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Immunodiagnosis of bunchy top viruses in abaca with polyclonal antibodies against their recombinant coat proteins

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ABSTRACT

Polyclonal antibodies against abaca bunchy top virus (ABTV) and banana bunchy top virus (BBTV) proteins are necessary for immuno-based detection of these two viruses in abaca (*Musa textilis* Nee). In this study, recombinant bunchy top viral coat proteins fused with a 6xHis tag at the N-terminus were expressed in *E. coli* BL21Star™ (DE3)pLysS strain and purified under denaturing conditions. Purified recombinant ABTV and BBTV coat proteins were used as antigens for the production of rabbit polyclonal antibodies. IgG was purified and evaluated by Direct Antigen Coating (DAC)-ELISA and further optimized by testing primary to secondary antibody dilution combinations. Analysis of ABTV and BBTV-infected abaca samples using the optimized DAC-ELISA assay showed that the anti-ABTV CP IgG can react to BBTV and that anti-BBTV CP IgG can react to ABTV, hence, a cross-reaction. The study demonstrates the advantage of using recombinant DNA technology for mass production of antigens for antibody production. Although specificity of the polyclonal antibodies may have been compromised when renatured recombinant proteins were used as immunogens, the ability of the purified IgGs to detect positive abaca samples reveals that the DAC-ELISA can be routinely used for screening disease-free abaca planting materials.

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KEYWORDS
Abaca bunchy top virus; banana bunchy top virus; recombinant coat protein; polyclonal antibody

Introduction

Abaca (*Musa textilis* Nee) is known for its high fiber tensile strength which contributes significant economic value to the Philippine fiber industry (Spencer 1951). The abaca fiber, commercially known as Manila

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Supplemental data for this article can be accessed here.

hemp, is not only prized for its great mechanical strength but is also valued for its lightness, long fiber length and high resistance to degradation in seawater. These characteristics make abaca the fiber of choice for the production of specialty paper products, cordage, handicrafts and automobile body parts (Spencer 1953; Lalusin and Villavicencio 2015). However, abaca fiber yield is severely reduced with the infection of two viruses namely the abaca bunchy top virus (ABTV) and the banana bunchy top virus (BBTV). The average incidence of these viruses in Bicol and Eastern Visayas regions were 5.19% and 8.16%, respectively, leading to a total estimated economic loss of 31 million Php (Raymundo et al. 2002; Halos 2008).

Bunchy top (BT) disease, which is caused by ABTV and BBTV, is widely regarded as the most destructive disease in abaca (Sharman et al. 2008). ABTV- and BBTV-infected abaca plants exhibit severe stunting, bunching of leaves, chlorotic flecks in leaves and leaf curling which lowers fiber yield and quality (Raymundo et al. 2001). The ABTV and BBTV viruses both belong to the genus *Babuvirus* family *Nanoviridae* and are mainly transmitted by the vector *Pentalonia nigronervosa* Coq. (Sharman et al. 2008). The genome of both ABTV and BBTV is composed of six circular single-stranded DNA (ssDNA) components, each having an approximate length of 1.0–1.1 kb. Each component has an open reading frame which codes for a single putative protein: DNA-R for replication initiation protein (Rep), DNA-S for coat protein (CP), DNA-C for cell-cycle link protein (C-link), DNA-M for movement protein (MP), DNA-N for nuclear shuttle protein (NSP) and DNA-U3 for a protein with no known function.

To ensure the production of virus-free abaca planting materials, it is essential to develop high quality antisera for large-scale screening of plantlets by immunodiagnostic methods such as enzyme-linked immunosorbent assay (ELISA). ELISA is one of the most widely used serological techniques to diagnose plant viruses (Noorani et al. 2015). The ELISA technique is suitable for the detection of viruses from large number of samples (Clark et al. 1986) and can be applied in various formats such as direct double antibody sandwich ELISA (DAS-ELISA) (Clark and Adams 1977) and direct antigen coating ELISA (DAC-ELISA) (Clark and Bar-Joseph 1984). Although more specific molecular diagnostic tools such as PCR are available, this method requires high quality DNA that is costly and cumbersome for screening large quantities of samples. The major requirement of any ELISA format is the availability of specific antibodies against plant viral proteins that can be used to screen large number of samples.

The production of polyclonal antibodies against recombinant proteins cloned from plant viruses has been extensively done in various plant viruses that belong to different families such as faba bean necrotic yellows virus (Kumari et al. 2001), banana bunchy top virus (Abdelkader

et al. 2004), carnation etched ring virus (Raikhy et al. 2007) and grapevine fanleaf virus (Sokhandan et al. 2015). The use of bacterial system *E. coli* in recombinant protein production provides a simple, fast and inexpensive method to produce high amounts of proteins with minimal post-translation modifications (Hartley 2006). Thus the production of recombinant proteins in prokaryotic systems provides an easier, faster and economical mode of polyclonal antibody production against plant viral proteins as compared to the conventional plant virus purification process. The study therefore aims to use expressed recombinant ABTV and BBTV CP as the antigen to produce efficient polyclonal antibodies against ABTV CP and BBTV CP for virus detection. Antibody titer shall be evaluated through serial dilution of raw antisera, which shall be purified and evaluated for its diagnostic performance using DAC-ELISA.

Materials and methods

Virus source, sample collection, DNA extraction

Healthy and ABTV/BBTV-infected abaca plants were maintained in a greenhouse at the Bureau of Plant Industry (Manila, Philippines). Total DNA was extracted using the modified cetyl-trimethylammonium bromide (CTAB) method (Gawel and Jarret 1991). Briefly, 1 g of tissue sample was ground into a fine powder using liquid nitrogen and homogenized in 7 mL of CTAB buffer (0.1 M CTAB, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB and 0.2% β-mercaptoethanol added before use) supplemented with 2% polyvinylpyrrolidone. Six hundred microliters of the homogenate was transferred to a 1.5 mL tube and incubated at 65 °C for 45 min with inversion every 10 min. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed thoroughly. Mixture was centrifuged at 15,000 × g for 2 min at room temperature (RT) and supernatant was recovered and chloroform: isoamyl alcohol extraction was repeated. Eight hundred microliters of 95% ethanol was added to the supernatant and incubated at −20 °C for 1 h. Mixture was centrifuged at 15,000 × g for 2 min at RT and pellet was washed twice with 75% ethanol. Total DNA pellet was resuspended in 50 µL of nuclease-free water. The quality and yield of extracted DNA were checked by measuring absorbance and determining the A260/A280 ratio using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). DNA concentrations were adjusted to 10 ng/µL prior to use in PCR.

Cloning and sequencing of coat protein genes

Primers targeting the full open-reading frame (ORF) of ABTV CP and BBTV CP were designed based on the sequences found in GenBank and

Table 1. Primers designed and used to clone and detect ABTV and BBTV CP.

Primer Name	Target Sequence	Primer Sequence (5′ to 3′)	Length (in bp)	GenBank Accession No.
ABTV3F	ABTV CP	AGATCTATGGCGAGGTATCCCAAGAAAT	28	EF546804.1
ABTV3R		GGTGACCTATTTCGTAGGCATATCATTGT	29	
BBTV3F	BBTV CP	CTAGATCTCAGATGGCGAGGTTTCCGAGG	29	AB250958.1
BBTV3R		CCAAATAACATACAACACTCGGTCACCTG	29	

were analyzed using the IDT oligoanalyzer tool (Table 1). Full coding sequences were amplified by PCR (10 µL) consisting of 1X Titanium *Taq* (*TiTaq*) buffer, 0.25 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.5 µM each of forward and reverse primers, 0.05X of *TiTaq* polymerase (Takara Clontech) and 0.5 µL of undiluted DNA template. The PCR condition used was: initial denaturation of 95 °C for 2 min, and 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 7 min. Amplicons of the expected size were ligated into the pGEM-T-Easy vector (Promega) following the manufacturer's protocol and ligation reactions were transformed into *E. coli* DH5α cells. Positive transformants were confirmed through colony screening PCR (Woodman et al. 2016) using gene specific primers (Table 1). Verified and purified plasmids were sent for sequencing (Macrogen) to confirm the identity of amplicons. Sequences were analyzed through BLAST (Altschul et al. 1990) and multiple sequence alignment through Multalin (Corpet 1988) while amino acid sequences were deduced through ExPASY translate tool (Gasteiger et al. 2003). Confirmed ABTV and BBTV CP sequences were reamplified with the gene-specific primers and subcloned into the pEXP5-NT/TOPO® vector (Invitrogen) through TOPO-TA-cloning following the manufacturer's protocol. Ligation reactions were transformed into *E. coli* DH5α and plasmids were verified through sequencing. Derived amino acid sequences were used to predict the theoretical molecular weight of proteins through the ExPASY pI/MW tool.

Expression and purification of recombinant coat proteins

Expression was carried out in *E. coli* BL21Star™(DE3)pLysS strains (Invitrogen) and purification of the fusion proteins was done via Ni-NTA gravity flow chromatography kit (Invitrogen) following the manufacturer's protocol. pEXP5-NT/TOPO-ABTVCP or pEXP5-NT/TOPO-BBTVCP was transformed into the BL21Star™(DE3)pLysS and positive transformants were screened with PCR using gene specific primers. Positive single colonies were used to inoculate overnight cultures consisting of LB broth supplemented with 34 µg/mL of chloramphenicol and 50 µg/mL of ampicillin and incubated at 37 °C with 200 rpm shaking. Two hundred fifty microliters of overnight culture was used to seed a new

5 mL culture and cells were allowed to reach an optical density (OD_{600}) of 0.4-0.5 before induction. Fusion proteins were expressed by induction with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and incubation at 37 °C for 6 h. Proteins were analyzed at three time points 2 h, 4 h and 6 h post-induction and cells (500 µL) were harvested (12,000 × g for 5 min at 4 °C) for each time point. Total protein of cell pellets were analyzed by adding 80 µL of 1× SDS treatment buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol, 0.002% Bromophenol Blue) and homogenate was vortexed for 30 sec and boiled for 10 min in a digital dry bath (Labnet) to completely lyse the cells. Proteins were analyzed through SDS-PAGE analysis with 5% stacking gel and 12% resolving gel.

Purification was done under denaturing conditions following the manufacturer's protocol (Invitrogen). In brief, 50 mL cell cultures were harvested by centrifugation at 8,000 × g for 10 min at 4 °C. The bacterial pellet was suspended in 6 M guanidinium lysis buffer. Mechanical cell lysis was done through sonication (Branson Sonifier) for a total of 20 pulses (at 20% output) with 30 sec duration for each pulse and 30 sec rest on ice after every pulse. Unwanted precipitates were removed by centrifugation at 10,000 × g for 20 min at 4 °C with the resulting supernatant loaded into the Ni-NTA column. The whole cell lysate was allowed to bind for at least 30 min under room temperature with constant shaking. Subsequent wash and elution steps were done using 8 M urea buffer with decreasing pH levels from pH 8.0 to pH 4.0. Purified proteins were confirmed through SDS-PAGE analysis and western blot using mouse monoclonal antibody against 6xHis tag (Calbiochem) at 1:2,500 dilution as primary antibody and goat anti-mouse IgG conjugated with alkaline-phosphatase (Roche) at 1:5,000 dilution as secondary antibody.

Production of antiserum against ABTV CP and BBTV CP

To remove excess urea from the purified recombinant ABTV and BBTV coat proteins, elution fractions were pooled and dialyzed using Pur-A-Lyzer™ Maxi 6000 Dialysis Kit (Sigma) overnight at 4 °C with three changes of 1 liter PBS with 4 h each buffer change. Concentration of purified recombinant ABTV and BBTV coat proteins was determined through Bradford assay (Bradford 1976) with bovine serum albumin (BSA) as the standard. Polyclonal antibodies against ABTV and BBTV rCP were produced by immunization of two New Zealand white rabbits in a span of two months with biweekly intervals of immunization for a total of four immunization shots. Purified rCP in 200 µg was emulsified with an equal volume (1:1 v/v) of complete Freund's adjuvant (Sigma) for the first injection and

injected intramuscularly. For succeeding injections, rCP (150 µg) emulsified with equal volume (1:1 v/v) of incomplete Freund's adjuvant (Sigma) was used. The collected blood was allowed to clot for 1 h at room temperature then overnight at 4 °C. Antisera were collected through centrifugation at 8000 × g at 4 °C for 20 min and stored at 4 °C until use.

Polyclonal antibody titer evaluation

The titer of the polyclonal antibodies were evaluated by employing direct antigen-coating ELISA (DAC-ELISA) (Clark and Bar-Joseph 1984) with modifications using the raw antisera. Samples were prepared by grinding in carbonate coating extraction buffer (0.1 M carbonate buffer, pH 9.6, 2% PVP, 0.25% sodium sulfite and 0.25% sodium diethylcarbamate) in 1:10 weight per volume (g/mL) ratio of sample to buffer. Samples in 200 µL volumes were dispensed in the 96-well plates and incubated at 4 °C overnight in a humid chamber. Plates were emptied and washed three times with PBS-T (PBS with 0.05% Tween-20) at 3 min interval per wash. Wells were blocked with 400 µL of blocking buffer composed of 0.5% w/v skimmed milk (Scharlau) in PBS-T, for 1 h at room temperature. Raw antisera was serially diluted in enzyme diluent buffer (PBS-T with 2% PVP and 0.25% skimmed milk) from 1:200 to 1:51,200. Primary antibody was dispensed in 200 µL volumes in each well and incubated at 37 °C for 2 h. Plates were then emptied and washed three times with 1XPBS-T. Goat anti-rabbit IgG alkaline phosphatase-conjugated (Biorad), as secondary antibody, was diluted to 1:5,000 in enzyme diluent buffer. The secondary antibody was dispensed at 200 µL per well and plates were incubated at 37 °C for 2 h. Plates were emptied and washed three times with PBS-T. Two hundred microliters of pNPP substrate (HiMedia) at 2 mg/mL concentration in 0.1 M diethanolamine buffer (9.7% diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was dispensed into wells and reaction was allowed to proceed in the dark at 37 °C and reactions were stopped by dispensing 50 µL of 3 M NaOH per well. Absorbance was recorded at 405 nm using a microplate reader (Varioskan). Data was analyzed quantitatively by measuring absorbance values and comparing their means with a computed threshold value (TV) = mean negative controls + three times the standard deviation of the negative controls. Samples were considered virus-positive if their mean absorbance values exceeded the TV.

Purification of IgG

IgG was acquired through sodium sulfate precipitation of raw/crude antisera (Page and Thorpe 1996). To 5 mL of crude antisera, equal volume of 36% (w/v) of sodium sulfate was added. Precipitates were

collected by spinning down the whole suspension at 8,000 rpm for 15 min at 25 °C. Pellets were washed twice with 18% (w/v) of sodium sulfate. The final pellet formed was resuspended in 1.5 mL of phosphate buffered saline (PBS). Excess sodium sulfate salts were removed by dialyzing the solutions using Pur-A-Lyzer™ Maxi 6000 Dialysis Kit (Sigma) overnight at 4 °C with three changes of 1 liter PBS with 4 h each buffer change. Dialyzed IgG was centrifuged at 8,000 rpm for 10 min at 25 °C to remove unwanted precipitates and concentration was read using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). Stock IgGs were diluted to 1 mg/mL working concentrations for use in DAC-ELISA.

Validation of anti-ABTV CP and anti-BBTV CP IgG for use in DAC-ELISA

To validate the developed DAC-ELISA protocol using the purified IgG, abaca and banana samples from various abaca growing regions were collected and subjected to the optimized DAC-ELISA protocol at 1:250 dilution of primary antibody and 1:5,000 dilution of secondary antibody. DAC-ELISA results were confirmed through PCR detection of each virus. Total DNA was extracted by the CTAB DNA extraction method and gene specific primers (Table 1) were used to amplify and detect the ABTV and BBTV CP segments. PCR reactions were done in 10 µL volumes consisting of 1X Titanium *Taq* (TiTaq) buffer, 0.25 mM of dNTPs, 0.5 µM each of forward and reverse primers, 0.05X of TiTaq polymerase (Takara Clontech) and 0.5 µL of undiluted DNA template. PCR amplification was done as follows: initial denaturation of 95 °C for 2 min, and 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and final extension of 72 °C for 7 min. The PCR products were analyzed by electrophoresis (0.5X TAE, 100 V) in a 1.0% agarose gel which was stained using GelRed™ (Biotium) and visualized using the AlphaImager gel documentation system. PCR detection was done in triplicates.

Results

Cloning and sequencing of coat protein genes

PCR amplification of ABTV CP and BBTV CP ORF using gene specific primers yielded an approximately 500 bp band for both viral genes. Cloning and sequencing of respective PCR products confirms that the sequences amplified were identical to database coding sequences of ABTV (Supplementary Figure 1) and BBTV CP genes (Supplementary Figure 2). The 513 bp ABTV CP ORF showed 100% nucleotide sequence identity with the Philippine ABTV isolate Q1108 (GenBank Accession No. EF546804.1) and 87% sequence identity with the Malaysian ABTV

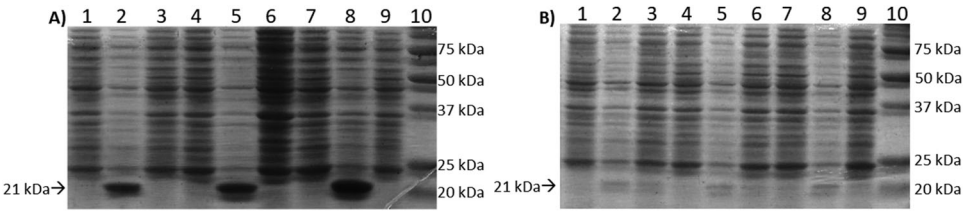

Figure 1. SDS protein analysis of recombinantly expressed ABTV CP and BBTV CP in *E. coli* BL21Star™(DE3)pLysS. Induction of cells with 1 mM IPTG in LB media to express (A) ABTV CP and (B) BBTV CP at 37 °C for 2 h, 4 h and 6 h post-induction. Lanes 1, 4 and 7: untransformed BL21Star™(DE3)pLysS at 2 h, 4 h and 6 h post-induction with 1 mM IPTG. Lanes 2, 5, and 8: BL21Star™(DE3)pLysS transformed with pEXP5-NT/TOPO-ABTV CP or BBTV CP at 2 h, 4 h and 6 h post-induction with 1 mM IPTG. Lanes 3, 6 and 9: uninduced BL21Star™(DE3)pLysS transformed with pEXP5-NT/TOPO-ABTV CP or BBTV CP at 2 h, 4 h and 6 h time points. Lane 10: PrecisionPlus™ All Blue Protein Prestained Standard (Biorad).

isolate Q767 (GenBank Accession No. EF546810.1). The 513 bp BBTV CP ORF showed 99.81% nucleotide sequence identity to the ORF of the Philippine BBTV isolate MS6_PH_2008 (GenBank Accession No. KM607522.1) and also showed 99.81% nucleotide sequence identity to the segment S of Taiwan BBTV isolate Q624_TW_1996 (GenBank Accession No. KM607541.1). Amino acid sequence analysis of the ABTV CP showed 100% sequence identity to the abaca bunchy top virus coat protein (GenBank Accession No. ABP96957.1) while the BBTV CP amino acid sequence showed 100% sequence identity to the banana bunchy top virus coat protein (GenBank Accession No. AAQ01659.1).

Amino acid translation of the nucleotide sequences showed that both the ABTV and BBTV CP genes code for a 170 aa protein. Computational prediction of the isoelectric point (pI) and molecular weight of the proteins showed that both proteins have a theoretical protein mass of approximately 20 kDa with the ABTV CP having a pI of 9.74 with the BBTV CP having a pI of 9.75. The pEXP5-NT/TOPO vector appends a 6xHis tag and a few amino acid linkers at the N-terminus of the protein that adds about 1 kDa thus the expected protein size for both is around 21 kDa.

Expression and purification of recombinant coat proteins

Expression of ABTV CP in BL21Star™(DE3)pLysS with induction of 1.0 mM of IPTG showed that at 2 h of incubation at 37 °C, a 21 kDa protein was observed in setups transformed with the pEXP5-NT/TOPO-ABTVCP vector (Figure 1A). When comparing the intensity of the bands for 2 h, 4 h and 6 h incubation, there was an increase in band intensity of the 21 kDa band size. Expression of BBTV CP on the other hand was not as strong as ABTV CP (Figure 1B) although there was a slight

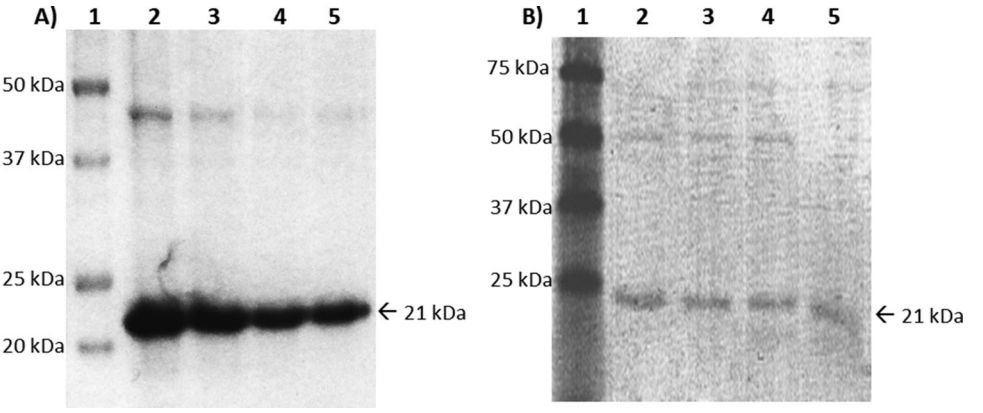

Figure 2. SDS protein analysis of purified recombinant 6xHis tagged-ABTV CP under denaturing conditions. A) SDS-PAGE and B) Western blot analysis. Lane 1: PrecisionPlus^{TM} All Blue Protein Prestained Standard (Biorad). Lanes 2 to 5: Elution fractions 1 to 4.

increase in protein expression when incubation was extended for 6 h. Proteins were initially purified under native conditions but no recombinant proteins were purified (data not shown). Recombinant ABTV CP and BBTV CP were therefore purified under denaturing conditions. SDS-PAGE analysis and western blot analysis of elution fractions under denaturing conditions confirmed that the eluted proteins were ABTV CP and BBTV CP tagged with 6xHis (Figures 2 and 3). Protein elutions were pooled together and excess urea was removed by dialysis. The resulting solution was subjected to the Bradford assay to estimate the protein concentration with BSA as the standard. Results showed that recombinant ABTV CP protein expression reaches 1 mg per 50 mL of BL21Star^{TM}(DE3)pLysS culture while for BBTV CP only 0.5 mg per 50 mL BL21Star^{TM}(DE3)pLysS culture was produced.

Polyclonal antibody titer evaluation

Antisera against ABTV and BBTV CP were serially diluted from 1:200 dilution to 1:51,200 dilution to determine the range of reactivity. Anti-ABTV CP allowed detection of ABTV-infected abaca samples from 1:200 dilution to 1:12,800 dilution (Figure 4A). The mean absorbance at 405 nm of the infected abaca sap decreased from 1.469 (1:200) to 0.294 (1:12,800). The readings of healthy controls were lower at all the dilutions from an absorbance value of 0.569 at 1:200 dilution to 0.169 at 1:12,800 dilution. ABTV-infected abaca sap was clearly distinguishable up until 1:12,800 dilution wherein the absorbance value of 0.294 was above the threshold value of 0.181. Anti-BBTV CP allowed detection of BBTV-infected abaca samples from 1:200 dilution to 1:6,400 dilution (Figure 4B). The mean absorbance at 405 nm of the infected abaca sap

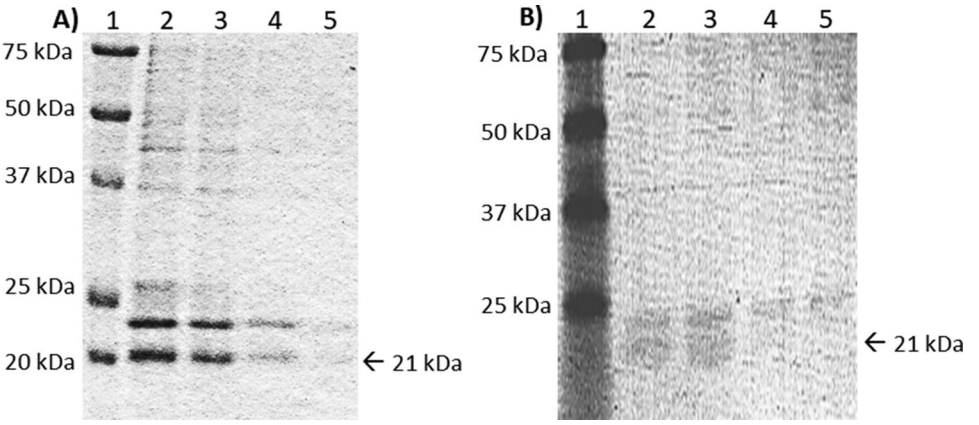

Figure 3. SDS protein analysis of purified recombinant 6xHis tagged-BBTV CP under denaturing conditions. A) SDS-PAGE and B) Western blot analysis. Lane 1: PrecisionPlus^{TM} All Blue Protein Prestained Standard (Biorad). Lanes 2 to 5: Elution fractions 1 to 4.

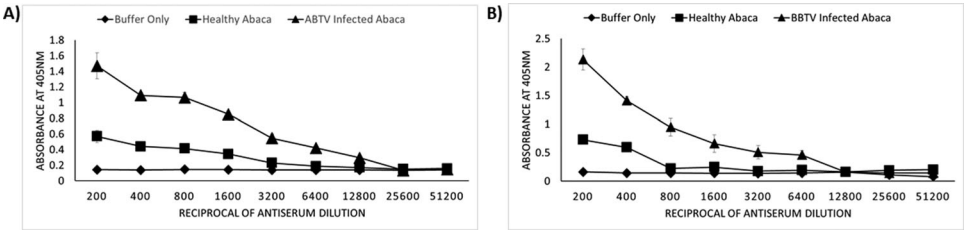

Figure 4. Anti-ABTV CP and anti-BBTV CP polyclonal antibody titer evaluation through DAC-ELISA of antibody serial dilutions A) anti-ABTV-CP rabbit polyclonal antibody and B) anti-BBTV CP rabbit polyclonal antibody. Healthy and ABTV or BBTV-infected abaca (1:10, w/v) were tested. Goat-anti rabbit AP-conjugate at 1:5000 dilution was used as secondary antibody.

decreased from 2.131 (1:200) to 0.161 (1:12,800). The readings of healthy controls were lower at all the dilutions from an absorbance value of 0.728 at 1:200 dilution to 0.161 at 1:12,800 dilution. BBTV-infected abaca sap was clearly distinguishable up until 1:6,400 dilution wherein the absorbance value of 0.460 was above the threshold value of 0.203.

Evaluation of purified IgG

Purified IgG concentrations were adjusted to 1 mg/mL to be used as primary antibody for DAC-ELISA. Serial dilutions (1:100, 1:200, 1:300 and 1:400) of the 1 mg/mL IgG as primary antibody was used to optimize the DAC-ELISA setups while secondary antibodies were diluted to 1:5,000, 1:7,500 and 1:10,000. Purified anti-ABTV CP IgG was able to detect ABTV-infected abaca samples at 1:100, 1:200, 1:300 and 1:400 dilution of 1 mg/mL purified IgG (Figure 5A). Purified anti-BBTV CP IgG was able to detect BBTV-infected abaca samples up to 1:300 dilution only (Figure 5B). Purified anti-ABTV CP IgG was also shown to react to

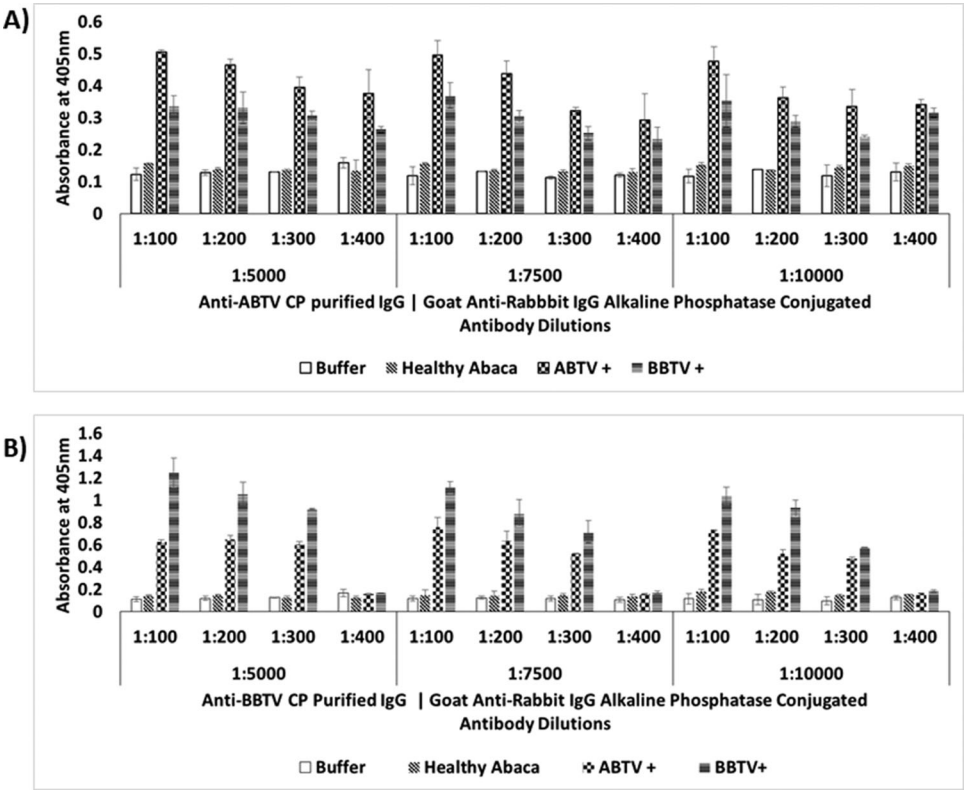

Figure 5. Optimization of primary and secondary antibody dilutions for DAC-ELISA detection of A) ABTV and B) BBTV using purified IgG as the primary antibody. Samples tested: A - carbonate coating extraction buffer only; B - healthy abaca sap; C - ABTV-infected abaca extract and D - BBTV-infected abaca extract.

BBTV-infected abaca samples up until 1:400 dilution. The same can also be observed in purified anti-BBTV CP IgG which also showed reactivity towards ABTV-infected abaca samples. This shows that there is a possible cross-reactivity between the anti-ABTV CP IgG to BBTV CP antigens and between the anti-BBTV CP IgG to ABTV CP antigens when using the DAC-ELISA format. Secondary antibody dilutions show that at 1:5,000 dilution, maximum 405 nm absorbance was observed for the both ABTV and BBTV CP detection through DAC-ELISA. A primary antibody dilution of 1:250 and secondary antibody dilution of 1:5,000 was therefore used in succeeding DAC-ELISA experiments for both ABTV and BBTV detection.

Validation of anti-ABTV CP and anti-BBTV CP IgG for use in DAC-ELISA

High cross-reactivity was observed when using anti-ABTV CP IgG and anti-BBTV CP IgG for DAC-ELISA in the detection of ABTV and BBTV in abaca (Table 2). Out of the 20 samples tested, 17 were positive for

Table 2. Detection of ABTV and BBTV in field-collected abaca samples by DAC- ELISA and PCR.

Method Detecting Target Samples	DAC-ELISA		PCR	
	ABTV CP	BBTV CP	ABTV CP	BBTV CP
Healthy PTC Abaca1	0.148[a] ± 0.002	0.144[a] ± 0.001	–	–
Healthy PTC Abaca2	0.166 ± 0.004	0.159 ± 0.002	–	–
Asymptomatic abaca	**0.267 ± 0.039**	**0.212 ± 0.003**	+	–
Asymptomatic banana	**0.327 ± 0.060**	**0.248 ± 0.013**	–	+
Banana 4	**0.538 ± 0.006**	**0.239 ± 0.013**	–	+
Abaca CDO 1	**0.441 ± 0.006**	**0.212 ± 0.003**	+	–
Abaca CDO 2	**1.494 ± 0.031**	**0.484 ± 0.056**	+	+
Lake Sebu 1	**0.673 ± 0.026**	**0.724 ± 0.053**	+	+
Lake Sebu 3	**0.505 ± 0.001**	**0.243 ± 0.009**	+	–
Mayon 9	**2.896 ± 1.376**	**1.732 ± 0.061**	+	+
Mayon 25	**2.291 ± 0.339**	**1.453 ± 0.123**	+	+
SSB1	**0.292 ± 0.034**	**0.256 ± 0.029**	+	+
SSB5	**0.207 ± 0.013**	**0.189 ± 0.005**	+	–
SSB6	**0.746 ± 0.138**	**0.549 ± 0.020**	–	+
SSB11	**0.278 ± 0.026**	**0.213 ± 0.008**	+	+
Davao 1	**0.492 ± 0.013**	**0.275 ± 0.009**	–	+
Davao 3	**0.287 ± 0.008**	**0.184 ± 0.008**	–	+
Davao 4	0.144 ± 0.003	**0.195 ± 0.003**	–	+
Davao 5	**0.289 ± 0.007**	**0.190 ± 0.011**	+	–
Davao 7	**0.674 ± 0.007**	**0.361 ± 0.035**	–	+
Threshold Value (TV)	0.176	0.165	N.A.	N.A.

[a]Values represent average of three 405 nm readings recorded after 3-hour incubation. Values in bold are positive for the ELISA. -, no band or negative; + presence of band or positive in PCR.

both ABTV while 18 were positive for BBTV when tested using DAC-ELISA. For PCR, 10 tested positive for ABTV CP detection while 12 tested positive for BBTV CP detection. Samples that were positive in PCR for ABTV CP were also found to be positive in ABTV CP DAC-ELISA test while samples that were positive in PCR for BBTV CP were also found to be positive in BBTV CP DAC-ELISA test.

Discussion

Abaca bunchy top virus and banana bunchy top virus cause significant reduction in fiber yield and quality in infected abaca plants. As the abaca is a vegetative propagated crop, development of high-throughput diagnostic method is a requirement for production and distribution of healthy and virus-free planting material. The prerequisite for the development of immunodiagnostic method is the availability of high-quality antisera. One way to develop high-quality antisera is to raise polyclonal antibodies against recombinant proteins derived from the genes of the respective plant virus. This study showed the successful cloning of CP gene of ABTV and BBTV and was characterized and expressed in *E. coli* BL21Star^{TM}(DE3)pLysS cells. Sequence analysis for both nucleotide and amino acid sequences of ABTV CP and BBTV CP showed high sequence identity to their respective Philippine isolates thus confirming the successful cloning of the full length coding sequence of ABTV CP and

BBTV CP. The coat protein gene of plant viruses is the mostly used antigen for the production of polyclonal antibody due to it being highly conserved among virus strains thus facilitating broad-based detection of plant virus strains (Čeřovská et al. 2006; Singh et al. 2014; Koolivand et al. 2016).

The pEXP5-NT/TOPO® bacterial expression vector includes a T7 promoter to facilitate initiation of protein expression in *E. coli* and an N-terminal 6xHis tag to facilitate purification and immunoblot detection. The choice of using and retaining the N-terminal 6xHis tag was based on the fact that it was not expected to have significant immunogenic properties (Mutasa-Gottgens et al. 2000; Kumari et al. 2001; Gulati-Sakhuja et al. 2009). These studies also proved the 6xHis tag to be effective in affinity purification of insoluble recombinant proteins from the inclusion bodies without altering the diagnostic potential of the polyclonal antiserum. Recombinant ABTV and BBTV coat proteins were successfully produced by inducing transformed BL21Star™(DE3)pLysS with 1 mM of IPTG. Incubation duration post-induction largely affected the amount of recombinant proteins produced and by increasing incubation time post-induction, more proteins were expressed for both ABTV CP and BBTV CP (Figure 1).

The successful purification of ABTV and BBTV rCP under denaturing conditions showed that the proteins are mainly secluded in the insoluble phase of the whole cell protein extract of *E. coli* (Figures 2 and 3). As with other studies on the production of recombinant viral coat proteins from other plant viruses such as faba bean yellows virus (Kumari et al. 2001), banana bunchy top virus (Abdelkader et al. 2004), pelargonium zonate spot virus (Gulati-Sakhuja et al. 2009), large cardamom crinkle virus (Vijayanandraj et al. 2013) and cherry virus A (Noorani et al. 2015), the fusion protein is shown to be compartmentalized in insoluble phase. Under such circumstances, purification under denaturing conditions would be best to release all proteins and gradually renature the proteins by dialysis. Recombinant plant viral proteins secluded into inclusion bodies help protect them from proteases and facilitate purification process but pose a challenge in protein renaturation. Numerous studies on the use of dialyzed denatured coat proteins as antigens for antiserum production have already been done (Kumari et al. 2001; Radaelli et al. 2008; Gulati-Sakhuja et al. 2009; Vijayanandraj et al. 2013; Sokhandan et al. 2015).

Evaluation of antibody titer by determining the ability of raw antisera to recognize target antigens in ABTV- or BBTV-infected abaca samples showed that rabbit anti-ABTV CP was able to react to ABTV-infected abaca samples until 1:12,800 dilution of raw antiserum while rabbit

anti-BBTV CP was only able to detect BBTV-infected abaca samples until 1:6,400 dilution of raw antiserum (Figure 4). The reason for the lower antibody titer of rabbit anti-BBTV CP as compared to the rabbit anti-ABTV CP antibody may be caused by the lower BBTV viral titer found in that specific BBTV-infected abaca sample.

Purification of IgG was done to enrich the overall IgG concentration as well as to provide a more stable form for the antibody preparations suitable for long term storage and distribution to remote diagnostic laboratories (Page and Thorpe 1996). Evaluation of purified IgG via DAC-ELISA confirmed the efficiency of the anti-ABTV CP and anti-BBTV CP rabbit IgG in detecting infected abaca samples (Figure 5 and Table 2). Although there is high cross-reactivity observed between the two antisera and against each other's respective antigens, this is expected as plant viral antigens belonging to the same virus family have been previously reported to have high antisera cross-reactivity due to the similarities in amino acid sequences and protein structure (Hull 2013). Nevertheless, the main objective for immunodiagnosis of asymptomatic abaca plants is to detect the presence or absence of virus diseases regardless of which specific bunchy-top virus is infecting it. The objective of specifically identifying the virus is an entirely other matter than can be better resolved by using more specific diagnostic tools such as PCR. The consistency of DAC-ELISA results and PCR results to detect ABTV and BBTV in abaca indicates that the anti-ABTV CP and anti-BBTV CP are applicable for the detection of ABTV and BBTV in field-collected abaca samples (Table 2).

This study demonstrates the advantage of utilizing recombinant DNA technology for mass production of antigens for immunization as an alternative for virus purification, which is laborious, inefficient and requires maintenance of live virus culture. The use of native ABTV and BBTV proteins from purified virus particles provides an advantage of retaining the native conformation of viral proteins. Thus antibodies produced against native viral protein would have a higher diagnostic specificity as opposed to recombinantly expressed proteins. As reported in previous studies and as demonstrated in this study, proteins overexpressed in *E. coli* have a tendency to be secluded in inclusion bodies. Thus it is inevitable that recombinant viral proteins will be purified under denaturing conditions which alters the overall native conformation of the viral protein. One way of preserving the soluble or native state of recombinant proteins overexpressed with *E. coli* is to fuse it with tags that improve recombinant protein solubility such as glutathione S-transferase (GST) (Wang et al. 2006; Singh et al. 2011) and maltose binding protein (MBP) (Agarwal et al. 2009; Phaneendra et al. 2014). This

strategy would allow the proteins to be expressed and purified in a soluble state thus preserving the native conformation of viral proteins but it would require cleavage of the large tags prior to use in immunization. Alternatively, the 6xHis tag can be retained but protein expression conditions such as incubation temperature and IPTG concentration may be optimized in combination as this can improve plant virus protein solubility in *E. coli* (Raikhy et al. 2007).

Nevertheless, this study proves that the recombinant DNA technology is an efficient and cost-effective approach for raising polyclonal antibodies against CPs of ABTV and BBTV. The developed antisera against ABTV and BBTV CP can be successfully used for the detection of ABTV and BBTV in asymptomatic abaca or banana plants to facilitate effective disease management within abaca plantations. Purified IgG can be further processed for alkaline phosphatase conjugation that can be used for various sandwich ELISA formats to improve specificity and sensitivity of the ELISA assay. Moreover, the issue of specificity to further differentiate or distinguish between ABTV of BBTV-infected abaca can be further addressed by succeeding research that will also utilize the initial findings from this current study.

Disclosure statement

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