Improved detection of *Banana Streak Viruses* and *Banana virus X* and study of their prevalence and molecular diversity in Guadeloupe

Amélioration de la détection des espèces virales BSV et BVX, étude de leur prévalence et de leur diversité moléculaire en Guadeloupe

Pierre-Yves Teycheney[1], Grégoire Le Provost[2], Nathalie Laboureau[2], Isabelle Acina[1], Frédéric Péréfarres[1], Marie-Line Iskra-Caruana[2], & Thierry Candresse[3]

[1]CIRAD, Station de Neufchateau, F-97130 Capesterre Belle-Eau, Guadeloupe – teycheney@cirad.fr – [2]CIRAD/UMR BGPI, Campus International de Baillarguet, F-34398 Montpellier, France – [3]UMR GD2P, F-33883 Villenave d'Ornon, France.

SUMMARY

Viral diseases hamper *Musa* germplasm movement, genetic improvement and mass propagation. Therefore, reliable and sensitive methods for detecting viruses infecting banana and plantain are needed. A simple single step multiplex immunocapture PCR (M-IC-PCR) assay was developed for the accurate detection of episomal *Banana streak virus* and a direct binding reverse transcription nested PCR (DB-RT-PCR) assay was developed for the detection of the recently described *Banana virus X* (BVX). Both methods were used for a large scale study of the prevalence and molecular diversity of both BSV and BVX in Guadeloupe.

RESUME

Les maladies virales limitent fortement les échanges, la multiplication de masse et l'amélioration génétique des bananiers et plantains. Aussi, il est important de disposer de techniques fiables et sensibles pour la détection des virus responsables de ces maladies. Un test de détection par multiplex immunocapture PCR (M-IC-PCR) a été développé pour la détection de copies épisomales du virus de la mosaïque en tirets du bananier (BSV), et un test de détection du virus X du bananier (BVX) par direct binding reverse transcription nested PCR (DB-RT-PCR) a également été développé. Ces outils ont été utilisés pour étudier la prévalence et la diversité des populations virales de BSV et de BVX présentes en Guadeloupe.

KEYWORDS

Banana streak virus, BSV ; *Banana virus X*, BVX ; Endogenous pararetrovirus, EPRV ; Multiplex immunocapture PCR, M-IC-PCR ; Direct binding reverse transcription PCR (DB-RT-PCR).

INTRODUCTION

Banana streak viruses (BSV) are mealybug-transmitted members of the plant pararetrovirus genus *Badnavirus,* infecting banana and plantain worldwide (Hull, 2000; Fauquet *et al.*, 2005). BSV infections cause characteristic chlorotic and necrotic leaf streak symptoms. Depending on infecting BSV species, highly susceptible banana cultivars can develop more severe symptoms, such as pseudostem splitting and necrosis (see figure 1), eventually leading to the death of infected plants (Lockhart and Jones, 2000; Daniells *et al.*, 2001). Interspecific *Musa acuminata* x *Musa balbisiana* genotypes, including a number of newly created hybrids, can produce BSV-infected propagules from virus-free source plants propagated by tissue culture (Dallot *et al.*, 2001). Likewise, infected progeny are often obtained following genetic crosses involving virus-free *M. acuminata* and *M. balbisiana* parents (Lheureux *et al.*, 2003). Such infections are correlated to the presence of endogenous BSV sequences called BSV EPRVs, which are integrated into the genome of *M. balbisiana* (Geering *et al.*, 2005), a widespread progenitor of natural and created hybrid banana and plantain species. Some infectious BSV EPRVs are assumed to lead to infectious viral particles through activation processes that are triggered by biotic and abiotic stresses such as *in vitro* culture (Folliot *et al.*, 2006).
Detection of episomal BSV by ELISA has been shown to lack sensitivity due to high backgrounds. Therefore immunocapture-PCR (IC-PCR) was adopted as the method of choice for detecting BSV, due to its high sensitivity and specificity. It combines immunological capture of BSV viral particles by a polyclonal antiserum (Ndowora, 1998) and amplification of part of the viral genome, using species-specific primers. Unfortunately, persistent background is encountered when indexing *Musa* species or hybrids harbouring the *M. balbisiana* genome that contains integrated BSV sequences. Such background results from the binding of *Musa* genomic DNA to the walls of the thin-walled tubes or microplates used for IC-PCR. In order to prevent this phenomenon, we developed a single step multiplex immunocapture PCR (M-IC-PCR) assay for the sole detection of episomal BSV (Le Provost *et al.*, 2006). *Musa* sequence tagged microsatellite site primers (STMS) were used in combination with BSV species-specific primers in order to allow the detection of false positives resulting from the presence of *Musa* genomic DNA, and immunocapture conditions were optimized in order to limit contamination by residual *Musa* genomic DNA.

Banana virus X (BVX) is a newly described member of the *Flexiviridae* family infecting banana (Teycheney *et al.*, 2005a). Although BVX has not been associated with any particular symptom so far, it is important to develop specific detection techniques, in order to prevent its spread through the use of infected plant material. Therefore, a direct binding reverse transcription-nested PCR (DB-RT-PCR) test was developed for the detection of BVX, using degenerate primers for the RT-PCR step and BVX-specific primers for the nested PCR step.

Both BSV and BVX detection tests were successfully used for a large scale study of the prevalence and molecular diversity of BSV and BVX in Guadeloupe.

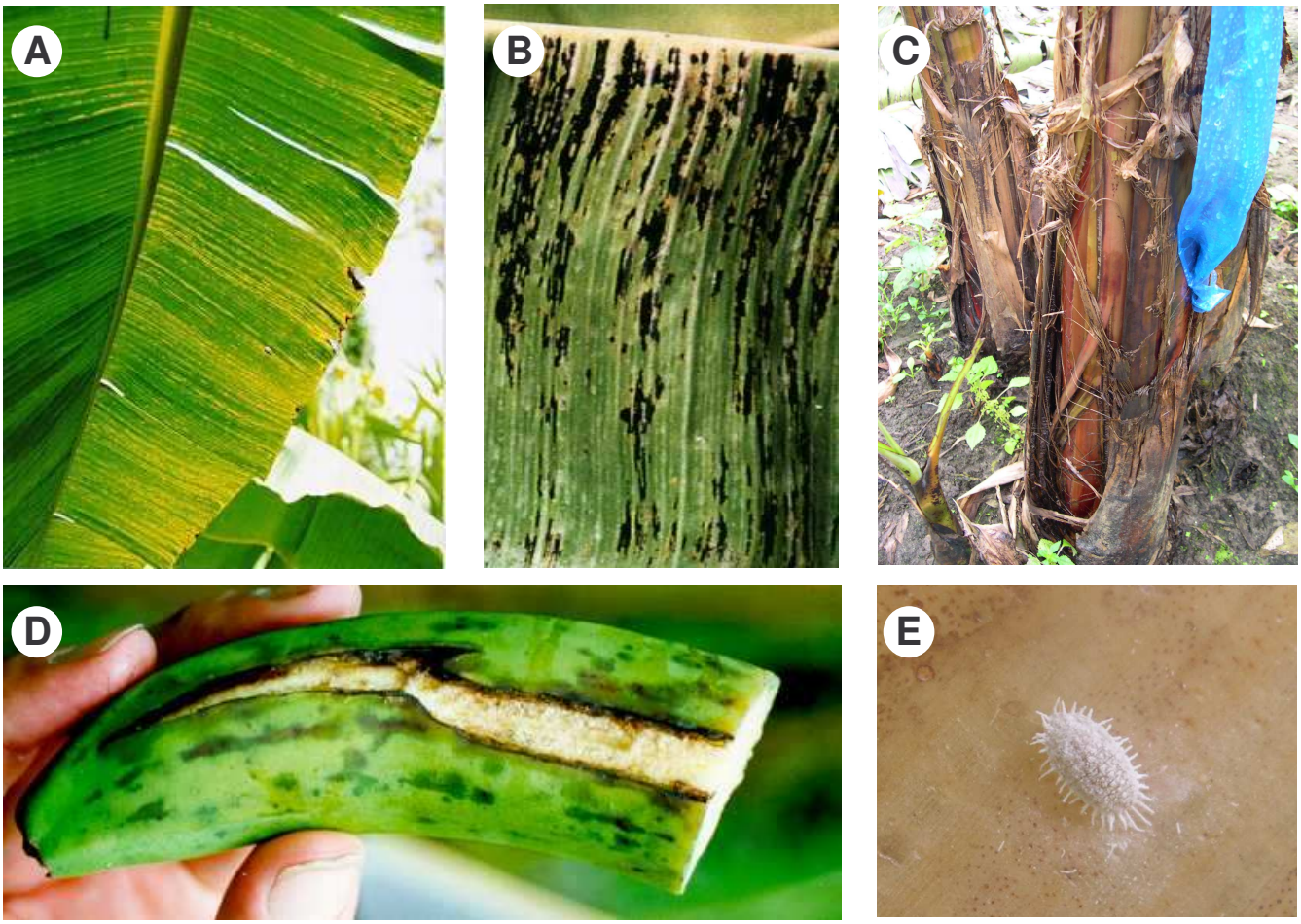

Figure 1 : BSV symptoms on banana leaves, pseudostem and fruit.
A: chlorotic leaf streak symptoms.
B : necrotic leaf streak symptoms.
C: pseudostem splitting.
D : symptoms on fruit.
E : mealybug feeding on the pseudostem of a BSV-infected banana plant.

MATERIAL & METHODS

Plant materials were collected from various locations in Guadeloupe. For the detection of BSV, 0.5g leaf samples were ground in 5ml of sample buffer (2% polyvinylpyrrolidone 40, 0.2% sodium sulfite and 0.2% bovine serumalbumine, prepared in PBS Tween). Polypropylene microplate wells were coated overnight at 4°C with 25 µl of IgG purified from a polyclonal antiserum (Ndowora, 1998) and diluted to 2 g/ml in standard carbonate coating buffer, then washed 3 times with PBS-Tween. Immunocapture was performed by incubating 25 µl of leaf extracts in Ig-coated microplate wells or tubes for 3 hrs at room temperature. Following several washes with PBS Tween, multiplex PCR was performed using BSV species specific primers targeting the RT/RNaseH domain of the viral genome and *Musa* sequence tagged microsatellite (STMS) primers targeting *Musa* genomic sequences (Lagoda *et al.* 1998, see table 1). PCR reaction mix of 25 µl contained 20mM Tris-HCl (pH 8.4), 50 mM KCl, 100 mM each dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1U *Taq* DNA polymerase (Eurogentech, Seraing, Belgium). PCR cycle conditions were an initial denaturation step at 94°C for 3min, then 35 cycles (94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min) followed by a final elongation step of 5 min at 72°C.

Table 1: Nucleotide sequence of primers used for the detection of BSV by M-IC-PCR.

Target	Name of primer	Primer sequence (5'-3')	Size of PCR product (bp)
BSOLV	RD-F1◊	ATCTGAAGGTGTGTTGATCAATGC	522
	RD-R1◊	GCTCACTCCGCATCTTATCAGTC	
BSGFV	GF-F1◊	ACGAACTATCACGACTTGTTCAAGC	476
	GF-R1◊	TCGGTGGAATAGTCCTGAGTCTTC	
BSMysV	Mys-F1◊	TAAAAGCACAGCTCAGAACAAACC	589
	Mys-R1◊	CTCCGTGATTTCTTCGTGGTC	
STMS	AGMI 025°	TTAAAGGTGGGTTAGCATTAGG	248*
	AGMI 026°	TTTGATGTCACAATGGTGTTCC	

◊ : published in Geering *et al.* (2000)
° : published in Lagoda *et al.* (1998)
* : size of PCR fragment amplified from *M. balbisiana* genomic DNA

For the detection of BVX, a direct binding reverse transcription nested PCR protocol was developed. Direct binding of viral particles was performed by incubating 50 µl of banana leaf extracts in thin-walled tubes or microplates overnight at 4°C. Plates or tubes were then washed 4 times in PBS-Tween and 1 time in sterile distilled water. RT-PCR was performed on directly bound viral particles, using the protocol and set of degenerate primers designed by Foissac *et al.* (2005) for the detection of *Flexiviruses*. A nested PCR was then performed on an aliquot of the RT-PCR mix, using a set of BVX-specific primers targeting the RNA dependant RNA polymerase (RdRp) domain of ORF1 (see figure 2).

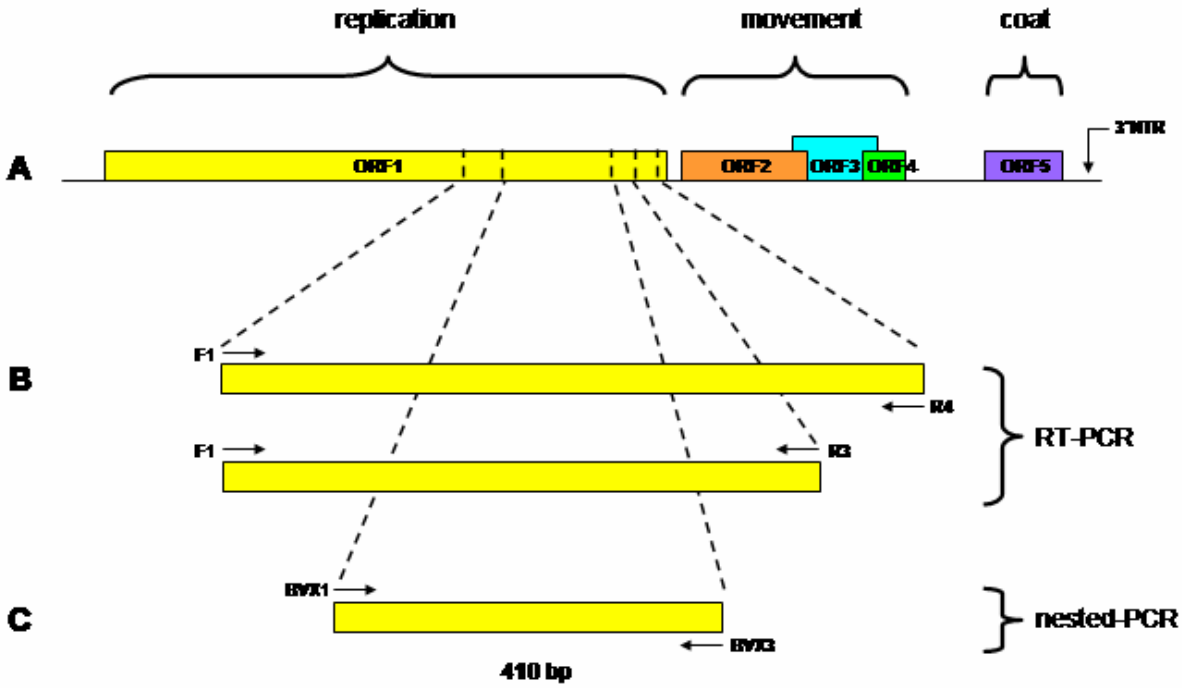

Figure 2 : Figure 1. Genetic organisation of BVX genomic RNA and regions amplified during DB-RT-nested PCR. Positions of the ORFs along BVX genomic RNA are indicated (A). Arrows show the position of the primers used for RT-PCR and nested PCR experiments. Degenerate inosine-containing primers F1, R3 and R4 were used for the RT-PCR step (B) whereas BVX-specific primer pair BVX1/BVX3 was used in the subsequent nested PCR step (C), giving rise to a 410 bp fragment.

RESULTS & DISCUSSION

Detection of BSV by M-IC-PCR

BSV detection by PCR-based techniques is hampered by the presence of BSV endogenous sequences (BSV EPRVs) in the genome of *Musa balbisiana* (Geering *et al.*, 2005). Indeed, BSV EPRVs can interfere with IC-PCR diagnosis and lead to background amplification of non episomal BSV sequences (Le Provost *et al.*, 2006). This phenomenon results from residual trapping on polypropylene thin-walled tubes or microplates of host *Musa* genomic DNA, which is present in leaf extracts. In order to eliminate background amplification of BSV EPRV sequences during IC-PCR, immunocapture conditions were optimised. Limiting immunocapture times was found to decrease the binding of residual *Musa* genomic to polypropylene below detection threshold by PCR. Furthermore, a multiplex PCR assay using STMS primers and BSV species-specific primers was developed in order to detect potential contaminations by *Musa* genomic DNA, which can lead to false positives in the course of IC-PCR. Figure 2 shows that the developed M-IC-PCR allows the specific detection of various BSV species.

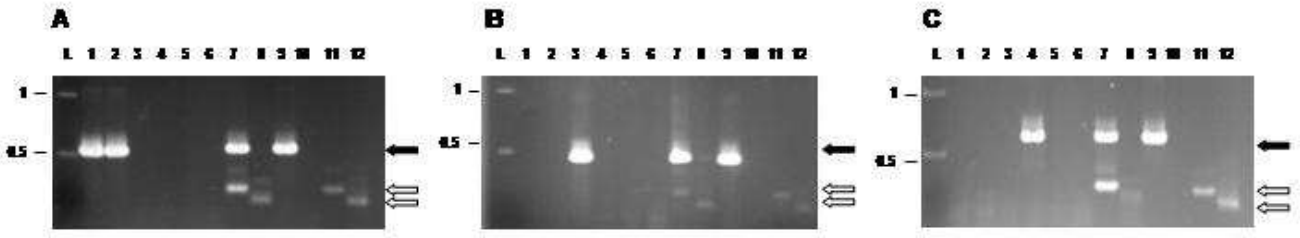

Figure 2: Detection of episomal BSOLV, BSGFV and BSMysV by M-IC-PCR.
Multiplex PCR reactions were carried out on immunocaptured viral particles using STMS primer pair AGMI 025 / AGMI 026 and either BSOLV species-specific primer pair RD-R1 / RD-F1 (A), BSGFV species-specific primer pair GF-R1 / GF-F1 (B) or BSMysV species-specific primer pair Mys-R1 / Mys-F1 (C). Prior to PCR, viral particles were immunocaptured for 3 hours at room temperature from 25 µl of leaf samples prepared from infected accessions Figue pomme (AAB, lane 1), Grande naine (AAA, lane 2), Guindy (AAB, lane 3), Langka (AAAB, lane 4), and uninfected PKW (BB, lane 5) and IDN-110 (AA, lane 6). Control PCR experiments were carried out on 50 ng of genomic DNA purified from cvs PKW (lanes 7, 9 and 11) or IDN-110 (lanes 8, 10 and 12) using BSV species-specific primer pairs and STMS primer pair (lanes 7 and 8), BSV species-specific primer pairs only (lanes 9 and 10) or STMS primer pair only (lanes 11 and 12). The filled arrow indicates BSV product, and the open arrows indicate STMS products.
L: 1kb DNA ladder (Sigma) with indicated marker sizes in kb.

Detection of BVX by DB-RT-PCR

Following the characterization of BVX in Guadeloupe (Teycheney et al., 2005a) sensitive and specific tools were needed for the detection of this virus. Therefore, a direct binding RT-nested PCR assay was designed. Figure 3 shows that the developed DB-IC-PCR allows the detection of BVX as sensitively as RT-PCr performed on total RNA extracted from infected plants .

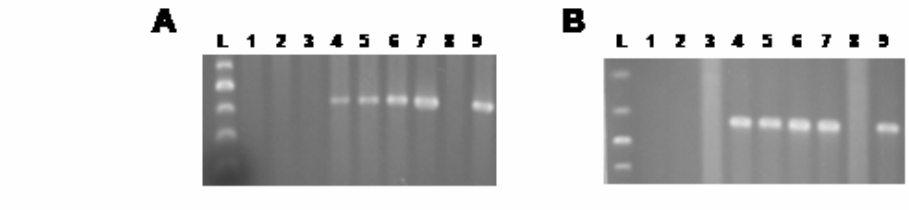

Figure 3 : Detection of BVX by DB-RT-PCR.
RT-nested PCR reactions were carried out using primer BVX1/BVX3 on directly bound BVX particles (A) or on total RNA extracted from the same samples (B). For DB-RT-PCR, viral particles were directly bound to thin-walled tubes or microplates overnight at 4°C from 50 µl of leaf samples prepared from uninfected accessions (lanes 1 to 3) and infected ones (lanes 4 to 7). Control PCR experiments were carried out on water (lane 8) and on cloned BVX sequence (lane 9) respectively.
L: 100 bp ladder (Promega).

Prevalence and diversity of BSV and BVX in Guadeloupe.

The prevalence of the main BSV species and of BVX in Guadeloupe was assessed by M-IC-PCR and DB-RT-PCR, respectively. Analysis of 432 leaf samples randomly collected in a wide range of locations confirmed that BSV is present at a low rate in Guadeloupe and that BSGFV is the predominant species on the island (see table 2): of the 39 positive samples, 38 were infected by BSGFV and only one by BSOLV, 33 were plantain (AAB genotypes) and 6 were dessert banana (Cavendish, an AAA genotype). Additional samples are currently being analysed in order to refine these preliminary results.

Table 2: Analysis of the prevalence of BSV in plantain and dessert banana in Guadeloupe

	Plantain	Dessert	Total
BSGFV	33	5	38
BSOLV	0	1	1
BSMysV	0	0	0
BSImV	0	0	0
Total	**33**	**6**	**39**

The presence of BVX was monitored in the same samples using DB-RT-PCR. This work showed that BVX is present at an even lower rate on the island, since only 4 of the analysed leaf samples were infected. No symptom could be associated with the presence of the virus, confirming previous observations (Teycheney *et al*., 2005a). Sequencing of the PCR product amplified following DB-RT-PCR is currently in progress, in order to get insights into the molecular variability of BVX in Guadeloupe. Previous data have showed that BVX displays a very low level of molecular diversity in the RdRp sequence that was analysed, contrary to the situation encountered for another member of the *Flexivirus* family infecting banana, *Banana mild mosaic virus* (Teycheney et al., 2005b).

CONCLUSIONS

Specific and sensitive methods were successfully developed for the detection of episomal BSV and BVX. They are being implemented for routine indexing of plant material prior to mass propagation, in order to avoid large scale distribution of infected plant material.

These detection methods are being used for a large scale study of the prevalence of both viruses in Guadeloupe. Preliminary results of this work indicate that both BSV and BVX are present on the island, at a very low rate and with no reported impact on banana and plantain production. Genotypes containing the *M. balbisiana* genome, such as most plantains, can become infected following either transmission of the virus by one its natural mealybug vectors or stress-mediated activation of BSV EPRVs. The low impact of BSV on plantain in Guadeloupe probably results from the fact that plantain is only vegetatively propagated there, and never mass propagated *in vitro* because of the risks of activation of BSV EPRVs by *in vitro* culture (Dallot *et al*., 2001; Folliot *et al*. 2006). Conversely, most dessert banana plantations in Guadeloupe use certified virus-fee vitroplants in order to avoid propagating (viral) diseases. These efforts certainly account for the low impact of viral diseases on banana and plantain in Guadeloupe.

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