.1	RdRp
Banana mild mosaic virus clone 4_3_39 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729494.1	RdRp
Banana mild mosaic virus clone 4_3_29 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729493.1	RdRp
Banana mild mosaic virus clone 2_3 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729492.1	RdRp
Banana mild mosaic virus clone 3_2 RNA-dependent RNA polymerase (RdRp) gene, partial cds	AY729491.1	RdRp
Banana mild mosaic virus isolate CP83.3 coat protein gene, partial cds	AY730758.1	CP
Banana mild mosaic virus isolate CP83.2 truncated coat protein gene, partial cds	AY730757.1	CP
Banana mild mosaic virus isolate CP83.1 coat protein gene, partial cds	AY730756.1	CP
Banana mild mosaic virus isolate CP82.3 coat protein gene, partial cds	AY730755.1	CP
Banana mild mosaic virus isolate CP82.2 coat protein gene, partial cds	AY730754.1	CP
Banana mild mosaic virus isolate CP82.1 coat protein gene, partial cds	AY730753.1	CP
Banana mild mosaic virus isolate CP78.3 truncated coat protein gene, partial cds	AY730752.1	CP
Banana mild mosaic virus isolate CP78.2 coat protein gene, partial cds	AY730751.1	CP
Banana mild mosaic virus isolate CP78.1 coat protein gene, partial cds	AY730750.1	CP
Banana mild mosaic virus isolate CP77.3 coat protein gene, partial cds	AY730749.1	CP
Banana mild mosaic virus isolate CP77.2 coat protein gene, partial cds	AY730748.1	CP
Banana mild mosaic virus isolate CP77.1 coat protein gene, partial cds	AY730747.1	CP
Banana mild mosaic virus isolate CP6.3 coat protein gene, partial cds	AY730746.1	CP
Banana mild mosaic virus isolate CP6.2 coat protein gene, partial cds	AY730745.1	CP
Banana mild mosaic virus isolate CP6.1 coat protein gene, partial cds	AY730744.1	CP
Banana mild mosaic virus isolate CP4.3 coat protein gene, partial cds	AY730743.1	CP

Banana mild mosaic virus isolate CP4.2 coat protein gene, partial cds	AY730742.1	CP
Banana mild mosaic virus isolate CP4.1 coat protein gene, partial cds	AY730741.1	CP
Banana mild mosaic virus isolate CP3.3 coat protein gene, partial cds	AY730740.1	CP
Banana mild mosaic virus isolate CP3.2 coat protein gene, partial cds	AY730739.1	CP
Banana mild mosaic virus isolate CP3.1 coat protein gene, partial cds	AY730738.1	CP
Banana mild mosaic virus isolate CP2.3 coat protein gene, partial cds	AY730737.1	CP
Banana mild mosaic virus isolate CP2.2 coat protein gene, partial cds	AY730736.1	CP
Banana mild mosaic virus isolate CP2.1 coat protein gene, partial cds	AY730735.1	CP
Banana mild mosaic virus isolate CP18.3 coat protein gene, partial cds	AY730734.1	CP
Banana mild mosaic virus isolate CP18.2 coat protein gene, partial cds	AY730733.1	CP
Banana mild mosaic virus isolate CP18.1 coat protein gene, partial cds	AY730732.1	CP
Banana mild mosaic virus isolate CP15.3 coat protein gene, partial cds	AY730731.1	CP
Banana mild mosaic virus isolate CP15.2 coat protein gene, partial cds	AY730730.1	CP
Banana mild mosaic virus isolate CP15.1 coat protein gene, partial cds	AY730729.1	CP
Banana mild mosaic virus RNA-dependent RNA polymerase gene, partial cds; nonfunctional triple gene block protein 2 and nonfunctional triple gene block protein 3 genes, complete sequence; and coat protein gene, complete cds	FJ179164.1	CP
Banana mild mosaic virus isolate TN2 coat protein gene, complete cds	KU378053.1	CP
Banana mild mosaic virus isolate TN1 coat protein gene, complete cds	KT780866.1	CP
UNVERIFIED: Banana mild mosaic virus isolate DA2 coat protein-like gene, partial sequence	JX183730.1	CP
UNVERIFIED: Banana mild mosaic virus isolate BF34 coat protein-like gene, partial sequence	JX183729.1	CP

UNVERIFIED: Banana mild mosaic virus isolate Y1 coat protein-like gene, partial sequence	JX183728.1	CP
UNVERIFIED: Banana mild mosaic virus isolate I9 coat protein-like gene, partial sequence	JX183727.1	CP
UNVERIFIED: Banana mild mosaic virus isolate S10 coat protein-like gene, partial sequence	JX183726.1	CP
UNVERIFIED: Banana mild mosaic virus isolate BO7 coat protein-like gene, partial sequence	JX183725.1	CP
UNVERIFIED: Banana mild mosaic virus isolate Z19 coat protein-like gene, partial sequence	JX183724.1	CP
UNVERIFIED: Banana mild mosaic virus isolate OM19 coat protein-like gene, partial sequence	JX183723.1	CP
UNVERIFIED: Banana mild mosaic virus isolate GA3 coat protein-like gene, partial sequence	JX183722.1	CP
Banana mild mosaic virus isolate AB19 coat protein gene, partial cds	JX014304.1	CP
Banana mild mosaic virus nonfunctional RNA-dependent RNA polymerase gene, complete sequence	FJ179163.1	CP
Banana mild mosaic virus isolate Ta1 coat protein gene, complete cds	AY366188.1	CP
Banana mild mosaic virus isolate F8 coat protein gene, complete cds	AY366187.1	CP
Banana mild mosaic virus isolate F2 coat protein gene, complete cds	AY366186.1	CP
Banana mild mosaic virus isolate c coat protein gene, complete cds	AY319333.2	CP
Banana mild mosaic virus isolate b coat protein gene, complete cds	AY319332.1	CP
Banana mild mosaic virus isolate a coat protein gene, complete cds	AY319331.1	CP
Banana mild mosaic virus isolate Q3.35T coat protein gene, complete cds	EF188275.1	CP
Banana mild mosaic virus isolate Q6.34 coat protein gene, complete cds	EF143979.1	CP
Banana mild mosaic virus isolate Q6.2T coat protein gene, complete cds	EF143978.1	CP
Banana mild mosaic virus isolate Q3.9T coat protein gene, complete cds	EF143977.1	CP
Banana mild mosaic virus isolate Q3.7 coat protein gene, complete cds	EF143976.1	CP
Barley yellow dwarf virus-PAV isolate BYDV-PAV-KS-SHKR, complete genome	KU170668	complete genome

Pepino mosaic virus isolate 1906 replicase, triple gene block protein 1 (TGBp1), triple gene block protein 2 (TGBp2), triple gene block protein 3 (TGBp3), and coat protein genes, complete cds	FJ457096	complete genome

Supplementary File S4

Primers and PCR programs used for sanger sequencing

Table 22: Primers and corresponding PCR programs used for the sanger sequencing in this study.

Primer name	Sequence (5'-3')	PCR program
73F1	TCCATTCTCGCTGCAATTAG	94℃ for 30s→(94℃ for 15s, 50℃ for 30s, 72℃ for 2min30s) X 40 cycles→ 72℃ 10min
510R1	TCGGGCTGAAAACAGGTTGA	
318F2	TGACACAGCTAGGGAGTTACTTG	94℃ for 30s→(94℃ for 15s, 52℃ for 30s, 72℃ for 2min30s) X 40 cycles→ 72℃ for 10min
1026R2	TGAATGTGAAAATATGGTGTAGGGT	
940F3	GGTGACATTTTGAGGACACGC	
1949R3	TCAGTATCCACAATTTCTACAAACTCA	
1726F4	AGCGAGAATTCTGACACTGCA	
2739R4	TGGCCCAATACTTCCAATTTCA	
2625F5	TCAAGAAGGTCATGGGTTGGA	
3346R5	TGTCACTGTCGTAGGAAGCT	
3250F6	CCCCCAGGCTATCTTGATTTGA	
4343R6	GCCATTTCTTCACCCATGCG	
4125F7	TGGACACATCACATCAATGCA	
5489R7	ATCCAGCCACTCCATGCAC	
6557F8	TGTGAATAGAACTTGTGTGTGTGA	94℃ for 30s→(94℃ for 15s, 48℃ for 30s, 72℃ for 2min30s) X 40 cycles→ 72℃ for 10min
6941R8	GGCGCTTGAACCATTGTGAT	
6827F9	AGAACTTGATTGGATTGGGGACT	94℃ for 30s→(94℃ for 15s, 52℃ for 30s, 72℃ for 2min30s) X 40 cycles→ 72℃ for 10min
7406R9	TCAACGAACATTTTTACGCGT	

Rq: The primers pairs in bold have been used to amplify the RdRp of the new species. The remaining two pairs have been used to amplify the CP of the species.

Supplementary File S5

Photo gel

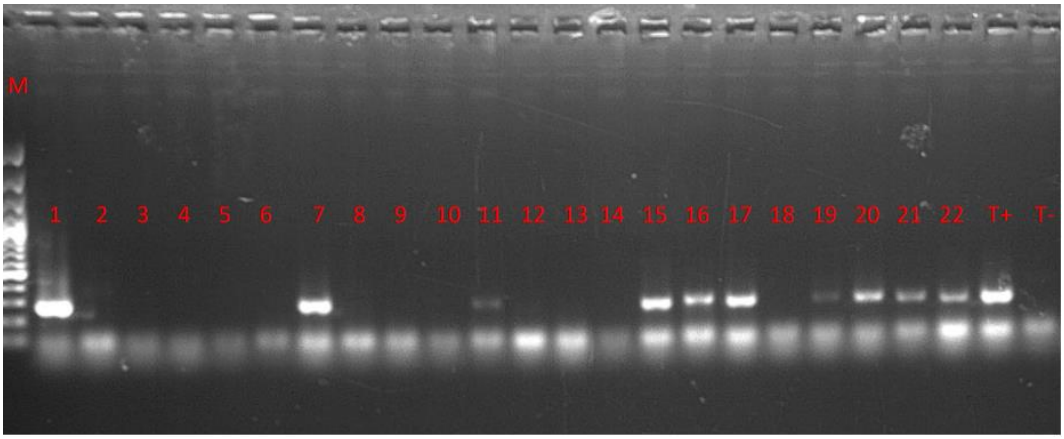

Figure 31: Gel electrophoresis of RT-PCR of banana accessions.

With M : 100 bp molecular weight marker, T+ refers to positive control and T- refers to negative control (healthy banana). Samples; from 1 to 7: ITC1564; from 8 to 14: ITC1541; from 15 to 22: eight plants from ITC 0099

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Chapter 5

General discussion and future perspectives

1. Discussion

Plant viruses are among the most important plant pests causing serious economic damages in crop production, up to 100% of yield losses in some cases. These pathogens are causing almost half of the emerging and the re-emerging plant pandemics all over the world (Jones, 2021). Furthermore, losses happen in either annual or perennial cultivated plants, and are capable of causing food shortages and famine. For instance, in 2014, the global economic impact of virus disease pandemics and epidemics was estimated to more than 30 billion USD per year. This impact has increased since then as the demand for plant products has also increased. Plant viruses are obligate parasites or holoparasites. Thus, they depend on their host for the replication of their genomes. Unlike bacteria and fungi, plant viruses hijack the host cellular machinery to their own advantage (Jones, 2021; Mehetre et al., 2021).

In the frame of the routine activities of the Virus Indexing and Sanitation Center (GHU) which is the Germplasm Health Unit (GHU) of Bioversity International, located at Gembloux Agro-Bio Tech, University of Liège (ULiège), this study aimed to focus on the detection and the identification of banana viruses. Firstly, several nucleic acid-based diagnostic tests were performed through this thesis in order to detect and identify viruses from different *Musa* accessions in different contexts, either *in vitro* and *in vivo* banana plants. Another objective was to assess the optimisation of the testing after thermotherapy used for banana viruses sanitation, and to evaluate the possibility of eliminating the greenhouse acclimation step of *in vitro* plants after sanitation. Finally, HTS technologies were performed and assessed for their use in routine detection of BanMMV from *in vitro* plants. They were compared to other conventional PCR methods. It should be noted that the evaluation of HTS technologies did not include the post-therapy testing process.

1.1. Effects of genetic diversity and evolution of plant viruses on the detection methods

In the past few decades, the emergence of new viral strains and/or new viral diseases in plants has gained great importance. For instance, 42 new Potato virus V (PVV) isolates have been discovered within the potato's Andean

domestication center in Peru (Fuentes et al., 2022). Similarly, 91 novel viruses have been discovered in fruit trees (Massart, Adams, et al., 2022). In addition, (Meng et al., 2017) have reported the discovery of Grapevine red blotch-associated virus (GRBaV) and Grapevine Pinot gris virus (GPGV) in grape material. Moreover, many new viruses have been discovered in wild asymptomatic plants (Roossinck et al., 2015).

The main factors driving this phenomenon are; the high genetic diversity and the rapid evolution of plant viruses, changes in vector population and agricultural changes, and climate change. In addition to these cited factors, global genomic features of the viruses impact also plant virus mutations and consequently the apparition of novel isolates (Duffy, 2018).

Viruses in general, and viruses with RNA genomes in particular, are able to generate large populations due to their great potential of high genetic variability and their rapid replication comparing to other group of replicons as RNA polymerases lack a proofreading activity, even though a recent study has reported remarkably similar substitution rates for DNA and RNA viruses (Rubio et al., 2020; Simmonds et al., 2019). We have reported through this thesis the discovery of two divergent isolates of BanMMV and a *Betaflexiviridae* species infecting banana, that were missed by published primers. This underlines the genetic diversity (mainly due to the accumulation of synonymous mutations) exhibited by BanMMV, especially at the nucleotide level. We have also reported the important intra-plant diversity by reporting the presence of highly divergent BanMMV isolates in a single plant (Bartola et al., 2020).

Although these findings are interesting, there is still a lot to do in order to confirm the high genetic diversity of BanMMV, such as some viruses like the Plum pox virus, Potato virus X or Potato virus Y that show very high genetic diversity with dozens of species (Cox & Jones, 2010; Karasev & Gray, 2013; Predajňa et al., 2012).

The genome variability of plant viruses, including the members of the *Betaflexiviridae* family among which is BanMMV, cannot be without impact on the detection methods. In fact, this diversity makes the molecular detection of plant viruses by specific primers particularly challenging. In same line, we showed through this thesis that it was impossible to detect different isolates of BanMMV with the same primers. Since it is highly challenging to develop a single protocol with inclusive primers able to detect all BanMMV isolates, we recommended the use of different primers to do so. However, this method can

be time-consuming, and unknown divergent isolates might exist. Thus, HTS technologies, that analyse the plant virome present in a sample with minimal bias and independently of the genome sequence of the target, can be a good alternative for routine indexing in the future. In addition, these technologies can generate several genomes of the virus, which allows the design of better diagnostic primers based on these genomes. In this study, we highlighted the very good performances of these technologies in detecting either known or unknow viruses.

1.2. What can make the diagnostic primers "obsolete" ?

In this study, a discrepancy was observed for the asymptomatic banana accession ITC0763. Filamentous viral particles were observed through electron microscopy while IC-RT-PCR, carried out for BanMMV detection, failed to amplify any fragment. The performing of HTS on this accession resulted on the identification of two complete genomes of BanMMV with 75.3% of identity. These divergent isolates were missed by the specific existing primer, which explained the false negative results obtained with molecular tests. This gives an insight into the "shelf life" of diagnostic specific primers. In fact, diagnostic primers become quickly "outdated" due to the high mutation rate in the virus genome. Thus, they can no more bind to the highly variable regions of the virus genome, due to the mismatches present in these regions. This results on the absence of amplification of the targeted genomic regions which leads to false negative results. In same line, (Mentes et al., 2022) have assessed the genetic variability of 53 existing SARS-CoV-2 primers and found out that the target regions of these primers in the analyzed samples were mostly susceptible to mutations. Therefore, they recommended the further investigation of the effects of these variations on the PCR sensitivity.

Every mismatch located within the primer sequence will definitely affect the PCR specificity by affecting the primer-template DNA duplex thermal stability. Nevertheless, the impact of mismatches depends on several factors such as the primer length and the number and position of mismatches. In fact, mismatches at the 3' end are considered high risk, since a single 3' end mismatch can lead to PCR reaction failure through the disruption of the nearby polymerase active site . The 3' end of a primer comprises the last 5 nucleotide

bases of the 3' end region. On the other hand, internal single mismatches far from the 3' end, or at 5' end can slightly affect the performances of the PCR assay. The efficiency of specific diagnostic primers is not constant over time. Thus, it is recommended to permanently monitor the performance of a PCR protocol through the use of complementary methods to PCR, such as electron microscopy or HTS, to confirm its results (in particular the negative ones) (Bru et al., 2008; Stadhouders et al., 2010).

1.3. HTS technologies are a double-edged sword

The use of HTS technologies in plant virus detection has presented numerous and clear advantages over classical diagnostic techniques. In fact, these without *a priori* technologies give a global idea about the viral status of a plant by analyzing the plant virome present in a sample with minimal bias and independently of the genome sequence of the target, which can also help to identify potential distant variants or novel viral species. Then, the sequence data issued from sequencing can be analysed at any time in parallel by one or more different scientists, for example, within multi-partners projects. Interestingly, these data can also be re-analysed once the global databases are expanded (Bester et al., 2021; Maree et al., 2018). Moreover, HTS could potentially greatly reduce time, material and personnel costs in comparison to conventional testing, as more samples can be processed at the same time for many viruses. Another interesting advantage of HTS remains its very high sensitivity (Rott et al., 2017). However, the cost can be a barrier to the use of these technologies in some laboratories. For instance, the costs might be detrimental in particular in case of hard to produce or highly sensitive biological assay reagents (Elmore et al., 2022; Mayr & Bojanic, 2009).

In this context, we have found out, through this thesis, that HTS had better diagnostic sensitivity than RT-PCR from the same tissues of banana *in vitro* plants (RNA extracts from pooled leaves or bases from at least four plants per accession). In fact, the average diagnostic sensitivity was of 65% and 100%, respectively for RT-PCR and HTS. These results suggest that a reliable diagnostic of BanMMV from in vitro plants can be used through HTS technologies (or RT-PCR if HTS is not available or is too expensive) and could be an efficient alternative for BanMMV testing after greenhouse cultivation. In addition, comparing to molecular tools, the very high inclusivity of HTS was also demonstrated, with the ability to detect distant

isolates of BanMMV missed by existing primers as well as the ability to identify a putative new species of *Betaflexiviridae* infecting banana.

In same line with these findings, several previous studies have shown that HTS technologies were equal or more sensitive than RT-PCR. For instance, a survey comparing detection methods of potato virus A and potato virus Y found out that HTS (based on sRNA) was 10 times more sensitive than qPCR (Santala & Valkonen, 2018). Similarly, HTS technologies (based on dsRNA) were equally sensitive to RT-PCR in barley and in fruit tress (Rolland et al., 2017; Rott et al., 2017). In another recent study, HTS test displayed higher analytical sensitivity, diagnostic sensitivity, diagnostic specificity and inclusivity than RT-PCR or IC-RT-PCR. Reproducibility and repeatability of virus detection were 100%. The same study showed that HTS test was able to detect at least two new viral species that are actually characterized (Rong et al., 2022). Other studies reported the discovery of new viral species during the evaluation of HTS performances on large scale testing (Pecman et al., 2017; Rott et al., 2017).

In brief, all the good performances described above allowed HTS to be considered as a good alternative for routine plant virus diagnostics. Nevertheless, as any technology, their use was associated with several pitfalls and challenges. These limitations need to be well understood before any validation or implementation of these technologies for routine plant virus diagnostics (Pallás et al., 2018).

A previous study on the validation of HTS for banana viruses detection, reported an important variability in reads number was noticed between replicates of the same/different batches, although the reproducibility and repeatability of the test reached 100% (Rong et al., 2022). This could be explained by the uneven distribution of viruses inside the plant tissues. Moreover, the presence of viruses in low titer can also be a limitation for HTS technologies as any other molecular tool (Bester et al., 2022).

Another serious issue of HTS, responsible of false positive results , is the contamination of samples with nucleic acids at different steps in the HTS protocols and within the platform. Even though false positives are less serious than false negatives, they are still a concern with HTS (Maree et al., 2018; Rott et al., 2017). In this context, HTS test displayed high false positive rate in the case of viruses that are integrated in the plant host genome (as partial or complete sequences). For instance, HTS detected viral reads from BSV

species (BSOLV, BSMYV, BSIMV, BSGFV and BSVNV) in samples containing B genome. Thus, it is compulsory to check this test with an IC-PCR test (Rong et al., 2022).

In order to optimize the use of HTS technologies and to avoid at best their limitations, some recommendations need to be followed. Indeed, it is important to choose the right HTS protocol that fits very well the purpose of the study. It can go up to performing a preliminary test that aims to compare protocols, before carrying out the study. Furthermore, an HTS-based result should be positive when the number of reads is above the detection threshold. Thus, it is necessary to determine this threshold that allows the distinction between potential contamination or infection. On the other hand, the use of controls with HTS tests, in particular, the alien control would strengthen the reliability of the results. An alien control is a new type of external control for plant pest detection, that helps to limit the false positive rate of an HTS test, by determining and/or adjusting the detection threshold. It can be used to replace or in addition to positive controls as it allows the evaluation and quantification of the cross-contamination burden (as a negative control) and the monitoring of target presence (as a positive control). An alien control might contain more than one alien target. In that case, it is preferable that it contains at least one of these target present in high concentration in order to better quantify and detect the alien contamination in the samples tested (Gauthier et al., 2022; Massart, Adams, et al., 2022; Rong et al., 2022).

However, the great importance of the addition of this control in an HTS test hides many limitations. Firstly, an alien control cannot determine the step at which the contamination happened. This can be solved by the addition of many alien controls at different steps but it requires higher costs. Secondly, the alien control is unable to identify one-time contamination events between two samples. Then, uncertainty can occur for low viral titre viruses such as BBTV. Indeed, the alien threshold can be a pitfall if it is not adjusted correctly. Low infection viruses can be missed by the high increase of the detection threshold in order to avoid false positives. Therefore, it needs to be adapted and optimized in the laboratory through the validation process of each HTS protocol (Rong et al., 2022).

1.4. Key phases for the reliable use of HTS technologies

The HTS process relies on eight different steps; firstly, the sampling then laboratory steps and bioinformatic components and finally, the target confirmation, interpretation and reporting. The laboratory steps include the nucleic acid extraction, the library preparation and the sequencing. Whereas, the bioinformatic steps include the analysis of raw reads, the identification of targets and the analysis of controls. The high complexity of some of these steps can negatively influence the reliability of the results. That's why it is highly important, for diagnostic and research laboratories, to have detailed guidelines on hand in order to ensure the reliability of the results provided by HTS technologies from any laboratory (Lebas et al., 2022).

The applications of HTS technologies in the detection of plant pathogens are very diverse. In fact, they can be used in the detection of known pests and/or the identification of novel viral isolates/ viruses (such is the case of this thesis) and their monitoring. Or, they can also be applied in order to study the virome composition at ecosystem scale (Maclot et al., 2020). Since the applications of these technologies are very large, it is recommended to select the HTS test protocol based on the intended use of this one, the time and the laboratory constraints and the availability of validated tests, before starting any step of their implementation as diagnostic tests. On the other hand, it should be noted that the risks associated with performing HTS tests need to be taken into account and analysed, as described in EPPO standard PM7/98 (2019). Indeed, there are the risks associated with any molecular test, such as the management of the laboratory or the documentation. But, there are also additional risks that are specific to HTS tests, such as those related to the sequencing platforms or the bioinformatics components. It is important that the risk analysis is performed by competent staff. These two steps are carried out during the test development and optimisation phase (Massart, Adams, et al., 2022).

Then, the use of reference material is recommended for the verification or the validation of HTS tests. Reference samples are used as external or internal controls (pooled or not) in order to continuously monitor the performance of the HTS tests, either for one specific step or the whole protocol of the HTS test. They should be produced by the diagnostic laboratory in a consistent manner following the EPPO standard PM 7/147 (2021). The reference

material include both biological reference samples and reference reads datasets. The stability of these references need to be checked for its identity and purity. In the case where a diagnostic laboratory is interested in using an already existing and validated HTS test, it should firstly check the risk analysis results and therefore assess the performance criteria of all its steps.

Furthermore, quality checks need to be conducted at three different levels which are the performance of the HTS test, the performance of the operator, and the performance of the laboratory. They help to ensure the validity of results and to investigate the origin if any problem occurs. Their types and frequency are defined during the risk analysis (Hébrant et al., 2017; Roenhorst et al., 2018).

2. Perspectives

2.1. Characterization of the 5'and 3' UTRs of the two BanMMV novel genomes

In this thesis, two whole genome sequences of BanMMV have been identified from ITC0763. They were deposited on GenBank under the following Genbank accessions numbers: MT872724 and MT872725. Their respective lengths are 7,336 nt and 7,311 nt making them quite near to the single complete genome of BanMMV whose length is 7,352 nt. The sizes of the UTR regions of these two novel isolates are 63 et 49 nt for the 5' end, and 95 and 67 nt for the 3' end, respectively for MT872724 and MT872725. The UTR sizes of the single reference BanMMV genome are 70 nt for the 5' end and 77 nt for the 3' end. Thus, it seems that the new genomes are unlikely to miss more than 20 nt in the 5' end and most likely less at the 3' end, in particular in the case of MT872724 for which the 3'UTR was longer than the one of the reference genome.

In order to check if we have obtained the complete genomes of the virus, it could be interesting to perform the RACE PCR procedure either at the 3'end or the 5'end. This system always begins with the reverse transcription step who aims to synthesize cDNA from the messenger RNA template, using a reverse transcriptase and a primer (an oligo-dT adapter), followed by PCR amplification of target cDNA. Then, the RACE products are sequenced by

High throughput Sequencing technologies. A cloning step can be added before the sequencing in most cases (Adamopoulos et al., 2022).

2.2. Biological characterization of the new Betaflexiviridae species

In this thesis, a new *Betaflexiviridae* species has been identified from ITC0528 with a genome of 7,364 bp, presenting a typical genome organisation of *Betaflexiviridae* members after annotation of its new contig. In addition, we have confirmed the detection of RdRp and CP sequences of this new species using targeted RT-PCR and Sanger sequencing. After performing these early steps of biological characterization, it could be interesting to consider the in-depth biological characterization of this new species using various experiments and investigations. This would help to know more about the symptomatology, transmission, host range of the new species, and its impact on either banana industry and/or the environment (Vazquez-Iglesias et al., 2022).

This in-depth characterization should fulfil the Koch's postulates that state the development of disease symptoms after the inoculation of a healthy host with the pathogen. In the case of BanMMV, this can be challenging as infection with this virus is very often asymptomatic. Moreover, according to the Bioversity International data, the accession ITC0528 (*Musa ornata* subsp. ornata) is a wild banana and has no known geographical origin. It was used in these experiments in the form of an *in vitro* plant. Thus, no symptoms of BanMMV infection were observed. The infection was confirmed through the observation of filamentous particles using electron microscopy. These are additional difficulties to this challenge.

In general, the most common technique of inoculation used, is the preparation of infectious clones. This method requires the complete viral genome sequence and offers precious information about symptomatology, host range studies and transmissibility. It was, for instance, developed to initiate studies on the pathogenicity of the blueberry virus S, a new virus from the genus *Carlavirus*, family *Betaflexiviridae*, Furthermore, this valuable information provided by this method helps to evaluate mutation/recombination rates in hosts plants. Then, inoculated plants from different species and cultivars

should be submitted to biotic or abiotic stresses in order to study their reaction in laboratory and/or greenhouses. Afterwards, The evaluation of the prevalence and distribution of the new species should be carried out through the organisation of large-scale surveys based on diagnostic tools. These surveys should be performed in either agricultural or natural ecosystems. They will also help to give an idea about the genetic variability of the new species (Massart et al., 2017).

2.3. Study of the functions of BanMMV TGB proteins

BanMMV is only known to infect species from the *Musa* genus. This virus is mainly transmitted through tissue culture or vegetative propagation. No natural vector has been identified, and mechanical transmission has not been successful. Virus transmission attempts made from a single infection in cv. Ducasse failed with mealybugs and aphids, via soil collected from around infected plants, and by root-to-root contact (Thomas, 2015).

Generally, plant viruses encode movement proteins to enable the passage of viral genome into adjacent cells via the plasmodesmata and in the long-distance virus movement via the phloem. These proteins are involved in the formation of sites for virus replication by using cell endomembranes (mostly the endoplasmic reticulum) (Arinaitwe et al., 2022; Lazareva et al., 2021).

As many RNA viruses from *Betaflexiviridae* family, the triple gene block (TGB) proteins of BanMMV would assist the viral cell-to-cell and long-distance movement of BanMMV. They would work together to complete the viral trafficking in infected plants (Igori et al., 2022; Verchot, 2022).

To date, there is no study describing the cell-to-cell movement of BanMMV in the plant. Thus, it could be really interesting to explore this research section by cloning the TGB genes of the virus and carrying out the subcellular localization of their proteins in *Nicotiana benthamiana*. Other techniques can be used in order to investigate the functions of TGB proteins in BanMMV, are the Biomolecular fluorescence complementation and the Co-immunoprecipitation that allow to characterize interactions faithfully between proteins (Ueki et al., 2011).

2.4. How can the sanitation process be improved?

Through this thesis, we found out that the sanitation rate of thermotherapy and meristem culture of banana *in vitro* plants was of 73%, as 30 out of 41 plants tested negative after thermotherapy, meristem culture and greenhouse acclimatization. In general, this sanitation process takes at least nine months from the placement of *in vitro* plants in the progressively rising temperature room, to the obtention of PCR results from *in vivo* leaves. Since this expensive process was time-consuming and labor-intensive, we have tried to accelerate it by assessing the reliability of PCR tests from *in vitro* plants right after thermotherapy in order to try to avoid the meristem culture and the acclimatization steps.

Unfortunately, we found out that among the 41 tested plants, only 22 presented the same results before and after greenhouse acclimatization, which makes a percentage of 53.7% of similar results in both situations. Thus, the removal of viral particles from all the plants is not guaranteed and it won't be possible to rely on this method. It is worth noting that the percentage of similar results might change if we test the virus detection in in vitro plants with a much more sensitive technique like HTS technologies.

On the other hand, it could be interesting to test a combination of treatments that includes chemotherapy to enhance the virus elimination rate of BanMMV from *in vitro* plants. Interestingly, (Meristem et al., 2023) have reported a 100% elimination of Onion yellow dwarf virus (OYDV), Garlic common latent virus (GCLV), Shallot latent virus (SLV) from garlic using a combination of shoot meristem culture, thermotherapy, and chemical treatment, with a high rate of plant regeneration of 66%. Indeed, the use of antiviral chemicals (like ribavirin or 8-azaguanine) can inhibit the virus replication and prevent its entry to the meristem from the neighbouring cells (Ramírez-Malagón et al., 2006).

Some other challenges were noticed throughout the experiments, such as the mortality of several plants during the heat process due to their sensitivity to high temperatures. In addition, some mechanical issues were observed while removing the meristem during meristem culture. In fact, it is not obvious to extract precisely the same dimensions of the excised tissue from all the *in vitro* plants. This has a direct impact on the elimination capacity of viral particles, as this latter depends on the size of the sampled tissue (Bettoni et al., 2016).

In this context, several studies have reported the numerous advantages and the good performances of cryotherapy over the conventional methods in eradicating viruses from many economically important crops such as banana, potato, plum, grapevine and apple (Bettoni et al., 2019; Vieira et al., 2015). Moreover, (Mathew et al., 2020) reported that cryotherapy has successfully eradicated the devastating bacterial pathogen, *Candidatus Liberobacter* asiaticus, from citrus. Then, (Bettoni et al., 2016) reported that the viruses eradication rate from grapevine using vitrification-based cryotherapy was of 97% against 12% with meristem culture.

In other words, cryotherapy is a promising biotechnological tool used to eliminate viruses and pathogens through cryopreservation methods, mainly vitrification techniques, by exposing the plants to liquid nitrogen (at -196°C) for a period . This ultra-low temperature is very efficient to eliminate infected cells. However, it causes some lethality rate for sensitive plants. Hopefully, this limitation can be compensated by the high proportion of virus-free regenerated-plants (Bettoni et al., 2016; Vieira et al., 2015).

In the case of *Musa* spp., some previous studies highlighted the interest in using cryotherapy to eradicate viruses from banana. Indeed, (Helliot et al., 2002) compared the cryotherapy with meristem culture for the elimination of viruses from banana, and found out that the cryotherapy was highly efficient. Then, it could be really interesting to update these experiments and run a large-scale survey in order to evaluate the efficiency of cryotherapy in eradicating banana viruses in particular BanMMV, and to see the impact of banana genotypes on it.

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