Development of Simple Molecular Protocols to Detect *Banana bunchy top virus* with the PhytoPaSS System

J.P. Busogoro[1], O. Dutrecq[2] and H. Jijakli[1]
[1] Plant Pathology Unit, Gembloux Agricultural University, 2 Passage des Déportés, 5030, Gembloux, Belgium
[2] DNAlis sprl: Spin-Off of the Gembloux Agricultural University, 20 Rue de la Croix Rouge, 5032 Bossière (Gembloux), Belgium

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Abstract

A new protocol was developed for the molecular detection of *Banana bunchy top virus* (BBTV) in banana. Fragments of banana tissue from BBTV-infected plants were collected by scraping leaves with a PhytoPASS abrasive membrane. The samples collected were stored at room temperature until tested by PCR. To detect BBTV, the PhytoPASS membrane covered by plant-tissue fragments was introduced into a 15-ml tube containing 1 ml of 4°C KAJI extraction buffer and swirled rapidly. The resultant crude suspension was diluted 100 times in distilled water prior to PCR amplification with previously described BBTV-specific primers. A specific DNA fragment was amplified suggesting that BBTV infection could be detected without purified DNA or the immunocapture step preceding the PCR reaction. Detection of BBTV in stored samples also suggests that the newly developed protocol could be used to analyse materials collected in different production areas. Because it involves the direct use of crude extracts, this simplified protocol allows quick virus detection with minimal risk of manipulation errors, misidentification, and cross-contamination when many samples are tested. Adopting this protocol would make the routine use of PCR for BBTV detection possible in all banana-growing regions.

INTRODUCTION

Banana bunchy top disease (BBTD), caused by *Banana bunchy top virus* (BBTV), is considered to be the most devastating virus disease of banana (Wanitchakorn et al., 1999). BBTV can cause extensive losses as infected plants do not usually produce fruit, and affected plantations quickly become unproductive. BBTV is widely distributed in the eastern hemisphere, but is not present in Latin America.

BBTV belongs to the *Babuvirus* genus in the family of the *Nanoviridae* (Bananej et al., 2007). Its genome consists of six circular single-stranded DNA components (Wanitchakorn et al., 1997). BBTV infection occurs via persistent transmission by the black banana aphid, *Pentalonia nigronervosa* Coq. (Beetham et al., 1999; Furuya et al., 2005). Vegetative transmission also occurs through the clonal multiplication of banana initiated from infected mother plants (Sharman et al., 2000; Su et al., 2003). The virus infects the phloem and induces leaf chlorosis, vein clearing, dwarfing and leaf atrophy (Karan et al., 1994).

As there is no effective resistance to BBTV in banana (Wanitchakorn et al., 2000), control of BBTD must be based mainly on the use of virus-free planting materials in combination with roguing of infected plants and strict quarantine barriers for areas where the virus is still absent. It is essential to detect infected plants, including those with latent infections. Sensitive BBTV detection methods are thus essential for successful disease management.

Different BBTV detection methods have been developed, including enzyme-linked immuno-sorbent assay (ELISA), immuno-sorbent electron microscopy (I-SEM) and polymerase chain reaction (PCR) (Su et al., 2003; Furuya et al., 2005). The ELISA technology is commonly used, but the efficiency of this serological method is limited by its low sensitivity. Different research teams have optimised the PCR method as it is more sensitive than the ELISA method (Furuya et al., 2005).

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Despite its high sensitivity, PCR is not used commonly on a large scale. A contributing factor may be the need for purified nucleic acids as template material (Furuya et al., 2005; Su et al., 2003; Wanitchakorn et al., 2000). Nucleic acids purification from banana tissues is time consuming and can lead to manipulation errors when high numbers of samples are analysed.

In this paper, we investigate a simplified protocol for the molecular detection of BBTV that does not use purified DNA prior to PCR amplification.

MATERIALS AND METHODS

Plant Materials and Preparation of Banana Samples for BBTV Detection

BBTV-infected banana plants from the Democratic Republic of Congo and from Burundi were grown in the greenhouse under a constant temperature of 24°C and a photoperiod of 16 hours per day. Leaves of each banana plant were scrubbed with the PhytoPASS (DNAlis sprl) abrasive membrane to detach plant tissues according to the PhytoPASS manufacturer's protocol (Fig. 1). The membrane covered with fragments of banana tissues was then introduced into a 15-ml tube containing 1 ml of 4°C KAJI extraction buffer (DNAlis sprl) and the preparation was vortexed at high speed (1500-2000 rpm for 30 sec) to yield the primary crude extract of banana. A green suspension of plant extracts was thus generated as shown on Figure 1.

Conservation of Plant Tissues on the PhytoPASS Surface

Samples harvested from a banana plant from the Democratic Republic of Congo were conserved on the PhytoPASS abrasive membrane to evaluate the feasibility of conserving the samples before molecular analysis. The samples were kept at room temperature on the PhytoPASS inside paper envelopes for 32, 40, 72 and 138 days. After this storage period, the samples were recovered and diluted in the above-mentioned KAJI extraction buffer prior to PCR.

Molecular Detection of BBTV

The primary extract was diluted 100 times in sterile distilled water and conserved by freezing at -20°C for future use as template for PCR amplification. PCR detection of BBTV was undertaken with the primer pairs (BBT1 & BBT2) (Thomson and Dietzgen, 1995). A PTC 200 thermocycler (Biozym, Landgraaf, the Netherlands) was used for the PCR amplification, in a final volume of 50 µl containing 5 µl of the 10x concentrated PCR reaction buffer (Roche), 200 µM of each dNTP, 0.5 µM of each primer and 5 µl of the 100x diluted plant crude extract suspension. Amplification conditions included a first denaturation step at 94°C for 5 min followed by 35 cycles of 30 sec at 94°C, 1 min at 52°C and 2 min at 72°C. These repetitive cycles were followed by a final elongation step performed at 72°C for 10 min. PCR products were separated by electrophoresis under a constant electric current of 120 mA in a 1% agarose gel containing ethidium bromide (1 µg/10 ml). The amplified products were visualised under UV light.

RESULTS AND DISCUSSION

Recovery of Crude Extracts and Efficiency of PCR Detection of BBTV

The preparation of the plant extract suspension was rapid and simple. The complete process from sampling to the preparation of crude extracts required less than 5 min.

Figure 2 shows the amplification patterns obtained for the two banana plants submitted to this analysis. In both cases, an amplified product was obtained, with the expected size of 349 bp (Thomson and Dietzgen, 1995) confirming that the plants are infected with BBTV.

The present assay showed for the first time that it is possible to detect BBTV infections by PCR with crude extracts prepared from samples collected with the

PhytoPASS system. The protocol simplifies and speeds up the preparatory steps needed before actual PCR reactions can be performed.

Classical protocols require nucleic acid purification before PCR amplification for BBTV detection. The purification of nucleic acids in turn requires homogenisation of leaf tissues in an extraction buffer and incubation of the preparation at high temperature for at least 1h (Su et al., 2003). Optimised protocols for nucleic acids purification still require a preliminary grinding step of banana leaves in liquid nitrogen before different incubation and centrifugation steps in extraction buffers (Furuya et al., 2005). These different steps are time and labour consuming and increase the risk of manipulation errors as well as cross-contamination between different samples. An alternative to nucleic acid purification was optimised to detect different banana viruses, including BBTV (Sharman et al., 2000). The proposed technology involves integration of an immuno-capture (IC) step preceding the PCR reaction to achieve immobilisation of virions on the PCR reaction tube wall. Although virus immobilisation overcomes limitations caused by PCR inhibitors in banana extracts, the procedure requires specific antibodies and remains time consuming.

The protocol described here significantly simplifies detection of BBTV in infected banana samples by using crude extracts for PCR amplification.

Virus Detection in Conserved Samples

The amplification profiles obtained for banana samples from the Democratic Republic of Congo harvested with the PhytoPASS system and stored at room temperature for different storage periods are illustrated in Figure 3. BBTV infections remained detectable for at least 4 months (the longest storage period tested) before PCR analysis.

CONCLUSION

This simplified detection protocol could be useful in the management of this important disease. It was shown that BBTV infections are easily detectable with the PCR technology using crude extracts as target materials. The developed protocol overcomes the need to purify nucleic acids or to integrate an immuno-capture step prior to PCR detection. Moreover, it allows the conservation of samples under common conditions, enabling easy transport of samples and thus bypassing the need to establish molecular laboratory capacities in a given producing region. These results could contribute to the development of a global banana health management programme.

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Figures

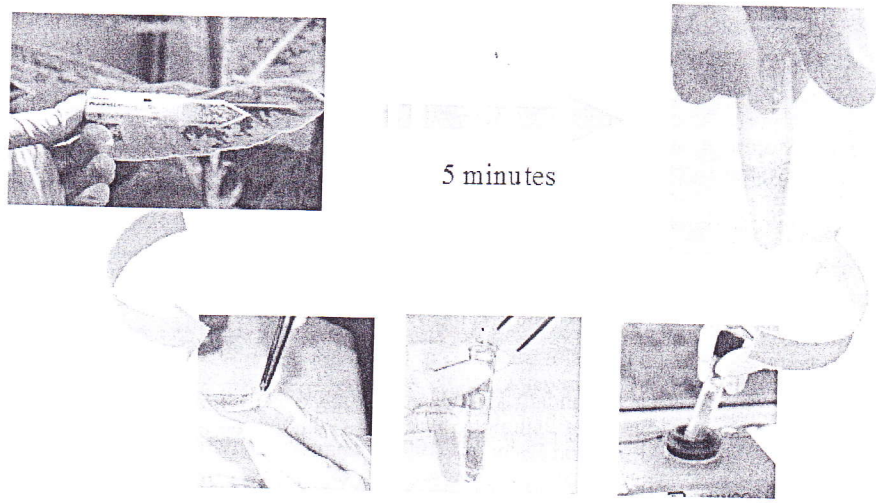

Fig. 1. Illustration of the banana sampling process using the PhytoPASS kit for PCR amplification using crude extracts to detect BBTV infections.

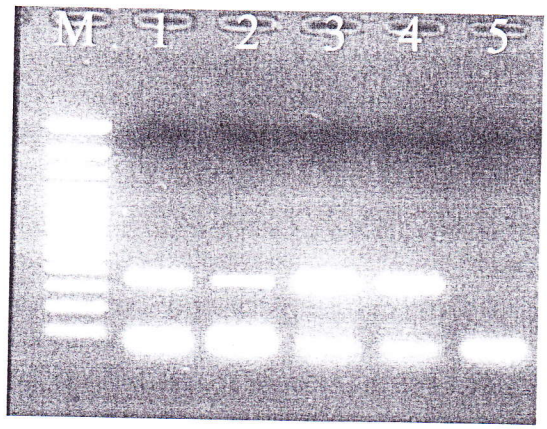

Fig. 2. Molecular detection profile of BBTV in banana tissue suspensions prepared according to the PhytoPASS protocol. Lane M: 100-bp ladder (Fermentas); lanes 1-2: samples collected from the plant from Burundi; lanes 3-4: samples collected from the plant from the Democratic Republic of Congo; lane 5: distilled water, negative control.

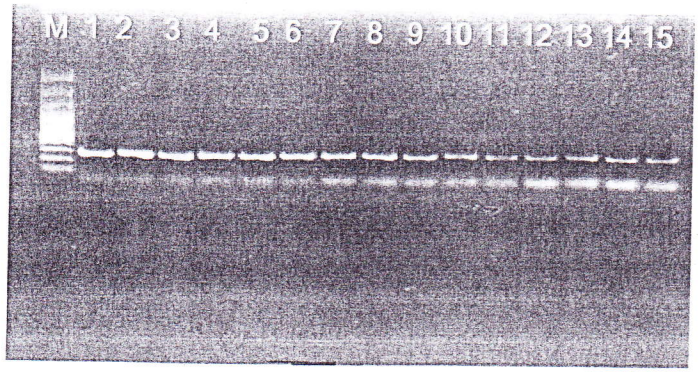

Fig. 3. Detection of BBTV in banana samples from an infected plant that were processed with the PhytoPASS system and conserved at room temperature. Lane M: 100 bp ladder (Fermentas); lanes 1-3: samples freshly collected; lanes 4-6: samples stored for 32 days; lanes 7-9: samples stored for 40 days; lanes 10-12: samples stored for 72 days; lanes 13-15: samples stored for 138 days.