VIRAL DISEASES OF PLANTAIN AND BANANA : CONSTRAINTS AND PROSPECTS

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Summary

Viruses cause destructive and economically important diseases on plantain and banana worldwide. They also hamper the exchange and diffusion of *Musa* germplasm. Most reported viruses infecting plantain and banana are transmitted vegetatively or by biological vectors and can therefore be managed through the control of such vectors. Meanwhile, *Banana streak virus* (BSV) is either transmitted by mealybugs or through stress-induced activation processes of viral sequences integrated into the genome of *Musa balbisiana*. Therefore its control is much more challenging.

Innovative and sensitive methods have been developed and implemented for the detection and control of two viruses infecting banana and plantain, *Banana Mild Mosaic Virus* (BanMMV) and BSV. They are now used routinely for the safe mass production of certified *Musa* germplasm. Meanwhile, recent breakthrough on the activation of BSV sequences in natural or created plantain hybrids following *in vitro* mass propagation have been registered, leading to new recommendations for a better control of BSV.

Introduction

Plantain and banana are susceptible to numerous pests and pathogens, including five reported and characterized viruses: *Banana bunchy top virus* (BBTV, a *Nanovirus*), *Banana bract*

mosaic virus (BBrMV, a *Potyvirus*), *Banana mild mosaic virus* (BanMMV, an unassigned *Flexiviridae*), *Banana streak virus* (BSV, a *Badnavirus*) and *Cucumber mosaic virus* (CMV, a *Cucumovirus*) (Jones, 2000). Some of these viruses such as BBTV and, to a lesser extent, BSV, cause major outbreaks of great economical importance. The impact of BBrMV, BanMMV and CMV seems somewhat lower although mixed infections involving them can lead to severe necrotic leaf symptoms. Nevertheless, all viruses infecting banana are severe constraints to exchanges of *Musa* germplasm. BSV is the major constraint for genetic improvement of banana. This situation results from the ability of BSV sequences integrated into the genome of *Musa balbisiana* to be triggered by various abiotic and biotic stresses, including genetic crosses, and to lead to infectious viral particles (Dallot *et al*., 2000).

The safe production and movement of *Musa* germplasm can only be achieved by the use of refined detection techniques and a careful management of *in vitro* production methods. Both problems were tackled through the development of highly sensitive methods for the detection of BanMMV and BSV and their implementation in *Musa* mass production schemes. Meanwhile, it has now been established that i*n vitro* multiplication is one of the main abiotic stresses triggering the production of episomal infectious particles of BSV in inter-specific banana hybrids, through the activation of BSV endogenous pararetrovirus sequences (BSV EPRVs) integrated into the genome of *Musa balbisiana* (Dallot *et al*., 2000). Nevertheless, mass production of vitroplantlets remains the most widely used method for diffusing wild *Musa* cultivars or new improved hybrid species. Therefore, there is a need to evaluate the effects of *in vitro* culture on the activation of BSV EPRVs and to assess the risk of spreading BSV through the diffusion of micropropagated plantain and banana vitroplants. We have studied which steps of *in vitro* culture are involved in the activation of BSV EPRVs, for natural and created plantain interspecific hybrid cultivars and checked whether all the hybrid cultivars studied here go through similar activation patterns during *in vitro* culture.

Results & discussion

Development of an immunocapture reverse transcription nested PCR (IC-RT-nested PCR) test for the detection of BanMMV

Currently available anti-BanMMV antisera are either very sensitive but highly strain-specific or not sensitive enough for the development of an ELISA test that would be both sensitive and polyvalent. Therefore, a method combining specific antigenic recognition of the virus by a polyclonal antiserum and PCR amplification of part of the viral genome by reverse transcription PCR (RT-PCR) was

developed. Immunocapture of BanMMV particles was performed on banana leaf homogenates with purified anti-BanMMV IgGs (kindly provided by B. Lockhart, University of Minnesota, USA) using the technique of Foissac *et al*. (2005) with minor modifications (Teycheney *et al*., manuscript in preparation), as shown on **figure 1**. Encapsidated viral genomic RNA (Gambley and Thomas, 2001) was released from the immunocaptured particles by heat treatment. First strand cDNAs were prepared using AMV reverse transcriptase. A 362 nt portion of BanMMV ORF1 encoding the viral RNA-dependent RNA polymerase (RdRp) was then amplified by reverse transcription nested PCR (RT-nested PCR) with inosine-containing degenerated primers, using the protocol, primers and conditions developed by Foissac *et al*. (2005). Sequence analysis of the PCR product confirmed the high specificity and polyvalence of this detection test. Implementation of this test at a large scale was first achieved at a local level, with the full indexing of CIRAD Guadeloupe's *Musa* collection, revealing that 8.2% (38/461) of the accessions were indeed infected. This test is now successfully used in the comprehensive indexing scheme of mother plants used for mass *in vitro* multiplication of banana vitroplants, allowing the safe production of virus-free certified plants. It is also now used for routine indexing in international indexing facilities.

The development of this test allowed for a study of the molecular diversity of BanMMV. This study showed that BanMMV displays a very high molecular diversity, and that the IC-RT-nested PCR test allows the detection of very divergent variants of the virus. It also provided for the first time evidence for a plant-to-plant transmission of BanMMV (Teycheney *et al*., submitted).

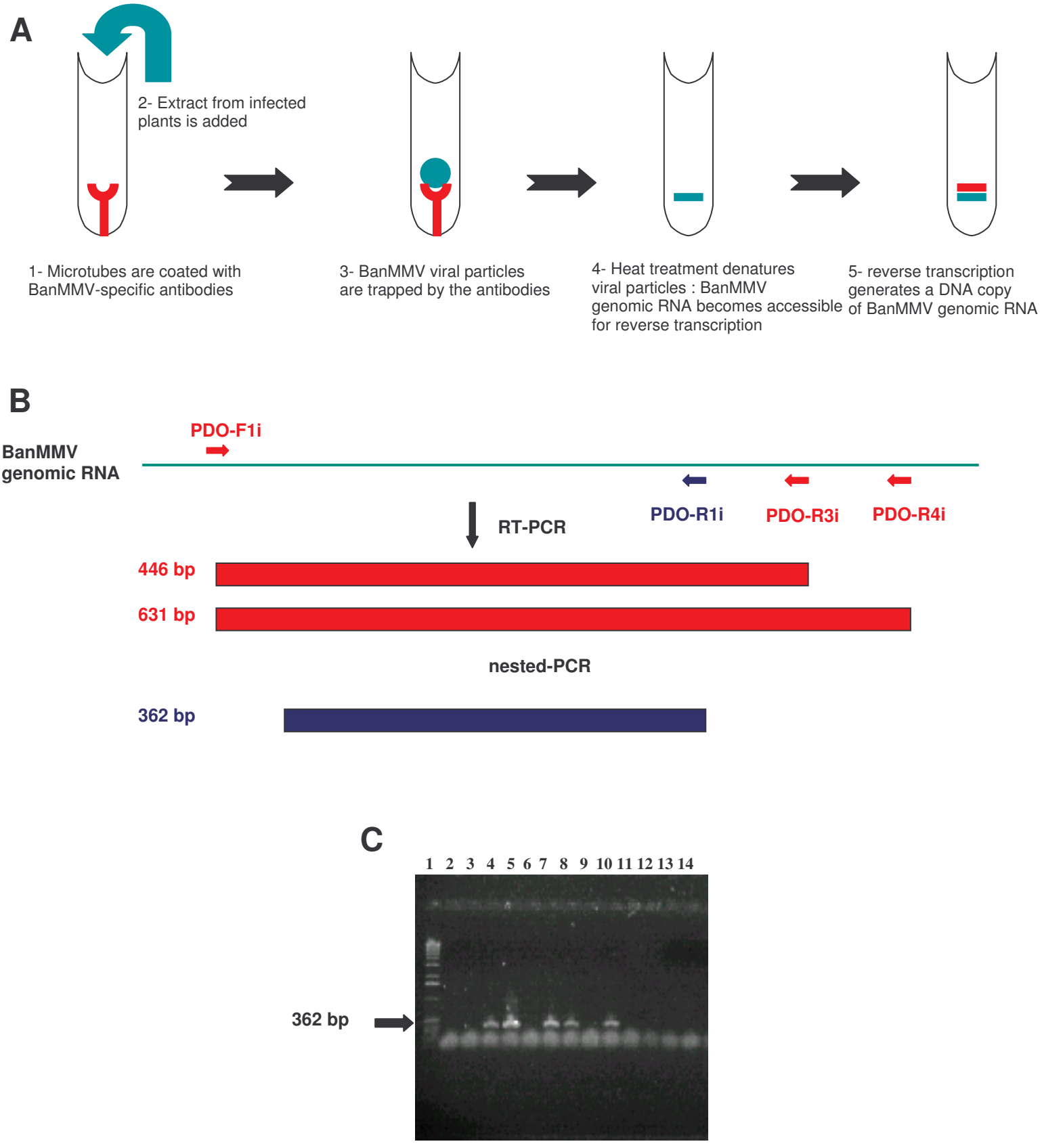

Figure 1 : Detection of *Banana mild mosaic virus* by immunocapture-RT-nested PCR

A : immunocapture-reverse transcription
B : polyvalent nested PCR process using degenerate primers containing inosine residues (PDO).
C : analysis of amplified fragments by electrophoresis on a 1% agarose gel
1 : DNA ladder
2 : virus-free control banana plant
3 – 14 : banana plant samples

Improved immunocapture-PCR (IC- PCR) test for the detection of BSV

Currently available anti-BSV antiserum is extremely polyvalent but not sensitive enough for the detection of BSV by ELISA test. Therefore, efficient diagnosis tools for the detection of BSV are based on immunocapture-PCR techniques. Following immunocapture of viral particles by polyvalent antibodies, part of BSV genomic DNA is amplified by PCR, using strain-specific primers. Unfortunately, the presence of BSV EPRVs in the genome of *M. balbisiana* can lead to the detection of false positives. Indeed, PCR products can result from 3 different situations: (i) amplification of sequences from episomal (encapsidated viral) DNA alone, (ii) amplification of both episomal and *Musa* genomic (EPRV) DNA sequences or (iii) amplification of *Musa* genomic (EPRV) DNA alone.

Existing IC-PCR protocols have been optimized in order to avoid putative contaminations by *Musa* genomic DNA and to minimize the amplification of EPRV sequences. In this regard, immunocapture proved to be a critical stage and incubation conditions were optimised. Furthermore, a multiplex PCR using both BSV specific primers and *Musa* specific primers allowed to detect contaminations by *Musa* genomic DNA, and to discard false positive resulting from such contaminations (see **figure 2**).

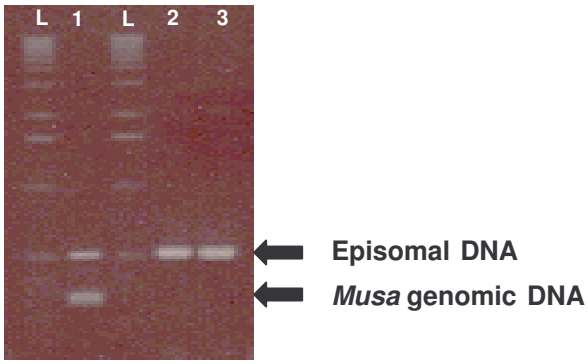

Figure 2 : Detection of *Banana Streak Virus* by immunocapture-PCR

L : DNA size ladder

1: infected sample contaminated by *Musa* genomic DNA

2 – 3 : infected sample free of *Musa* genomic DNA

This improved IC-PCR based detection test is now also used for routine indexing in indexing facilities.

Risk assessment of spreading BSV through *in vitro* multiplication of *Musa* germplasm

In vitro multiplication of *Musa* germplasm is often reported as the main abiotic stress leading to the production of episomal infectious BSV particles in plantain and banana varieties or cultivars harbouring some or all of the B (*M. balbisiana*) genome, through the activation of BSV EPRV sequences integrated into the B genome. Indeed, work performed by Dallot *et al*. (2001) has shown for the FHIA 21 hybrid species (AAAB) that 4 to 7 subcultures lead to the production of episomal BSV particles through activation processes in up to 58% of the produced shoots. Since mass production of vitroplantlets remains the method of choice for the diffusion of either *Musa* germplasm and new improved hybrid species, a better understanding of the effects of *in vitro* culture on BSV EPRV activation is necessary, in order to evaluate the risks of spreading BSV through the diffusion of vitroplantlets. To this aim, the behaviour of two natural triploid AAB plantains, Kelong Mekintu (KM) and Black Penkelon (PK), and the created tetraploid AAAB plantain CRBP 39 were studied during *in vitro* culture. Virus-free suckers were used for producing vitroplantlets, using the *in vitro* budding method described by Côte *et al*. (1990). For each of the three accessions, two (KM) or three (PK, CRBP39) distinct suckers were used in order to establish two or three different lines. During the successive multiplication (proliferation) subcultures, at least 40 shoots were randomly picked and indexed by IC-PCR for the presence of BSV (Goldfinger strain, noted BSV-Gf) viral particles. Results (shown in **figure 3**) were expressed as the percentage of shoots exhibiting BSV-Gf particles as a function of TPS (total produced shoots) values comprised between 50 to 8 000, which correspond to 5 to 12 proliferation subcultures.

For Black Penkelon and Kelong Mekintu cultivars and CRBP 39 variety, curve trends determined for BSV-Gf were similar for the three accessions studied and exhibited three main phases :

1. Immediately following the start of *in vitro* culture, the percentage of vitroplantlets indexed positive for BSV-Gf rapidly increased after the first subculture cycles. The maximum percentage of BSV Gf-positive plantlets ranged between 10 % (cv. Penkelon and CRBP 39) and 20 % (cv. Kelong Mekintu).
2. Following this increase step, a steady state phase was observed for the Black Penkelon and Kelong Mekintu cultivars.
3. Following this steady state phase, a decrease in the percentage of BSV Gf-positive plantlets was observed when increasing the number of subcultures for the three cultivars studied. This

was especially striking for CRBP 39 hybrid, for which values of zero (i.e below the sensitivity threshold of detection tests) were reached from TPS values of 4000 onwards.

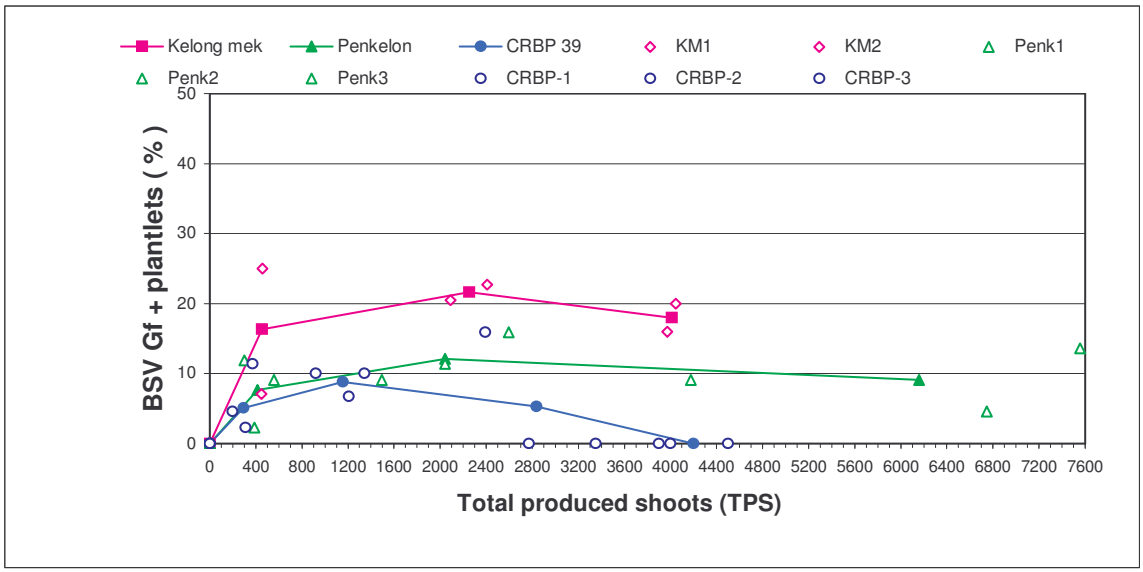

Figure 3: Relationship between the percentage of BSV-Gf (Goldfinger strain) -infected vitroplantlets and the number of vitroplantlets produced after *in vitro* culture.

TPS : total produced shoots.

Each point represents the percentage of BSV-Gf infected plantlets calculated from at least 40 plantlets. Filled symbols correspond to the mean percentage of BSV-Gf infected plantlets detected in the 2 (KM) or 3 (PK, CRBP39) lines studied for each cultivar.

Results indicate that vitroplantlets from either CRBP39 hybrid species and “natural” accessions KM and PK similarly display episomal BSV particles after *in vitro* multiplication. The observed increase in BSV-infected plantlets is unambiguously correlated with increasing numbers of *in vitro* sub-cultures. However maximum values for the percentage of BSV-infected plantlets slightly differ between the cultivars and hybrid studied. It ranges between 10% (for KM and PK) and 20 % (for CRBP39) in average and is always below 25%. Surprisingly, a decrease in the percentage of BSV-infected plantlets was observed for the highest values of TPS. This observation could result from two distinct biological phenomena : i) an initial activation of BSVEPRVs, ii) a concomitant decrease in the level of episomal particles in newly formed plantlets due to the well known dilution effect of viral particles

through *in vitro* culture, which results from the fact that cell multiplication outcompetes virus multiplication.

Conclusions

The development, improvement and implementation of detection methods are critical for the control of viral diseases. In order to increase the control of two of the main viral constraints hampering the movement of *Musa* germplasm and the genetic improvement of *Musa*, immuno-molecular methods for the sensitive detection of *Banana mild mosaic virus* and *Banana streak virus* were established or improved. These methods were implemented for large scale indexing and have now been adopted for routine screening of *Musa* germplasm. They allow the production of certified virus-free *Musa* germplasm. They have also been successfully used for characterizing the molecular variability of BanMMV and eventually lead to the identification of a new viral species infecting *Musa* spp (Teycheney *et al*., 2005).

Furthermore, research was carried out in order to assess the risk of spreading BSV through the diffusion of natural or created plantain interspecific hybrids harbouring the B genome and multiplied *in vitro*. For the three cultivars studied, it was shown that *in vitro* culture can lead to a substantial percentage of BSV-infected plantlets comprised between 10 and 20%, with natural and created hybrids behaving similarly. These results unambiguously show that vitroplantlets from both natural plantains and CRBP39 hybrid display similar patterns of activation during *in vitro* culture. Field scale experiments are now needed to assess the risk of spreading BSV through the diffusion of *Musa* germplasm harbouring the B genome and multiplied *in vitro*. The behaviour of vitroplantlets remaining uninfected after *in vitro* culture, when placed in field conditions, needs to be thoroughly evaluated. In order to develop a much needed comprehensive risk assessment scheme, the epidemiology and evolution potential of BSV populations will also have to be taken into account. Such studies, which are critical to the whole *Musa* community, will require the establishment of international partnerships through joint collaborative projects.

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