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PCR optimization for the detection of bunchy top virus of abaca (*Musa textilis* Nee) in Eastern Visayas Philippines

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ABSTRACT

The bunchy top virus in Eastern Visayas has serverly reduced abaca production. Early and accurate detection of plant viral pathogens is an essential and crucial component for disease management. At present, there are no standard PCR conditions in the Eastern Visayas region for detecting the bunchy top virus at an early stage using PCR. Thus, optimization for the detection was carried out to assist in disease management. Different annealing temperatures (57, 60 and 65°C), gel concentrations (1, 1.5 and 2%), and running conditions (80, 90 and 100 volts) were tested using My Taq™ DNA Polymerase (Bioline, USA). The annealing temperatures of 57°C and 60°C resulted in DNA amplification as indicated by the presence of bands but absence of bands at 65°C. The higher voltages of 90 and 100 volts resulted in smears and distorted DNA bands with 1% and 1.5% agarose; thus 2% agarose gel was used to resolve small DNA fragments (100bp to 3kb). Electrophoresis using 80 volts for 45min successfully separated the DNA bands. The amplification of the product with internal control primers indicated the absence of PCR inhibitors in the abaca-extracted DNA samples. This confirmed the negative PCR reaction as indicative of the absence of the virus. The optimized PCR conditions could be applied by students and researchers for the early detection of bunchy top virus in the National Abaca Research Center Germplasm collection and the region.

Keywords: bunchy top virus, PCR, Manila hemp, detection, abaca hybrid

INTRODUCTION

Abaca or Manila hemp (*Musa textilis* Nee) is endemic to the Philippines and is grown as a fiber crop. The fiber is used for various fabrics and yarns and has many other uses, including banknotes, lens-cleaning tissue, tea bags, and capacitor

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papers (Sharman et al 2008). The Philippines supplies 90% of the world's total abaca requirement, while the remaining 10% is supplied by Ecuador. The local industry exports an average of 13,178 metric tons of abaca fiber per year (Boguero et al 2016). One of the significant challenges facing the abaca industry in the Philippines is the infection with abaca bunchy top caused by *Abaca bunchy top virus* (ABTV) (Furuya et al 2006). ABTV has a genome consisting of six different ssDNA components, referred to as DNA-R, -S, -C, -M, -N, and -U3 (King et al 2012). The high incidence of abaca bunchy top disease (ABTD) in the Philippines was primarily confined to the Bicol Region, the top producer of abaca, but it subsequently spread to other abaca-producing regions in the country (Bajet and Magnaye 2002, Nuñez 2013). In 2003, the infection reached alarming levels in Eastern Visayas, Philippines, the second largest abaca-producing region, when two of its provinces (Leyte and Samar) were put in a state of calamity. The disease reportedly wiped out 16,737 hectares of a total 26,374 hectares of abaca plantations (Nuñez 2013).

Diagnosis based on the symptoms of virus diseases, particularly abaca bunchy top, is not always reliable and generally presumptive or not enough; thus, early detection at the molecular level is crucial for disease control. Abaca virus diseases usually occur as mixed infections of bunchy top and mosaic, and it is difficult to distinguish the symptoms induced by each. Serological and molecular-based detection of the virus is necessary since some plants are infected and yet asymptomatic (Sta Cruz et al 2016, Piamonte and Sta Cruz 2018). Early, rapid, and specific detection and identification of plant viruses are essential for effective plant disease management. However, without reliable disease diagnosis, control measures cannot be used appropriately (Sankarana et al 2010). Molecular-based techniques can overcome many of the shortcomings of conventional methods and PCR-based assays are generally more specific and faster than conventional techniques (Schena et al 2004).

Molecular detection by PCR has been used to detect *Banana bunchy top virus* (BBTV) in banana (Thomson and Dietzgen 1995). The virus is also detectable in abaca, (Furuya et al 2006, Piamonte and Sta Cruz 2018). However, the conditions still need to be optimized to improve sensitivity, reliability, and detection consistency. Generally, PCR is an error-prone assay producing inconsistent and false negative results especially when assay conditions are not optimized. Piamonte and Sta Cruz (2018) reported that plants had shown the bunchy top symptoms but the presence of BTV was not detected. However, the advantage of the PCR technique is that it can also be applied to non-culturable microorganisms, as the organism does not need to be isolated to be identified by PCR (Rubio and Ferriol 2020).

MATERIALS AND METHODS

Test Plant Preparations and Collection of Aphid Vector for BTVs Isolates

Three varieties were used in the study; var. Inosa (commercial check), abaca hybrid BC2-7 (BANDALA) as resistant check, and Pacol (parental). Thirty three-month-old disease-free plantlets of each variety were obtained from the tissue culture laboratory of the National Abaca Research Center (NARC), Visayas State University (VSU), Leyte, Philippines.

Diseased specimens, including the adult aphid vector *Pentalonia nigronervosa* Coquerel, were collected from major abaca growing areas in Eastern Visayas where records of BTV infection exist. These areas were VSU in Leyte (Leyte isolate), Sogod in Southern Leyte (Southern Leyte isolate), Catarman in Northern Samar (Samar Isolate), and Naval in Biliran Island (Biliran isolate).

Insect Transmission Assay

The collected adult *P. nigronervosa* were starved for approximately 2h. Groups of 20 were transferred to healthy test plants at the 3-5 leaf stage for an inoculation access period (IAP) of 4 days. The plantlets were immediately covered by 8ftx4ft nylon mesh. At the end of IAP, aphids were removed from the plants and then sprayed with broad-spectrum insecticide using the manufacturer's recommendations (Cypermethrin, 5 EC) to kill the viruliferous aphids. Leaf samples of test plants per genotype were collected every day for up to 14 days specifically the second and third leaf in the shoot (not too young or old) for the early detection of BTV. Plants were visually inspected daily for symptom development for 54 days.

Total Genomic DNA Extraction from Collected Samples

The total DNA of the abaca was isolated using the Dellaporta extraction method (Dellaporta et al 1983, Piamonte and Sta Cruz 2018). For extraction 2 fresh leaf punches (10.8mm diameter) per sample were collected in 2mL microtubes. These were ground in a mortar and pestle using 500µL Dellaporta extraction buffer (100mM Tris pH8.0, 50mM ethylenediaminetetraacetic acid (EDTA), pH8.0, 500mM Sodium chloride (NaCl), 10mM B-mercaptoethanol). The extracts were then placed in 2mL microcentrifuge tubes. Next 30µL of 20% SDS was added and incubated in a water bath for 10min at 65°C. After incubation, 160µL of 5M Potassium Acetate (KOAc) were added and vortexed for 2min and spun at 14,000rpm for 10min in a 2mL microcentrifuge (Heraeus PICO 21, Thermo Scientific). Afterwards, 450µL of supernatant was transferred into a new tube; then 225µL of isopropanol was added, vortexed in 2min, and spun at 14,000rpm for 10min. The supernatant was discarded, and the dried pellet was washed with 70% ETOH and spun at 14,000rpm for 5min. The extracted DNA was air-dried overnight at room temperature and the dried pellet was suspended in 50µL distilled H_2O.

Optimization of PCR Conditions for BTVs Detection

Optimization was done by comparing the annealing temperatures of 57°C (this study), 60°C (manufacturer's recommendation), and 65°C (reports/literature); gel concentrations of 1, 1.5 and 2%, and electrophoresis voltage conditions of 80, 90, and 100volts for 45min in 1X Tris Borate EDTA (TBE) buffer. In addition, undiluted and different dilutions of template DNA (1:10, 1:50 and 1:100) were also compared.

Preparation for PCR Reaction

The PCR mixture was made following the manufacturer's recommendation of My Taq™ DNA Polymerase (Bioline, USA). The reactions were performed using 10µL aliquots of the DNA extracts with the following stocks: 2µL of 5X My Taq™ reaction

buffer, 5mM dNTPs, 15mM $MgCl_2$, stabilizers, and enhancer; 1µL template DNA and 0.2µL of primers, 10µM each; 0.2µL of My Taq™ DNA Polymerase and 6.4µL of distilled H_2O

Virus Detection

The PCR mixtures were prepared as described above. PCR detected the presence of bunchy top virus using the primer pairs BBT-1 (5'CTCGTCATGTGCA AGGTTATGTCG-3'), and BBT-2 (5'GAAGTTCTCCAGCTATTCATCGCC-3') (Thomson and Dietzgen 1995, Sta Cruz et al 2016, Piamonte and Sta Cruz 2018). These primers are designed to amplify the 349bp fragment of the DNA-R (replicate). The PCR procedure was performed using Thermal Cycler (BOECO, TC-TE). The thermo-cycling scheme comprised initial denaturation at 95°C for 1min with one cycle; 35 cycles of 15s at 95°C (denaturation). The modifications of annealing tempetatures and gel concentrations were performed as described to find the optimum conditions for the protocol. DNA was visualized by staining the gel with gel red nucleic acid stain (1µL per 50mL, Biotium USA) and photographed on the gel documentation system (ENDURO™ GDS, Labnet International).

Validation of BTV Negative Samples

The PCR amplification of an internal control DNA was carried out using the *Musa*-tagged microsatellite site primers AGMI025 (5'-TTA AAG GTG GGT TAG CAT RAG G-3') and AGMI026 (5'-TTT GAT GTC ACA ATG GTG TTC C-3') to validate the virus from BTV negative samples in DNA extracts. This amplifies 248bp fragments of the host DNA (Mansoor et al 2005, Piamonte and Sta Cruz 2018, Parac et al 2021). PCR amplification was carried out using the protocol mentioned above.

RESULTS AND DISCUSSIONS

Optimized PCR Conditions for BTVs Detection

The optimized conditions amplified the target of interest, which resulted in a clear band indicating a positive presence of the virus. All the test plants from the Inosa variety, both asymptomatic and symptomatic, yielded positive reactions using the optimized PCR conditions established by this study.

Gel Concentrations and Running Conditions

In this study, the use of 1 and 1.5% gel concentrations resulted in poor DNA resolution. Smiley to fuzzy bands were encountered, particularly with small DNA fragments, and resulted in a thermal diffusion of the bands (Figure 1). Likewise, the DNA marker was not accurate in determining the fragment size when compared to the amplified DNA. It was observed that using higher voltages, eg, 90–100volts, could smear or distort the DNA fragments on the 1 and 1.5% gel (Table 1). Lower concentrations of gel, however, were fragile and therefore hard to handle. For this experiment, 2% gel electrophoresis was used to resolve small DNA fragments of 100bp to 3kb. However, it was observed that at the higher the voltage the DNA travelled faster through the gel. If the voltage is too high it can melt the gel or cause

smearing and distortion of DNA bands. In this study, 80 volts were used since the DNA samples migrated as shown by the tracking dye with precise amplification of bands.

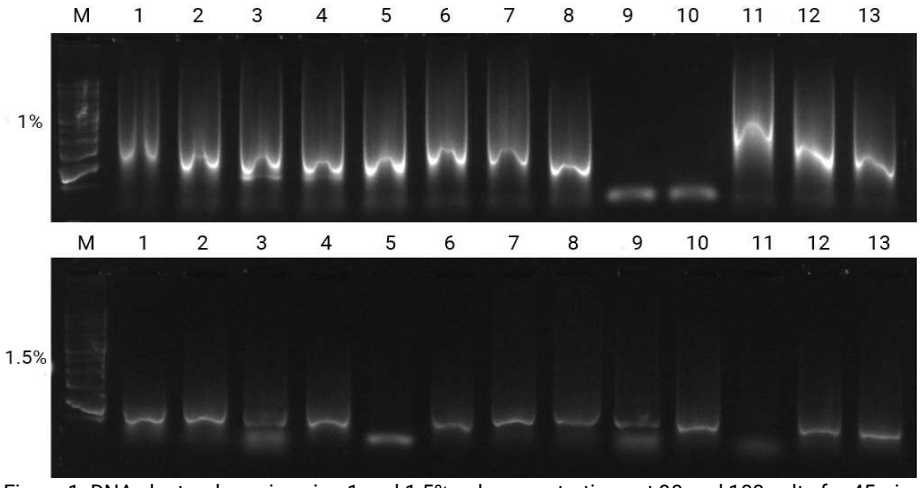

Figure 1. DNA electrophoresis using 1 and 1.5% gel concentrations at 90 and 100 volts for 45min, Lane M; 1kb HyperLadder™ (Bioline, USA); Lane 1-8, positive sample of bunchy top virus at 90 volts, Lane 9-13, at 100 volts showing smiley to fuzzy and distorted bands.

Table 1. Bands observed using different gel concentrations and voltages in optimizing PCR conditions

Gel concentrations (%)	Voltages		
	80	90	100
1	Smiley to fuzzy bands		
1.5			
2	Clear bands		Distorted bands

Electrophoresis uses an electrical field to move the negatively charged DNA toward a positive electrode through an agarose gel matrix. Thus, shorter DNA fragments migrate through the gel more quickly than longer ones. Some factors can affect the migration of nucleic acids: the dimension of the gel pores (gel concentration), the size of DNA being electrophoresed, and the voltage used (Lucotte and Baneyx 1993). These factors were also encountered by Lee et al (2012), during the separation of DNA fragments in agarose gel electrophoresis.

Annealing Temperatures

The annealing temperatures of 57°C and 60°C resulted in the amplification of DNA as indicated by the presence of bands in figure 2, lanes 1-3 and lanes 4-6, respectively. At 65°C no amplified DNA bands as shown in figure 2, lanes 7-9. Amplification of DNA as affected by annealing temperatures is summarized in table 2. As per the manufacturer's recommendation of My Taq™ DNA Polymerase (Bioline, USA), the reduction by 5°C and 7°C of optimal primer melting temperature for annealing (65°C) was used and resulted in the amplification of bands at 57°C and 60°C. The primer sequence and buffer properties of My Taq™ DNA Polymerase

(Bioline, USA) and the National Center for Biotechnology Information (NCBI) primer blast had an optimal melting temperature of around ±62°C (manufacturer's recommendation). During the annealing phase of PCR, the reaction temperature needs to be sufficiently low to allow both forward and reverse primers to bind to the template, but not so low as to enable the formation of undesired, non-specific duplexes or intramolecular hairpins, both of which reduce reaction efficiency (Owczarzy et al 2008). The findings of this research confirmed the findings of Xie and Hu (1995) who reported that BBTV infections were detected in banana plant tissue and aphid samples using the PCR with annealing temperatures of 57°C and 58°C. The optimal annealing temperature depends primarily on the length and base composition of the PCR primer and can vary from 50°C to 72°C or even higher. Also, salt concentrations affect the melting temperature (Tm). Increasing the annealing temperature decreases the chance of specific PCR products, but nothing is amplified if the temperature is too high (Lee et al 2012). A similar result was obtained with the increase of annealing temperatures by Sipos et al (2007). Amplification of mismatch primer with increasing annealing temperatures from 59.9 to 61°C detected the mismatch template. In addition, it is noteworthy that even a low annealing temperature of 57°C had no negative effect on the PCR products during the amplifications since this temperature could clearly amplify the positive bands of BTV.

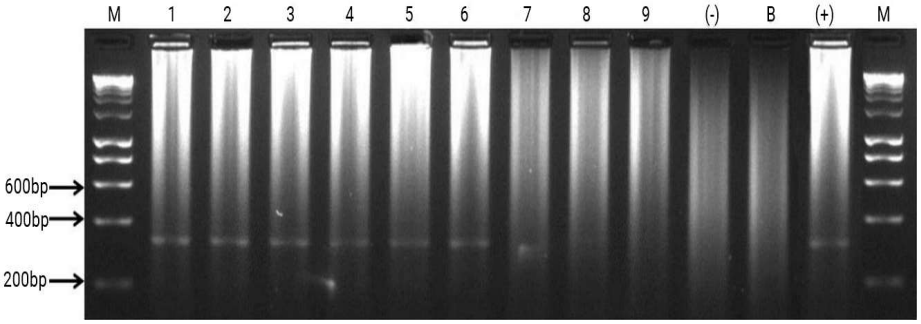

Figure 2. Optimization of annealing temperatures by PCR using the positive bunchy top virus in 2% agarose gel; Lane M: 1kb HyperLadder™ (Bioline, USA); Showing amplified bands in Lane 1-3, 57°C; Lane 4-6, 60°C; non-amplified in Lane 7-9, 65°C. Lane (-) negative control; Lane B-Blank, water only; Lane (+) positive control

Table 2. Amplification of DNA as affected by annealing temperatures

Annealing temperatures (°C)	Amplification
57	Amplified
60	
65	Non-amplified

Dilution of template DNA

Both the undiluted and dilution of 1:10 resulted in the band's amplification in lanes 5-6 (Figure 3). Succeeding dilutions of 1:50 (lanes 7-8) and 1:100 (lanes 9-10) resulted in a thinner band (Table 3). Preliminary experiments using further dilution

template DNA resulted in thinner or no bands; hence only dilutions of 1:10, 1:50 and 1:100 were used. Using a small amount of leaf tissue (2 punches 10.8mm diameter per 2mL microtube) appeared to contain fewer inhibitors. Based on the findings of Sta Cruz et al (2016), increasing the dilution of the template DNA could increase the number of BBTV-positive samples in PCR. Thus, template DNA dilutions of 1:10 up to 1:20 can increase the sensitivity of detection. In Piamonte and Sta Cruz's (2018) study, they used 0.5g of leaf tissue that would contain higher amounts of inhibitors; thus, dilution was needed. In addition, BBTV detected by PCR using DNA extracted by the Dellaporta and Sarkosyl method was the most sensitive. In their study the BBTV was detected 100% in DNA-extracted samples using various amounts of diluted extract (100, 10, 1 and 0.1ng) and pure extract (28.6%). The Piamonte and Sta Cruz (2018) study also showed that the DNA extracted by the Dellaporta method appeared to contain fewer inhibitors than those extracted by the CTAB or Sarkosyl methods. Our results obtained from the dilution were also confirmed using an internal control DNA of *Musa*-tagged microsatellite primers.

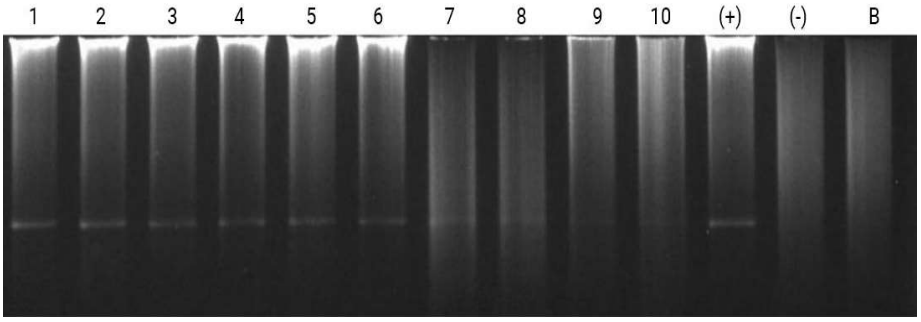

Figure 3. DNA electrophoresis of undiluted and diluted template DNA from BTV positive samples; Lane M: 1kb HyperLadder™ (Bioline, USA). Lanes 1-4, undiluted; Lanes 5-6, diluted 1:10; Lanes 7-8, diluted 1:50, and Lanes 9-10, diluted 1:100. Lane (+) positive control; Lane (-) negative control; Lane B- Blank, water only

Table 3. Bands observed of diluted and undiluted template DNA

Dilution	Bands observed
Undiluted	Amplified band
1:10	
1:50	Thinner band
1:100	

Validation of Optimized PCR Conditions for the Detection of BTVs in Abaca

The optimized PCR conditions were further validated for BTV from transmission assays. BTV was successfully detected based on optimized PCR conditions. Using the initial denaturation at 95°C for 1min with one cycle, 35 cycles each for the denaturation at 95°C for 15s, annealing for 15s at 57°C, and 10s for the extension at 72°C. Amplified DNA products were resolved on 2% agarose gel at 80 volts for 45min. For the Inosa variety, the BTV was detected early after inoculation by aphids; the virus was recognized as early as 5 DAI (Figure 4). The development of BTV was compared using the optimized PCR conditions outlined above. Detection of the virus was confirmed at 5 DAI in Leyte (Figure 4-B) and Southern Leyte isolates

(Figure 4-D), indicating the presence of clear bands from lanes 5-14. In addition, BTV was detected at 6 DAI in lanes 6-14, and 7 DAI in lanes 7-14 in Samar (Figure 4-A) and Biliran (Figure 4-C) isolates, respectively. No BTV was detected in either the abaca hybrid BC2-7 (BANDALA) or the Pacol until 54 DAI.

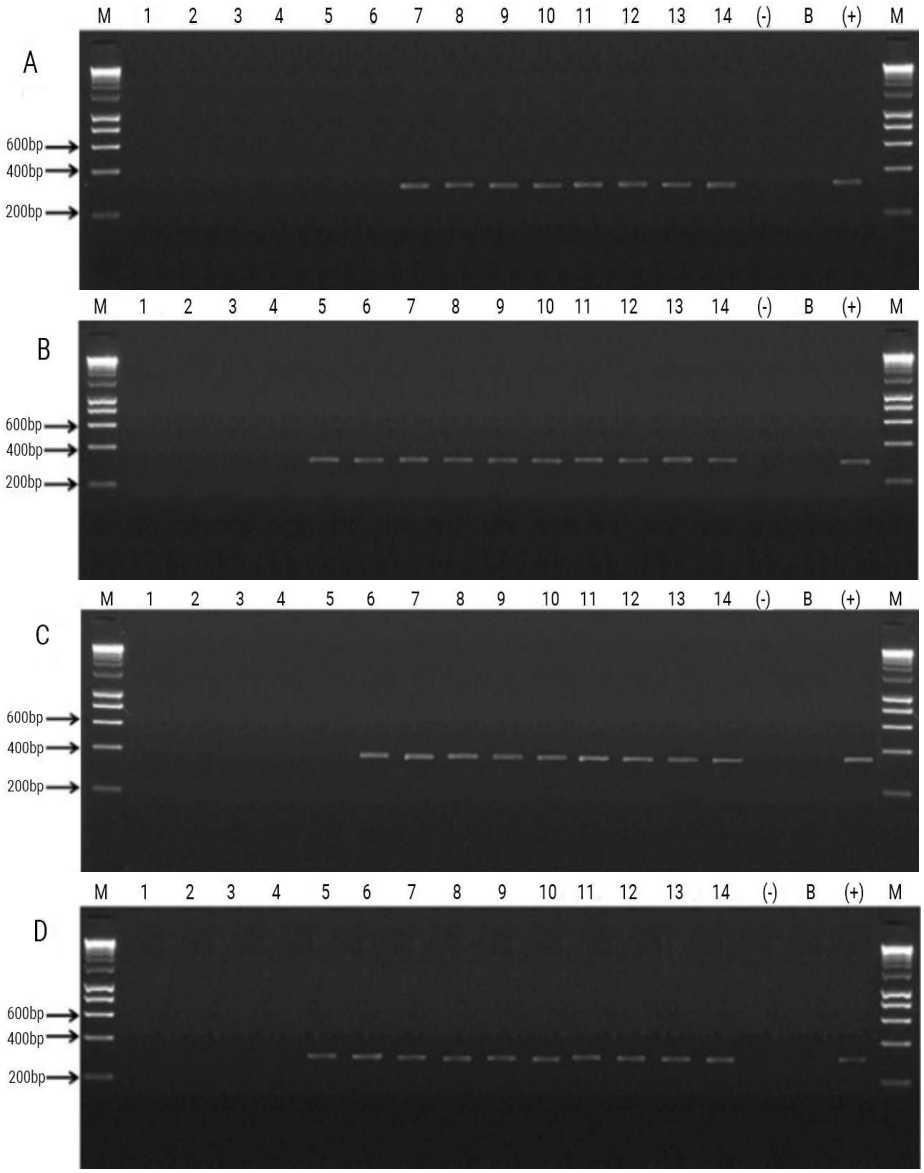

Figure 4. Amplified DNA (349bp) of BTV using the optimized conditions with the primer pair BBT-1 and BBT-2, based on Thomson and Dietzgen (1995) from 1 DAI to 14 DAI in Inosa: A- Biliran isolate (Lane 1-6, BTV negative, Lane 7-14, BTV positive) B-Leyte isolate (Lane 1-4, BTV negative, Lane 5-14, BTV positive) C-Samar isolate (Lane 1-5 BTV negative, Lane 6-14, BTV positive) and D-S. Leyte isolate (Lane 1-4, BTV negative, Lane 5-14, BTV positive: Lane M: 1kb DNA ladder (HyperLadder™, Bioline USA); Lane (-) negative control, Lane B- Blank, water only; Lane (+) positive control

The absence of infection in abaca hybrid BC2-7 (BANDALA) and Pacol was confirmed by PCR analysis using BBT-1 and BBT-2 (Figure 5). Amplification of 349bp was detected in positive samples of Inosa (Lane +). This primer pair has been designed for the Australian isolate and used to amplify the DNA-1 component of BBTV isolates (also referred to as the DNA-R, replicate) irrespective of their origin (Thomson and Dietzgen 1995, Sta Cruz et al 2016). The virus was not detectable in any test plants of the abaca hybrid BC2-7 (BANDALA) or its parental Pacol, which remained symptomless until 54 DAI in the screenhouse evaluation (Figure 6). Likewise, Piamonte and Sta Cruz (2018) and Parac et al (2021) did not detect the virus from their field samples using their sensitive and reliable detection of BTVs by PCR.

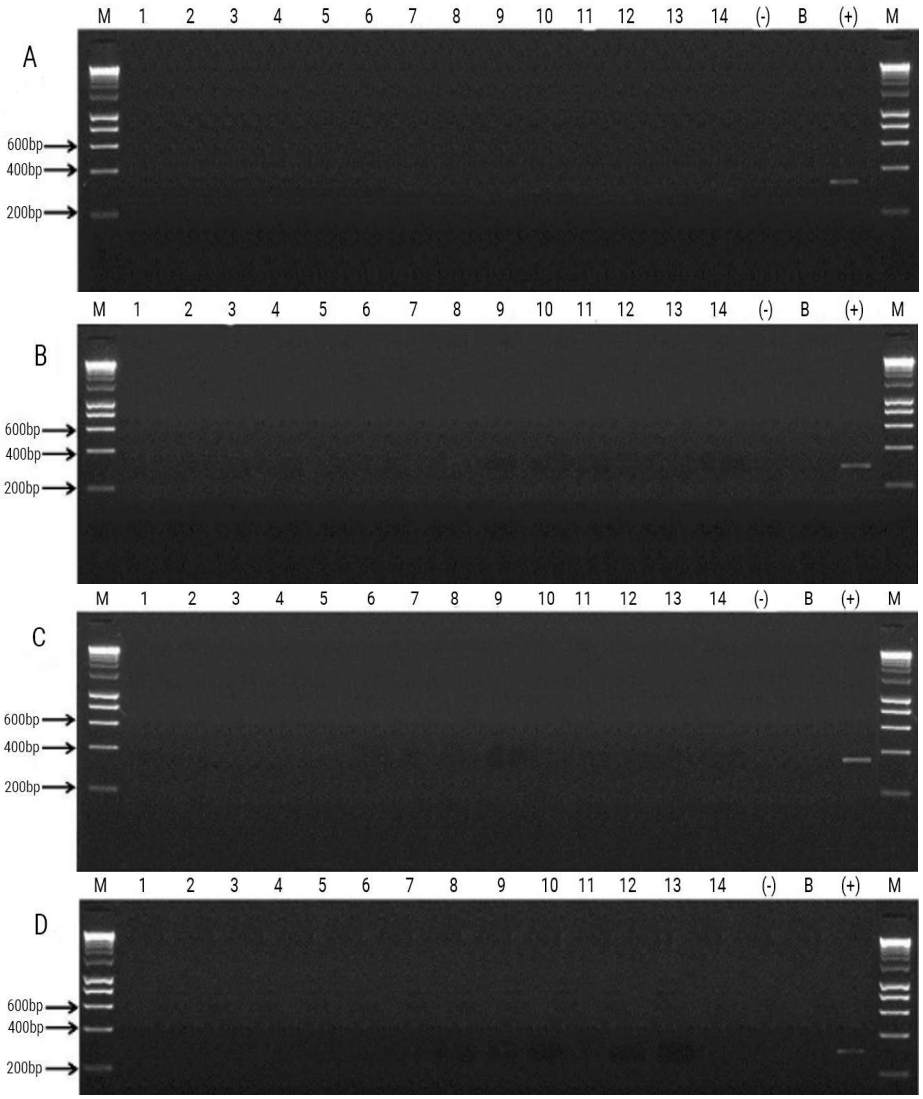

Figure 5. Negative amplification result of BTV using the optimized conditions with primer pair BBT-1 and BBT-2, based on Thomson and Dietzgen (1995) in abaca hybrid BC2-7 (BANDALA) and Pacol: A-Biliran isolate, B-Leyte isolate, C-Samar isolate and D-Southern Leyte isolate: Lane M; 1kb HyperLadder™ (Bioline, USA); Lane 1 (1DAI)- 14 (14 DAI) negative of BTV.); Lane (-) negative control, Lane B- Blank, water only; Lane (+) positive control

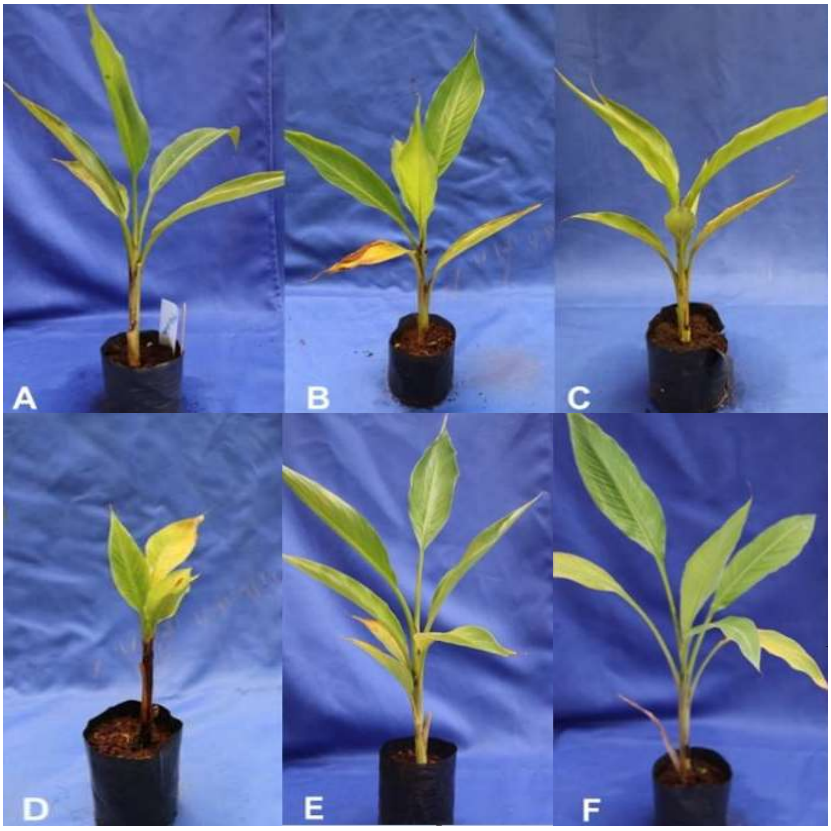

Figure 6. Test plant used in the study at 54 DAI; A-C: uninoculated, A-Inosa, B- Pacol, C-Abaca Hybrid BC2-7 (Bandala) and D-F: inoculated; symptom expressed and positive of BTV in D-Inosa, and BTV negative in E- Pacol and F- Abaca Hybrid BC2-7 (Bandala)

Validation of BTV Negative Samples Using Internal Control

The purpose of the amplifying internal control, using the *Musa*-tagged microsatellite site primers AGMI025 and AGMI026, was to confirm the BTV negative result in the abaca hybrid BC2-7 (BANDALA), Inosa, and Pacol as analyzed by PCR. The host DNA was detected by amplifying 248 bp using an internal control (Lagoda et al 1998, Piamonte and Sta Cruz 2018). The BTV negative samples of Inosa as shown in figure 4 showed positive amplification of the host DNA shown in figure 7. In figure 7 lanes 1-6, these samples were BTV negative from Biliran isolate (Figure 4-A: Lanes 1-6). The same result was observed from Leyte isolate (Figure 7, Lanes 7-10), Samar isolate (Figure 7, Lanes 11-15), and Southern Leyte isolate (Figure 7, Lanes 16-19). These isolates were shown to be BTV negative in figure 4-B and 4-D, Lanes 1-4 (Leyte and S. Leyte) respectively, and figure 4-C, Lanes 1-5 in Samar. Analysis by PCR using an internal control confirmed that the negative reaction of BTV was due to the absence of the virus and not the presence of PCR inhibitory compounds. The positive amplification was confirmed to be due to the absence of the BTV DNA and not the absence of template DNA in the reaction.

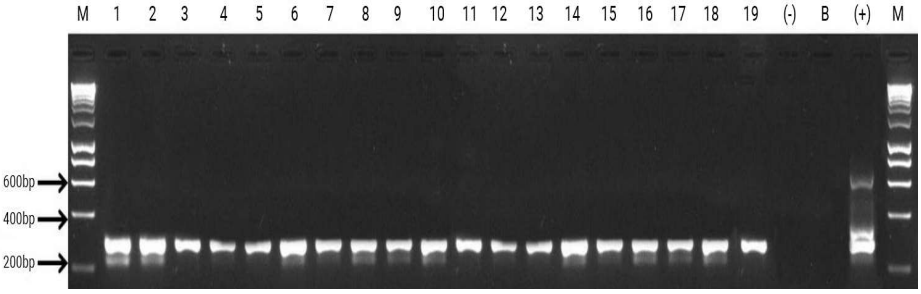

Figure 7. Amplification result of host DNA (248bp) from Inosa of *Musa*-tagged sequence by PCR using the primer pair AGMI025 and AGMI026 in Inosa (BTV negative detected in Figure 4): Lane M: 1kb DNA Ladder (HyperLadder™, Bioline, USA); Lanes 1-6: Biliran isolate; Lanes 7-10: Leyte isolate: Lanes 11-15: Samar isolate; Lanes 16-19: Southern Leyte isolate; Lane (-) negative control, Lane B-Blank, water only; Lane (+) positive control of host DNA

Positive amplification of the *Musa*-tagged sequence was detected from abaca hybrid BC2-7 (BANDALA) and Pacol, and these samples tested negative for BTV from 1 DAI to 14 DAI. The fact that the BTV negative samples were positive for the *Musa* sequence, indicates that the template DNA did not contain PCR inhibitory substances that may have given false-negative results. Thus, the negative reaction of the abaca hybrid BC2-7 (BANDALA) and Pacol was confirmed to be due to the absence of the virus. Parac et al (2020) and Piamonte and Sta Cruz (2018) also used this internal control confirming that the virus had not been detected in the abaca hybrid BC2-7 (BANDALA) and Pacol while the *Musa* sequence was detectable in all abaca hybrid test plants.

CONCLUSION AND RECOMMENDATIONS

This research has successfully established the optimized PCR conditions for detecting BTV. The conditions of annealing temperature of 57°C, 2% of agarose gel, and 80volts of electrophoresis for 45min resulted in a precise amplification of 349bp bands in BTV-positive samples using the primer pairs of BBT-1 and BBT-2. The amplification of a product with the internal control primers demonstrates the absence of PCR inhibitors in the DNA samples. It provides a high level of confidence in a diagnostic PCR reaction that is negative for the presence of BTV. These optimized PCR conditions can be used for virus indexing of abaca planting materials, and can be utilized for virus resistance screening of the NARC Abaca Germplasm collection. For future studies using this protocol dilution of 1:10 to 1:100 is required as a preliminary assay when amplifying DNA from BTV positive samples. To sum up, these optimized PCR conditions can be used as a reliable tool for detecting BTV in *Musa* families.

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