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Evidence that the proliferation stage of micropropagation procedure is determinant in the expression of *Banana streak virus* integrated into the genome of the FHIA 21 hybrid (*Musa* AAAB)

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Summary. *Banana streak virus* (BSV) is causing increasing concern in almost every producing area of banana and plantain (*Musa* spp.) worldwide. This situation appeared partially linked to some breeding lines and micropropagated hybrids. A complete BSV sequence integrated into the genome of a triploid plantain has been recently characterised and it has been hypothesised that it could give rise to infectious virus via recombination. In this study, we evaluated the effect of a routine micropropagation procedure on the expression of BSV in the FHIA 21 tetraploid hybrid. The widespread presence of integrated sequences and the absence of episomal BSV in thirty FHIA 21 "mother plants" selected for micropropagation were first confirmed by specific PCR and IC-PCR tests. The proliferation stage of the procedure, characterised by an intensive production of neoformed buds, appeared determinant in BSV expression whereas the rooting and acclimatisation stages had little or no effect. The duration in culture and the way of subdividing the clumps of proliferation influenced greatly the percentage of episomal BSV infections, reaching 58% of infected micropropagated lines after six *in vitro* subcultures. These data suggest that the expression of episomal BSV observed during the *in vitro* procedure is correlated with the presence of an integrated form.

Introduction

First described in the Ivory Coast [19, 36], banana streak disease has been identified during the past few years in almost every producing area of banana

and plantain (*Musa* spp.) worldwide and is causing increasing concern. The causal agent, *Banana streak virus* (BSV), is a badnavirus characterised by a circular dsDNA genome of approximately 7.4 to 8 kb replicated by reverse transcription [20, 21].

The use of recent developed molecular BSV detection tools such as direct PCR seemed to indicate the widespread presence of badna-viral sequences in *Musa* plants, but disease symptoms and/or viral particles detected by ISEM and ELISA were not systematically associated [8, 17, 28]. These data suggested a possible integration of full or partial BSV sequences into the *Musa* genome, as previously observed with partial sequences of single-stranded DNA of a geminivirus [3, 4] and suggested for *Petunia vein clearing* caulimovirus [30, 31]. Partial "dead" badnavirus sequences integrated into the *Musa* genome were first discovered by LaFleur et al. [17]. More recently, the presence of BSV full sequences integrated into the nuclear DNA of the triploid plantain cultivar "Obino l'Ewaï" was shown using distinct methodologies [11, 26]. Even though this integrant appeared particularly complex with a scrambled region consisting of inverted and non-contiguous viral sequences, the authors suggested that this structure might give rise to infectious episomal virus via homologous recombination and labelled it "potentially activateable sequence".

It was hypothesised that *in vitro* micropropagation and hybridisation might be possible triggers for infection due to the appearance of episomal BSV in some tissue culture and breeding lines deriving from healthy parents [26]. However, the presence of episomal BSV in the parents was not systematically investigated by reliable and sensitive detection tests. Moreover, no data are currently available about the specific effect of the different stages of *in vitro* micropropagation procedure on such BSV expression.

In this study, we evaluated the effect of a routine *in vitro* micropropagation procedure on the suspected expression of BSV deriving from integrated sequences, using as starting plant material 30 episomal BSV free suckers of the tetraploid hybrid AAAB cv. FHIA 21. These plants had no tissue-culture ancestry in their background history. The appearance of episomal BSV infection during each stage of the procedure (proliferation, rooting, acclimatisation..) was monitored by a specific and sensitive immunocapture-PCR (IC-PCR) test.

Our results clearly demonstrate the determinant effect of the proliferation stage of *in vitro* micropropagation for early BSV expression in the FHIA 21 clumps of proliferation.

Materials and methods

Plant material

Thirty symptomless plants of the FHIA 21 tetraploid hybrid (AAAB) were selected at the FHIA institute in Honduras as mother plants. They were never micro-propagated by tissue culture and directly derived through conventional vegetative propagation for five years. The presence of the previously characterised BSV integrated sequence and the absence of episomal virus were confirmed by PCR and IC-PCR tests.

In vitro *micropropagation procedure*

Two suckers were selected from each mother plant and the shoot tips (60 explants) were micropropagated according to a routine procedure of banana *in vitro* culture as described by Vuylsteke [34] and adapted by Acuña [1]. This procedure followed three stages: (1) the shoot tips were trimmed and transferred to the culture medium for the *initiation stage*, (2) the formation of multiple shoots and buds (clumps of proliferation) was obtained during the *proliferation stage* by subdividing the newly formed shoots on a medium with cytokinin, this stage is constituted by 6 cycles of multiplication, (3) shoot development and rooting of individual plants (plantlets) were obtained during the *rooting stage*. Finally, the plantlets were transferred to nursery for the acclimatisation stage.

One and 2 mg/l of BA (6-benzylaminopurine) were used in the MS medium [24], for the initiation (one month) and proliferation (three to six cycles of three weeks each) stages respectively. During the rooting stage, the plantlets were grown in a MS medium without BA. During the stages of initiation and rooting, the photoperiod was fixed at 12 h of light (15500 Lux) and 12 h of darkness. The plant material in proliferation was maintained in darkness. In both cases, the temperature was fixed at $27+/-1\,^{\circ}C$.

Experimentation protocol

Two distinct experiments were performed as described in "*in vitro* micropropagation procedures" with 30 explants (micropropagated lines) in each experiment. In the first experiment, the multiplication was obtained by subdividing longitudinally all of the growing shoots or bud clusters as classically performed in a routine procedure. The second experiment was performed as the first one, except that the clumps of proliferation were submitted to a less intensive subdividing procedure at each proliferation cycle, resulting in the production of 30% less shoots.

In both experiments, the explants were tested for the presence of episomal BSV at the *proliferation*, *rooting* and *acclimatisation* stages of the micropropagation procedure.

During the stage of proliferation, the detection tests were performed after three and six cycles. For each micropropagated line, samplings were constituted by a pool of the upper part of all the shoots cut from all the clumps of proliferation. Only negative lines were submitted to six cycles.

After the first three cycles of proliferation, three to five shoots of each micropropagated line were isolated, transferred for four weeks to the routine medium and then acclimated in nursery. The same procedure were performed after the next three cycles of proliferation.

A total of 266 plantlets and 225 plants were individually tested, during the rooting and acclimatisation stages respectively.

PCR and IC-PCR tests

The presence of the previously characterised potentially "activateable BSV sequence" [26] was checked up by PCR on total *Musa* DNA extractions (DNAeasy, QIAGEN), using the mixed primers MusaT3–2 5′GGCTTATGATGCTGACCACAT3′ and BSV510.2 5′GCCATAAAAACGAACCTTGCT3′ located on the *Musa* and BSV genomes respectively, as described by Ndowora [25].

An immunocapture PCR (IC-PCR) procedure was developed in order to specifically detect encapsidated BSV DNA (episomal BSV) which could derive from the integrated sequences. The viral particles were specifically trapped in 200 μl PCR microtubes using anti BSV antibodies PMx R2-2C [27], at a concentration of 5 μg/ml in sterile carbonate buffer.

After a 4 h incubation, immunocapture was performed with 50 µl of plant extract (1/5 in PBS-T-PVP 2%-DIECA 0.45%) incubated overnight at 4 °C.

Using the BSV-Onne sequence (accession number AJ002234) very similar to the integrated sequence previously described in the Obino l'Ewaï plantain, we designed specific primers BSVcl1 5′ATGGCCTTAATAGTCTTTCGTGAT3′ and BSVcl2 5′GGTGGCGCTGAGGATGTG3′ located in the 5′ terminal part of the ORF3 (capsid protein gene) of BSV genome [9, 11, 28]. The PCR reaction mix contained: 1.7% of Triton X100, 1 × Taq Promega buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X100), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 1 U Taq DNA polymerase (Promega). The following PCR amplification scheme was used: denaturation stage at 94 °C during 5 min followed by 35 cycles (denaturation for 30 sec at 94 °C, primer annealing for 30 sec at 55 °C and primer extension for 1 min at 72 °C) and a final extension at 72 °C for 10 min.

In some cases, IC-PCR was performed using degenerated badnavirus primers Badna2 5′TAYATTGATGAYATWYTDGT3′ and Badna3 5′CATCBSTYTCWATKATVATG3′, deriving from those previously described by Thomson et al. [33].

Southern hybridisation

The specificity of the amplified fragments was confirmed by Southern hybridisation. Following electrophoresis through a 1.5% agarose gel, the products were transferred onto a nylon membrane (Zeta-probe membrane, Biorad). A biotinylated probe was synthesised by random priming of a PCR product of a BSV-Onne *Pst1* clone, using the BioPrime Labeling system (Life Technologies). Hybridisation was performed according the protocol described by the membrane supplier. A coloured revelation of the hybridised fragments was performed using a BCIP/NBT substrate solution.

Results

Widespread presence of integrated BSV sequences in healthy FHIA 21 hybrids

The presence of integrated BSV sequences and the absence of episomal virus were determined in each of the 30 FHIA 21 "mother" plants used as starting material for *in vitro* micropropagation by PCR, ELISA, IC-PCR and ISEM (immunosorbent electron microscopy) techniques.

For each plant, a specific product with the expected size (1500 bp) was amplified with the mixed MusaT3-2 and BSV510.2 primers, whereas no amplification was visible with total DNA extracted from healthy Grande naine and Williams cultivars (AAA). This result demonstrated the widespread presence of BSV integrated sequences in this tetraploid hybrid (Fig. 1).

In order to detect episomal BSV only, an IC-PCR test was developed and first optimised with known field plants. The procedure combining a serological and molecular detection allowed a reliable discrimination of the samples rather than those obtained through the strict observation of the presence or absence of symptoms and/or viral particles detected by ELISA and ISEM. Applied to the FHIA 21 mother plants, IC-PCR tests using both degenerated and specific primers clearly demonstrated the absence of episomal BSV in each of these 30

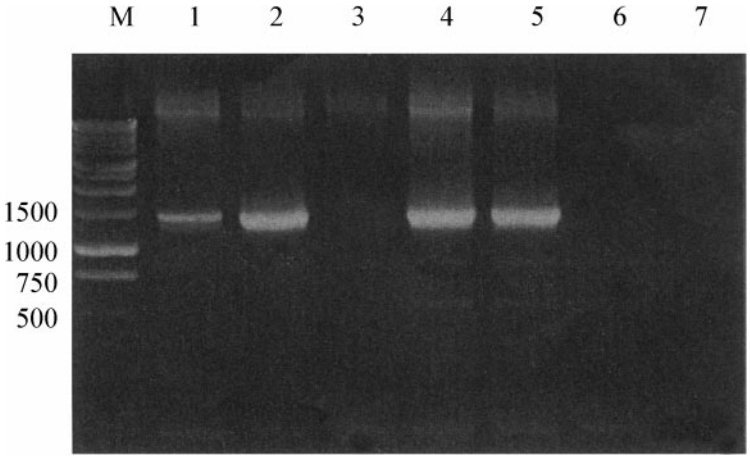

Fig. 1. PCR detection of the "potentially activateable" BSV integrated sequence in the FHIA 21 hybrid using mixed primers MusaT3/BSV510.2. *M* Molecular markers of 1 kb (Promega). The 1500 bp fragment is indicated. Specific amplification of a 1500 bp fragment with MusaT3-2 and BSV510.2 primers from total DNA extractions of the FHIA 21 "mother plants": *1, 4* FHIA 21 plant 1 with 100 and 200 ng of total DNA; *2, 5* FHIA 21 plant 2 with 100 and 200 ng of total DNA; *3, 6* "Grande Naine" cultivar; *7* buffer control

plants. These results were confirmed by TAS-ELISA and ISEM, following the protocols previously described [2, 27] (data not shown).

Early BSV expression during the proliferation stage

BSV expression was followed in 26 micropropagated lines in both experiments instead of 30, due to initial medium contamination and loss of micropropagated material.

Applied to the shoots produced by the clumps of proliferation, IC-PCR using specific BSVcl1 and BSVcl2 primers allowed a clear discrimination of the samples and thus, a reliable detection of episomal BSV as it was previously observed with field samples. Strong and specific amplified fragments of the expected size (494 bp) were obtained, even when applied to an amount of only 100 mg or less of proliferated plant material (Fig. 2).

BSV virions were detected very early in the clumps of proliferation after only three cycles of multiplication. Thus, 23% and 4% of the micropropagated lines were found BSV infected in the first and second experiments respectively. Finally, the frequency of BSV infected micropropagated lines increased with the number of cycles and consequently with the time of proliferation, reaching 58% (first experiment) and 15% (second experiment) after six cycles of multiplication (Table 1).

IC-PCR tests performed on each of the 73 plantlets regenerated from the proliferation clumps found infected after three and six multiplication cycles confirmed the presence of episomal BSV. However, a strong heterogeneity of infection was

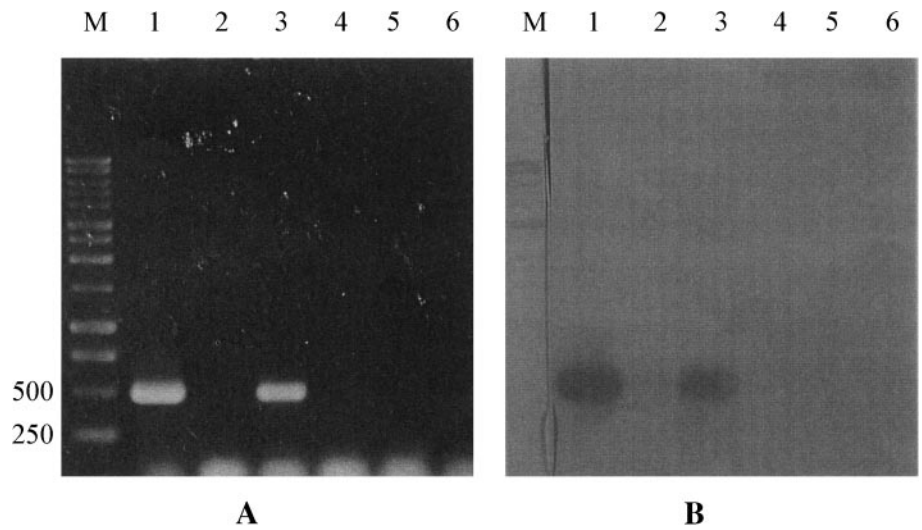

Fig. 2. Specific detection of episomal BSV by discriminant IC-PCR procedure. **A** Analysis in ethidium bromide stained agarose gel of amplified fragments obtained by IC-PCR using BSVcl1 and BSV cl2 primers: *1* Field sample of BSV infected FHIA 21 hybrid; *2* field sample of healthy FHIA 21; *3* BSV infected micro-propagated FHIA 21; *4* healthy micropropagated FHIA 21; *5* healthy "Grande Naine" banana; *6* buffer control. **B** Southern blot analysis of the previously IC-PCR amplified fragments. A biotinylated probe produced by PCR amplification of a BSV *Pst1* cloned fragment was used. A BCIP/NBT coloured system was adopted to detect the hybridised fragments. The markers of molecular size were stained with bromophenol blue

Table 1. Evolution of episomal BSV infections detected during the proliferation stage

Experiments	Number of proliferation cycles	
	3	6
1	6/26[1] (23%)	15/26 (58%)
2	1/26 (4%)	4/26 (15%)

[1]The number of infected micropropagated lines/number of micropropagated lines as well as the corresponding approximate percentage are indicated. Episomal BSV was detected by specific IC-PCR tests using BSVcl1 and BSVCl2 primers, performed directly on the clumps of proliferation after 3 and 6 cycles of proliferation

found between the plantlets issued from a same clump of proliferation (Table 2). In several cases, only one plantlet of the four or five regenerated appeared infected, suggesting a strict localisation of episomal BSV inside the clumps of proliferation. Further analysis confirmed the absence of BSV virions in these still "healthy" plantlets.

Table 2. Heterogeneity of infection between the plantlets issued from the same clump of proliferation

Experiments	Number of proliferation cycles			
	3		6	
	Infected clumps of proliferation[a]	Infected plantlets[b]	Infected clumps of proliferation	Infected plantlets
1	6	14/23	9	9/40
2	1	1/4	3	4/9

[a]The number of clumps of proliferation found infected by episomal BSV (IC-PCR test) is indicated after 3 and 6 cycles for both experiments

[b]The number of infected plantlets/number of plantlets tested by IC-PCR is reported. A minimum of three plantlets per clump of proliferation was analysed

Limited effect of the rooting and acclimatisation stages on BSV expression

Young shoots isolated from the clumps of proliferation still uninfected after 3 and 6 multiplication cycles in both experiments were submitted to a rooting and growing stage and individually tested by IC-PCR. Only one plantlet of the 190 tested in both experiments was found infected by episomal BSV (Table 3), indicating that the rooting of the plantlets has no significant effect on BSV expression in contrast to the proliferation stage.

225 plantlets remaining uninfected after the rooting stage were then transferred to nursery and again tested by IC-PCR. New episomal BSV infections were detected in a limited number of young plants, in two and three plants in the first and second experiment respectively. Each of these plants were issued from different micropropagated lines. These results reinforce the idea that BSV expression can occur in a very randomly way.

Table 3. Limited effect of the rooting stage on BSV expression

Experiments	Number of proliferation cycles	
	3	6
1	0/29[a]	1/51
2	0/34	0/76

[a]The total number of plantlets tested by IC-PCR is indicated. Approximately three plantlets for each micropropagated line still uninfected after three and six cycles of proliferation have been tested

At the end of all of the stages of the *in vitro* procedure, 65% (first experiment) and 35% (2nd experiment) of the micropropagated lines have produced at least one plant infected by episomal BSV.

Discussion

During the past few months, the detection in the field of *de novo* BSV infections in numerous micropropagated triploid and tetraploid *Musa* hybrids has led to the hypothesis of a possible effect of the *in vitro* multiplication procedure on BSV expression [6, 16]. Our study demonstrates for the first time the effect of a common shoot tip micropropagation procedure on the expression of *Banana streak virus* in an healthy tetraploid hybrid AAAB carrying integrated BSV sequences. Moreover, we identified the crucial stages of the procedure leading to the "expression" of the episomal virus and we quantified the frequency of such event.

We first confirmed, by a sensitive and adapted IC-PCR test, the absence of episomal BSV in the mother plant material used for *in vitro* micropropagation. Due to the presence of integrated BSV sequences in the *Musa* genome, PCR procedures failed to specifically detect the episomal virus. Very recently, Harper et al. [9] proposed an IC-PCR procedure to detect this form of the virus, by specifically trapping BSV particles with antibodies prepared from a pool of BSV isolates [27]. A similar procedure was developed in this paper, with our own primers located in the CP coding region of the BSV genome. Applied to the starting plant material put in culture, this detection test using both specific and badnavirus polyvalent primers confirmed the absence of episomal BSV. The presence of the previously characterised integrated sequence was also verified by PCR using mixed BSV/*Musa* primers. These primary analysis constitute a strong guarantee for the results obtained during this experimentation.

The proliferation stage was found clearly determinant in the appearance of episomal BSV and the difference in the percentage of BSV infected micropropagated lines between both experiments suggests that the intensity of subdividing applied to the proliferation clumps may modulate the frequency of BSV expression from integrated sequences. The increase of infected micropropagated lines with the duration of culture as well as the high heterogeneity of infection between the plantlets deriving from the same micropropagated line suggest that episomal BSV expression occurs at random and in a non-simultaneous way in the different cells constituting the proliferation clumps. These different aspects of BSV expression during the proliferation stage are coherent with the known effect of tissue culture on plant genomes. It is now well accepted that genetic modifications are at the origin of most of the phenomenon of somaclonal variation [14, 18, 32, 35] and are sometimes related to retrotransposon activation as a response to "stressful" conditions [7, 12, 13]. Thus, it has been previously demonstrated that the duration of culture induced an increase in the number of retrotransposon copies in tobacco and rice [12, 13]. Similarly, the number of proliferation cycles as well as the way of budding and proliferation of *Musaceae* modulate the frequency of somaclonal variation events in banana and plantain cultivars [5, 32].

These data as well as the confirmation of the absence of episomal BSV in the "mother plants" before tissue culture strongly argue for an integrated origin of the episomal form detected during the stage of proliferation. Other lines of evidence such as the genomic homogeneity between the described integrated sequence and deriving episomal BSV genomes in contrast to the existing variability among "common" field BSV isolates [23] support this hypothesis for other *acuminata/balbisiana* hybrids (Lockhart, unpublished results). Moreover, the appearance of episomal BSV associated with some genetic crossings during the selection of new diploid and triploid *acuminata/balbisiana* hybrids (Lheureux et al., personal communication) reinforce a genetic basis of such episomal BSV expression.

During the last few months, reported cases of integration of pararetrovirus sequences in plants have increased greatly. A multiple integration into the genome of *Nicotiana tabaccum* of partial sequences of an uncharacterised pararetrovirus has been reported at the end of last year [15]. There is no evidence for reconstitution of an infectious virus from these sequences, but another very recent study [22] has shown that an episomal form of *Tobacco vein clearing virus* may arise from its integrated sequences into the genome of *Nicotiana edwardsonii*.

At this stage of the study, the exact molecular phenomenon implicated in BSV expression in the clumps of proliferation of FHIA 21 has not been identified. However, it can be hypothesised that the increased chromosome breakage, single base changes or alterations in DNA methylation associated with the proliferation stage of *in vitro* culture [29] may have led to the activation of an integrated sequence. At that time, it is not clear if the complex BSV integrant previously described in the triploid plantain Obino l'Ewaï [11, 26] is at the origin of the episomal BSV infection detected in the FHIA 21 studied material. Such a molecular characterisation of the BSV integrant(s) has not been performed for this or other tetraploid hybrids. Moreover, the high percentage of infected micropropagated lines obtained at the end of the tissue culture procedure is not completely consistent with the hypothesised relatively rare molecular mechanism of homologous recombination necessary to the production of infectious episomal BSV. The previous studies showed that the nuclear genome of Obino l'Ewaï contained multiple BSV integrants [11] and the presence of another complete BSV sequence with a most simple organisation in the FHIA 21 and other hybrid genomes cannot be totally excluded. Other facts seem to distinguish the FHIA 21 hybrid from Obino l'Ewaï. Among them, the FHIA 21 hybrid appears more disposed to produce episomal BSV when submitted to other stress conditions such as culture in pot under greenhouse conditions (our own observations and Lepoivre, personal communication).

Field observations and analysis will be performed in further experiments to evaluate the stability of the "healthy" status of the remaining FHIA 21 plants after the regeneration stage. Moreover, this first study produced interesting characterised plant material, micropropagated or kept under screenhouse conditions, available to further investigate more precisely the molecular phenomena at the basis of BSV expression during tissue culture.

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