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Serological and Molecular Detection of Mixed Bunchy Top and Mosaic Virus Infections in Abaca (*Musa textilis* Nee)

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Diagnosis based on symptoms does not clearly distinguish diseases of abaca caused by mixed infections of different viruses causing bunchy top and mosaic diseases. Reliable diagnosis requires more sensitive serological and molecular detection methods. Thus, the occurrence of mixed bunchy top and mosaic virus infections in two abaca-growing areas in the Philippines was determined through serological and molecular virus detection. Abaca leaf samples collected from seven locations in the Bicol and Eastern Visayas regions were analyzed for the presence of *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV), *Sugarcane mosaic virus* (SCMV) and *Cucumber mosaic virus* (CMV). Serological detection by enzyme-linked immunosorbent assay (ELISA) of samples from all locations revealed the presence of BBTV, BBrMV, SCMV and CMV. BBTV, BBrMV, SCMV and CMV were detected in 92%, 71%, 62% and 26%, respectively, of samples from the Bicol region. Likewise, these viruses were detected in most samples from the Eastern Visayas at frequencies of 100% for BBTV, 86% for BBrMV, 91% for SCMV and 85% for CMV. Infection occurred mostly as a combination of two to four viruses detected in samples from both symptomatic and asymptomatic plants. Diseased plants exhibited varying symptoms, and that symptoms of single and mixed infections were not distinguishable. Molecular detection by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) confirmed the presence of these viruses. BBTV was detected by PCR using DNA component specific primers, but detection was not consistent among the primers used. BBT1 and BBT2 primer pair detected the most number of BBTV positive samples. BBrMV was detected by RT-PCR using the virus specific primer pair bract 1 and bract 2. Detection of BBTV by ELISA was not always consistent with PCR. The sensitivity of BBTV detection by PCR was affected by the dilution of the template DNA.

Key Words: abaca mosaic, bract mosaic, bunchy top, serological and molecular detection

Abbreviations: ABTV – *Abaca bunchy top virus*, BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosasic virus*, ELISA – enzyme-linked immunosorbent assay, PBS-T – phosphate buffer saline-tween 20, PCR – polymerase chain reaction, RT-PCR – reverse transcription polymerase chain reaction, SCMV – *Sugarcane mosaic virus*

INTRODUCTION

The Philippines is the major producer of abaca fiber and supplies about 85% of the total abaca requirement in the world (PCARRD 2003). Abaca production is also a source of livelihood for the Filipino farmers. However, the abaca industry is faced with various constraints, and virus diseases such as abaca bunchy top, bract mosaic and abaca mosaic have been one of the major factors contributing to decreased crop productivity (FIDA fact sheet, 2013, unpublished data). Abaca bunchy top was first observed in the country in 1915 (Ocfemia 1926; Ocfemia 1930), and continues to cause severe damage and significant yield decline (Magnaye 1989; Raymundo et al. 2001, FIDA 2010, unpublished data). Abaca plants infected with bunchy top are typically stunted, and produce undersized suckers with short, narrow, stiff and upcurled leaves, and chlorotic to necrotic leaf margins (Ocfemia 1926; Ocfemia 1930; Raymundo 2000; Bajet and Magnaye 2002).

Due to the similarities of symptom and virus transmission by a common aphid vector, *Pentalonia nigronervosa,* in abaca and banana, abaca bunchy top was thought to have been caused by *Banana bunchy top virus* (BBTV), the causative virus of bunchy top disease in banana (Magee 1953). Later findings involving electron microscopy, serological and molecular virus detection, and nucleotide sequence analysis have provided evidences that BBTV causes abaca bunchy top (Bajet and Magnaye 2002; Furuya et al. 2006). The virus was also shown to be transmissible to abaca by inoculation using *P. nigronervosa* (Ocfemia 1930; Ocfemia and Buhay 1934). BBTV belongs to the Family *Nanoviridae* and the Genus *Babuvirus* with genome consisting of six circular single-stranded DNA components of about 1 to 1.1 kb (Harding et al. 1993;

Burns et al. 1995; Karan et al. 1997; ICTVdB 2006a), which is encapsidated in isometric particles of 18–20 nm in diameter (Harding et al. 1991). These components, named as DNA-R, -U3, -S, -M, -C and –N (Vetten et al. 2005; Natsuaki and Furuya 2007), contain an open reading frame encoding a putative protein except for DNA-R with two genes (Harding et al. 1993; Burns et al. 1995; Beetham et al. 1997; Beetham et al. 1999), and share two common regions, the stem-loop and the major common regions.

Later, a new and distinct virus species named *Abaca bunchy top virus* (ABTV) was found associated with an isolate (Q1108) from Albay Province, Bicol Region in the Philippines (Sharman et al. 2008). ABTV has genome characteristics similar to those of BBTV (Sharman et al. 2008). However, ABTV lacks an internal ORF in its DNA-R and DNA-U3 components which are found in some BBTV isolates. BBTV and ABTV are considered separate species with 79–81% amino acid sequence identity for the putative coat protein, and a mean of 63% overall nucleotide sequence identity across all DNA components, both of which were less than the nanovirus species demarcation values of 85% and 75% for the coat protein and nucleotide sequence identities, respectively (Vetten et al. 2005; Sharman et al. 2008).

The mosaic diseases of abaca in the country are those attributed to *Banana bract mosaic virus* (BBrMV), *Sugarcane mosaic virus* (SCMV), and *Cucumber mosaic virus* (CMV). Banana bract was first documented in 1989 and is widespread in banana in the country (Magnaye and Espino 1990; Bajet and Magnaye 2002). The diagnostic symptom appears as dark spindle to mosaic discoloration on the bract of the inflorescence. The leaves of severely infected banana have discontinuous dashes or streaks along the primary vein that extend from the midrib to the edge of the lamina (Bajet and Magnaye 2002). BBrMV is transmitted by aphid vectors such as *Aphis gossypii* and *Rhopalosiphum maydis* (Magnaye and Espino 1990) and *P. nigronervosa* (Muñez 1992). The virus can also be transmitted through vegetative propagation. BBrMV is a member of the Family *Potyviridae,* Genus *Potyvirus* with genome consisting of positive sense single-stranded RNA (ICTVdB 2006b) and virions approximately 725 nm long (Thomas et al. 1997). In 2000, BBrMV was isolated from naturally infected abaca in the Bicol region (Sharman et al. 2000). Infected abaca has symptoms similar to those of BBrMV-infected banana. The viral coat protein gene has 98% nucleotide sequence identity with that of the BBrMV banana isolate, indicating that the virus causing abaca bract mosaic is a strain of BBrMV (Ramirez and Aquino 2014).

Abaca mosaic was first reported in the country in 1934 (Calinisan 1934). Infected plants have various symptoms described as alternate green and yellow streaks, spindle-shaped patterns on leaves, and mottling on petioles and leaf sheath (Calinisan 1939; Bajet and Magnaye 2002), mosaic, chlorotic stripes and streaks (Gambley et al. 2004). Based on the similarity in particle morphology, aphid transmission and serological relationship with *Sugarcane mosaic virus (*SCMV), it was shown that a potyvirus, initially named abaca mosaic virus, causes abaca mosaic (Eloja and Tinsley 1963; Bajet and Magnaye 2002). Later, the virus was considered as a strain of SCMV having close similarity in the core protein amino acid sequence, and in the nucleotide sequence of the 3' untranslated region which fall above the threshold value of 90% and 80%, respectively (Gambley et al. 2004). The N terminal region of the coat protein has a unique amino acid repeat motif which is not present in other SCMV strains and BBrMV. It is also serologically distinct and has low nucleotide sequence similarity with BBrMV (Thomas et al. 1997; Gambley et al. 2004). The virus is transmitted by several aphid species such *A. gossypii, R. maydis* and *R. nympheae* (Ocfemia and Celino 1938; Celino 1940; Celino and Ocfemia 1941), and also by vegetative and mechanical means (Bajet and Eloja 1968, as cited by Bajet and Magnaye 2002).

The third mosaic disease is caused by *Cucumber mosaic virus* (CMV) (Furuya et al. 2006; Natsuaki and Furuya 2007). It was first reported to be affecting banana in 1950 (Castillo 1952; Bajet and Magnaye 2002), but does not usually have a major impact on banana production. Infected plants have mottling symptom that runs from the midrib to the margin of the leaf, and is characterized by the presence of light yellowish streaks running parallel to the veinlets and comes in various shapes and sizes (Castillo 1952; Bajet and Magnaye 2002). CMV belongs to the Family *Bromoviridae*, Genus *Cucumovirus* with genome consisting of positive sense single-stranded RNA which is encapsidated in spherical-shaped particles (ICTVdB 2006c). The virus is transmitted by aphid species such as *Aphis gossypii* and *Rhopalosiphum maydis* (Magnaye and Eloja 1968).

The disease identification method used in surveys and monitoring of abaca virus diseases in the country is usually based on visual assessment of symptoms (Raymundo et al. 2001). However, symptom-based diagnosis would not clearly distinguish diseases in abaca caused by different viruses particularly in mixed infections. Although the virus causing abaca bunchy top still needs to be ascertained, whether or not it is BBTV or ABTV or possibly both viruses, identification of the disease requires molecular detection as these two viruses differ only in their molecular characteristics. Identification of mosaic diseases also requires serological and molecular detection because the symptoms they induce are similar and indistinguishable. Differences in their molecular characteristics are known, and serological and molecular detection methods are available. BBTV, BBrMV, SCMV and CMV have been detected in abaca using serological or molecular methods (Gambley et al. 2004; Furuya et al. 2006). Although mixed infections of BBrMV and SCMV (Gambley et al. 2004), and BBTV, BBrMV and CMV (Furuya et al. 2006) were detected in abaca, only few samples were analyzed.

This study aimed to establish a reliable diagnosis of bunchy top and mosaic diseases of abaca in mixed infections by using serological and molecular virus detection methods. In this study, BBTV, BBrMV, SCMV

and CMV were detected by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR) in abaca leaf samples from the Bicol and Eastern Visayas regions, two of the three major abaca-growing areas in the Philippines. The results of ELISA and PCR or RT-PCR detection were compared to determine which would be the more reliable method for detection of abaca viruses. The study also determined the most efficient primers for BBTV detection by PCR and BBrMV detection by RT-PCR. Accurate diagnosis and knowledge on the occurrence of these viruses must be considered in the current virus resistance breeding and abaca rehabilitation programs in the country.

MATERIALS AND METHODS

This study was conducted at the Crop Protection Cluster, College of Agriculture, University of the Philippines Los Baños, College, Laguna, Philippines in 2012–2013. The occurrence of mixed abaca bunchy top and mosaic virus infections in two abaca-growing areas in the Philippines was determined.

Survey of Abaca Virus Diseases and Collection of Abaca Leaf Samples from Bicol and Eastern Visayas

Surveys were conducted in abaca farms in three locations in the Bicol region, namely, Tabaco, Albay; Casiguran, Sorsogon; and Ocampo, Camarines Sur, and in four locations in Leyte province, namely, Tanauan, Abuyog, Baybay and Kanangga. The plants were assessed visually and samples from 196 symptomatic and 33 asymptomatic plants were collected and analyzed by ELISA and PCR or RT-PCR.

Virus Detection by ELISA

Generally, the conditions for virus detection by ELISA followed the previously established protocol for BBTV (Su 1999; Bajet and Magnaye 2002) with some modifications. Detection of CMV followed the protocol for BBTV detection by Su (1999) while BBrMV and SCMV followed the manufacturer's (Agdia®) protocol with modifications as explained in the succeeding section. The positive and healthy negative control samples were obtained from Agdia. In addition, another set of healthy control from tissue-cultured plant and positive control from virus isolates that have been maintained in the greenhouse were also included.

BBTV was detected by indirect ELISA following the method described by Bajet and Magnaye (2002), except that commercial polyclonal antibody from Agdia® was used. The leaf sample was homogenized at 1:5 dilution in Tris-Na-DIECA buffer (0.5 M Tris buffer, pH 7.5, 0.1% sodium diethyldithiocarbamate, trihydrate, 5% sucrose, 0.5% skim milk). The use of Tris-Na-DIECA buffer for tissue homogenization was optimized by Su (1999) and adapted by Bajet and Magnaye (2002). However, some of the components such as 0.2% bovine serum, 0.2 M potassium phosphate buffer (pH 7.4) and 0.5% sodium sulfite in the Tris-Na-DIECA buffer formulated by Su were not included in the buffer described by Bajet and Magnaye (2002). Then 100 µL of the sap of each sample was loaded in each well of the ELISA microtitre plate and incubated at 4 °C overnight. After incubation, the plate was washed thrice for 5 min interval using 1X phosphate buffer saline-tween 20 (PBS-T) (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, and a pH of 7.4). Then 200 µL blocking buffer (1% skim milk in 1X PBS) was added in each well and the plate was incubated at room temperature for 1 h, and washed as described above. Then 100 µL of the diluted primary antibody (1:200) (Agdia) in 1X PBS with 0.2% skim milk was loaded in each well, incubated at 37 °C for 2–3 h, and washed. The goat anti rabbit-IgG alkaline phosphatase (GARAP) (Sigma) conjugate diluted at 1:1000 in 1X PBS-T was loaded in each well, incubated for 2–3 h at 37 °C, and then washed. The substrate p-nitrophenylphosphate (Sigma) diluted at 1 mg-ml in substrate buffer (10% diethanolamine buffer, pH 9.8) was loaded in each well and the plate was incubated at room temperature for 30–60 min to allow the color reaction to take place. The absorbance reading of each sample was taken at 405 nm using an ELISA reader. The sample was considered virus-positive if the absorbance reading was greater than two times the average absorbance value of the three healthy control samples. CMV was also detected by indirect ELISA using the commercial CMV polyclonal antibody (Agdia) in the sap extract homogenized in Tris-Na-DIECA.

BBrMV and SCMV were detected by direct ELISA using the Agdia commercial antibodies following the manufacturer's instructions except that Tris-Na-DIECA buffer was used instead of 1X Agdia general extraction buffer (1X Phosphate buffer saline-tween (PBS-T, 0.13% sodium sulfite, 2% polyvinylpyrrolidone, mw 24,000–40,000, 0.02% Sodium azide, 0.2% egg albumin and 2% tween-20). The wells of the ELISA microtitre plate were coated with 100 µL capture antibody diluted at 1:200 in carbonate coating buffer and the plate was incubated at 37 °C for 3–4 h, and washed using PBS-T as described above. Then 100 µL of the sap extracted in Tris-Na-DIECA buffer at 1:5 dilution instead of the general extraction buffer (Agdia) was loaded in each well, and the plate was incubated at 4 °C overnight, and then washed with PBS-T. In addition, the antibody enzyme conjugate diluted at 1:200 in 1X ECL buffer (Agdia) was loaded in each well and the plate was incubated at room temperature for 2 h, and then washed again. The addition of substrate and the determination of positive reaction followed the same method as in indirect ELISA described above.

Virus Detection by PCR or RT-PCR

The total nucleic acid from abaca leaf samples was extracted as described by Su (1999). BBTV was detected by PCR and BBrMV and SCMV by RT-PCR following the conditions specified in the published literatures with some modifications as described below. The positive control was obtained in sample from symptomatic plant

which had been previously tested by PCR to be virus-infected. The negative control was obtained from a tissue-cultured plant and had been confirmed to be virus-free.

Four primer pairs, namely, BBT1 and BBT2 (Thomson and Dietzgen 1995), F3 and FPCR4 (Karan et al. 1994), CR-SL (F) and CR-SL (Su et al. 2003), and J02 and G01 (Furuya et al. 2006) were used for BBTV detection. The PCR conditions followed the procedure described in the literature except that amplification was done using undiluted and 1:10-1:20 diluted template DNA. The samples that were negative using the undiluted template DNA were retested using DNA diluted at 1:10-1:20. Generally, the PCR reaction mixture was prepared in a 10 µL reaction volume consisting of 1X PCR Buffer, 1.5–4 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2–1 µM forward primer, 0.2–1 µM reverse primer, 1.0 U Taq DNA polymerase, 1 µL template and DEPC water. All amplifications were carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems). PCR amplified products were analyzed by electrophoresis, and the gel was stained with gel red (Biotum) and visualized by the Alpha Imager Mini Analysis System (Alpha Innotech).

The two-step RT-PCR system was followed using the Superscript III Reverse Transcriptase (Invitrogen). The cDNA synthesis was carried out in a 20 µL reaction volume following the manufacturer's protocol. PCR amplification was done using the cDNA, and following the conditions specified in the published literatures. The primer pair bract 1 and bract 2 (Rodoni et al. 1997; Sharman et al. 2000) and the P1 and P2 (Su 1999) were used for BBrMV detection. Bract 1 and bract 2 are BBrMV-specific primers while P1 and P2 are potyvirus-degenerate primers. The potyvirus primer pair U341 and D341 (Langeveld et al. 1991) and the virus-specific primer pair ABAMVUF1 and ABAMVUR1 (Gambley et al. 2004) were used for SCMV detection.

RESULTS AND DISCUSSION

Viruses Detected by ELISA

Abaca leaf samples of symptomless and symptomatic plants from the Bicol region were positive to BBTV, BBrMV, SCMV and CMV in ELISA using the commercial virus antibody (Table 1). BBTV was detected in 85% of the samples from Albay; 91% from Sorsogon; and 100% from Camarines Sur. In this study, the presence of ABTV was not tested because of the unavailability of the antibody specific to the ABTV. BBrMV was detected in 77%, 82% and 54% while SCMV was detected in 48%, 73% and 66% of the samples from Albay, Sorsogon and Camarines Sur, respectively. Fewer samples (6–46%) were found positive to CMV. Likewise, abaca samples from Abuyog, Baybay, Kanangga and Tanauan in Leyte were positive to BBTV, BBrMV, SCMV and CMV in ELISA (Table 1). The occurrence of these viruses in all four locations was high with 100% for BBTV, 67–94% for BBrMV, 78–100% for SCMV and 72–97% for CMV. Since the sample collection did not follow a particular sampling pattern, the values obtained indicated only the occurrence of the viruses, and did not reflect the disease incidence in the field.

Mixed Virus Infections

Infection occurred mostly as mixed infection of two to four viruses (Table 2) in symptomatic and asymptomatic plants. In the Bicol region, mixed infection of BBTV, BBrMV and SCMV was highest at 27%, then at 22% for BBTV and BBrMV. The disease also occurred as mixed infections of BBTV + SCMV (9%), BBTV + CMV (2%), BBrMV + SCMV (3%), BBTV + BBrMV + CMV (3%), BBTV + SCMV + CMV (6%), and BBTV + BBrMV + SCMV + CMV (17%). Thus, 89% of the total plants tested (124 symptomatic) from the Bicol Region had mixed infections. The occurrence of single infection was very low with a mean of 7%, 0.7% and 0.7% for BBTV, BBrMV and SCMV, respectively. Likewise, 98% of the plants tested (72 symptomatic and 33 asymptomatic) from the Visayas had mixed infections.

Table 1. Occurrence of abaca viruses in the Bicol and Eastern Visayas regions of the Philippines as detected by enzyme-linked immunosorbent assay.

Location	No. of Samples Tested	Virus-Positive Plants (%)			
		BBTV	BBrMV	SCMV	CMV
Bicol[1]					
Tabaco, Albay	54 (all symptomatic)	85	77	48	6
Casiguran, Sorsogon	44 (all symptomatic)	91	82	73	27
Ocampo, Camarines Sur	26 (all symptomatic)	100	54	66	46
Mean		92	71	62	26
Eastern Visayas[1]					
Abuyog, Leyte	18 (all asymptomatic)	100	67	78	72
Baybay, Leyte	32 (7 asymptomatic)	100	94	97	75
Kanangga, Leyte	26 (all symptomatic)	100	92	88	96
Tanauan, Leyte	29 (8 asymptomatic)	100	90	100	97
Mean		100	86	91	85

[1]Samples were collected from three sites located in different towns and provinces in the Bicol region and from four sites in different towns of Leyte province in the Eastern Visayas.
BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*

Mixed infected plants may show symptom of single infection only or a combination of symptoms due to other viruses. For instance, some plants infected with BBTV together with BBrMV, SCMV or CMV had bunchy top symptom only (Fig. 1a–1d) or in combination with mosaic disease symptoms (Fig. 1e). The BBTV symptoms ranged from mild to severe bunchy top appearance associated with slight to very narrow and stiff leaves and chlorotic to necrotic edges on the leaf margins only (Fig. 1a–1d). The other symptoms observed were hardened light green areas or stripes originating from the midrib to the leaf margins, leaf distortion and leaf rolling (Fig 2). Mosaic symptoms such as alternate light and dark green stripes (Fig. 3a), light green and dark green dashes (Fig. 3b) and spindle-shaped patterns on the leaf (Fig. 3c) which have been described in the literature were observed in this study. These plants were mostly mixed infected with three to four viruses.

Virus Infection of Asymptomatic Plants

The asymptomatic plants (Fig. 4) were also found to be infected as shown by their positive reactions in ELISA. All the samples tested were positive to BBTV while the frequency of occurrence for BBrMV, SCMV and CMV was 67%, 88% and 67%, respectively (Table 3). About 70% of the plants were mixed infected with BBTV, BBrMV, SCMV and CMV (data not shown).

Viruses Detected by PCR

Banana bunchy top virus. Using the BBT1 and BBT2 primers, the expected amplicon size of 349 bp was obtained from bunchy top-infected abaca from both Bicol and Visayas sites and positive control samples, but not from the healthy abaca and negative controls (Fig. 5). BBTV was detected in 33 of 40 Bicol samples and in 33 of 50 Visayas samples (Table 4). Our results support and extend the use and application of the BBT1 and BBT2 primers that were designed from the Australian isolate, and it can be used to amplify the DNA-1 (also referred to as DNA-R) component of BBTV isolates irrespective of its origin (Thompson and Dietzgen 1995). Nucleotide sequence analysis of BBTV DNA-1 and DNA-6 revealed that there are two groups of isolates, the South Pacific (including Australia) and the Asian group (including the Philippines) (Karan et al.1994; Karan et al. 1997). The BBTV DNA-1 is associated with BBTV isolates from both the South Pacific and the Asian group (Karan et al. 1994).

The F3 and FPCR4 primers were also designed from the DNA-R component of the BBTV South Pacific group (Karan et al. 1994), but detection using this primer gave results that are different from those of BBT1 and BBT2 (Table 4). The primer pair J02 and G01 designed to amplify the DNA-4 (DNA-M) component from the Philippine isolate (Furuya et al. 2006) amplified BBTV from the Eastern Visayas only but not from the Bicol samples (Table 4). Our results supported the findings of Furuya et al. (2006) that abaca bunchy top is associated with BBTV as detected using the primers J02 and G01. However, detection using the same primer was only 50% efficient compared with BBT1 and BBT2. The primers CR-SL (F) and CR-SL (R) designed on the stem loop common region of the viral genome also detected the virus, but in lesser number of samples (Table 4). Among the primers used in the study, the BBT1 and BBT2 primer pair was the most efficient for BBTV detection.

Abaca bunchy top virus. Detection of ABTV was not successful using the primers designed by Sharman et al. (2008), except SLCR2 and CRMB. Amplification using SLCR2 and CRMB was obtained from the positive control samples of bunchy top-infected abaca and banana, but not from any of the Bicol and the Eastern Visayas samples. PCR amplification using the published abaca primers needs further optimization, e.g. dilution of the template DNA. As shown below, the efficiency of amplification using the BBT1 and BBT2 primers increased with dilution of the template DNA. The negative result must be confirmed in future test using an internal PCR amplification control.

Banana bract mosaic virus. Amplification by RT-PCR using the BBrMV-specific primer pair bract 1 and bract 2 gave the expected amplicon size of 604 bp (Fig. 6). Likewise, using the primer pair P1 and P2, the expected amplicon size of 324 bp was obtained from samples of bract mosaic-infected abaca. The primer pair P1 and P2 is a potyvirus degenerate primer and it may detect both BBrMV and SCMV. RT-PCR amplification

Table 2. Occurrence of single and mixed virus infections in abaca as detected by enzyme-linked immunosorbent assay.

Location	No. of Samples Tested	Virus-Positive Plants (%)										
		BBTV alone	BBrMV alone	SCMV alone	BBTV + BBrMV	BBTV + SCMV	BBTV + CMV	BBrMV + SCMV	BBTV + BBrMV + SCMV	BBTV + BBrMV + CMV	BBTV + SCMV +CMV	BBTV + BBrMV + SCMV + CMV
Bicol												
Tabaco, Albay	54	4	2	0	35	7	0	4	33	2	0	4
Casiguran, Sorsogon	44	5	0	2	16	2	2	5	41	2	5	18
Ocampo, Camarines Sur	26	12	0	0	15	19	4	0	8	4	12	30
Mean		7	0.7	0.7	22	9	2	3	27	3	6	17
Visayas												
Abuyog, Leyte	18	0	0	0	0	11	0	0	17	0	22	50
Baybay, Leyte	32	3	0	0	0	3	0	0	19	0	0	75
Kanangga, Leyte	26	0	0	0	0	0	4	0	4	0	4	80
Tanauan, Leyte	29	0	0	0	0	0	0	0	4	0	10	86
Mean		0.75	0	0	0	4	1	0	11	0	9	73

BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*

Fig. 1. Mixed virus infections with bunchy top symptom only or in combination with mosaic: **a**) Severe bunchy top, severely narrowed and stiff leaves with distinct chlorotic to necrotic edges of leaf margins; **b-c**) Severe bunchy top with narrow and stiff leaves and moderate chlorotic and necrotic leaf margins; **d**) Severe bunchy top with very severe necrotic symptoms on the leaf blade; and **e**) Mixed symptom of bunchy top and mosaic consisting of hardened dark green stripes along the veins, leaf distortion and leaf rolling. **a-d**, mixed infections of BBTV, BBrMV, SCMV and CMV; **e**, mixed infections of BBTV, BBrMV and SCMV. (BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*).

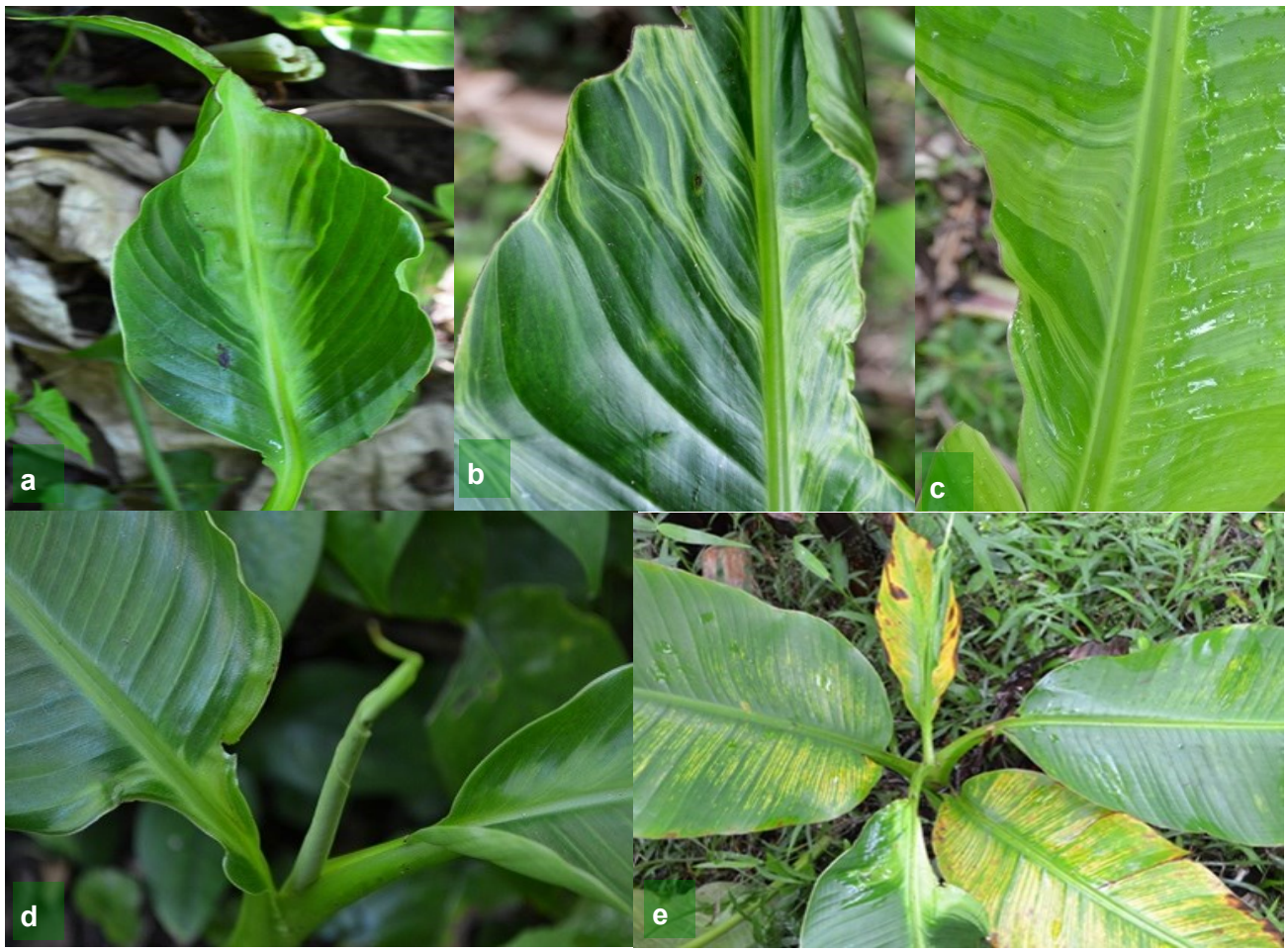

Fig. 2. Other symptoms associated with abaca virus diseases a) hardened light green areas originating from the midrib to the leaf margins; b-c) hardened light green stripes along the veins with leaf distortion and rolling; d) hardened light green stripes on the leaf and severe rolling of the emerging leaf and; e) chlorotic streaks and stripes along the veins. Figures a, b, c, d) mixed infected with BBTV, BBrMV, SCMV and CMV; and e) BBrMV and SCMV. (BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*).

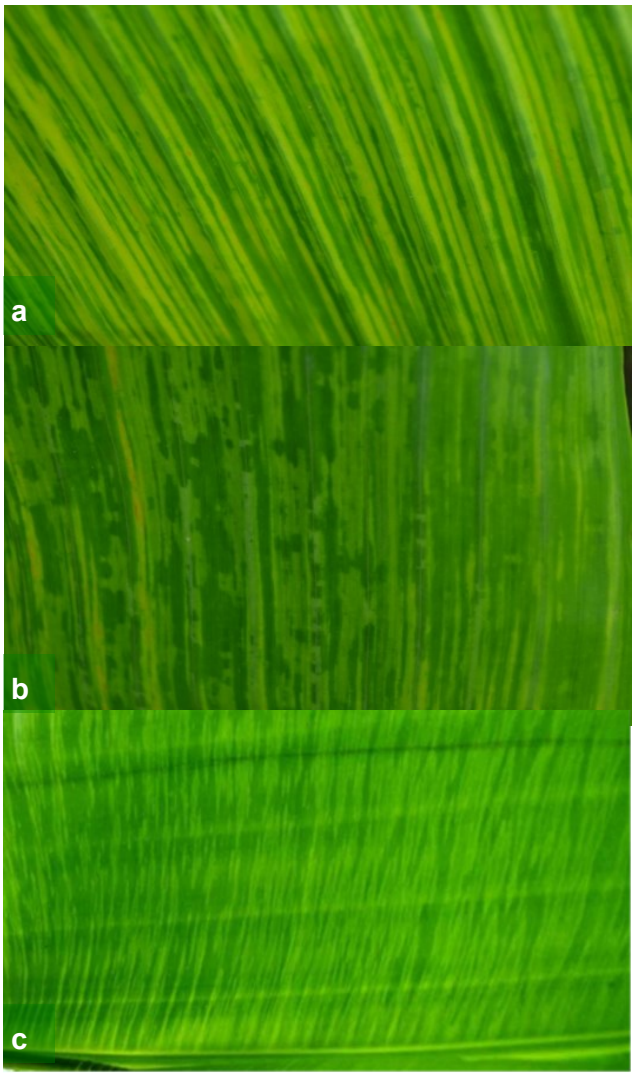

Fig. 3. Various symptoms of mosaic in mixed virus infections: **a-b**) BBTV, BBrMV, SCMV, CMV; **c**) BBTV, BBrMV, SCMV. (BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*).

Fig. 4. Asymptomatic abaca plants mixed infected with: **a**) BBTV, BBrMV and SCMV; and **b**) BBTV, BBrMV, SCMV and CMV. (BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*).

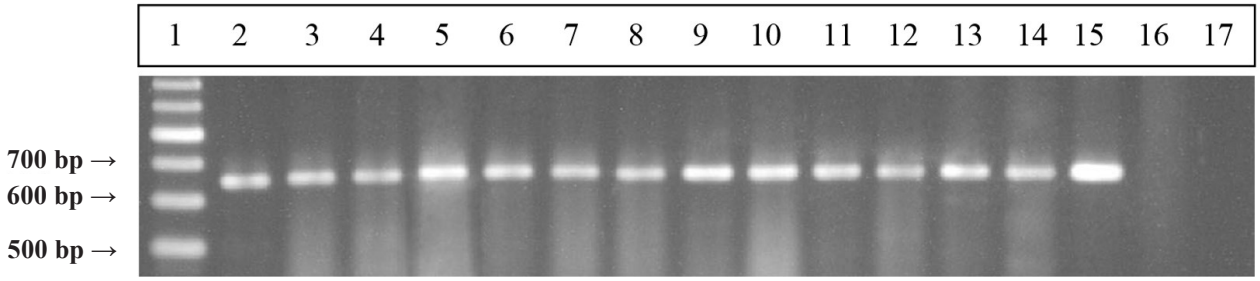

Fig. 5. Detection of *Banana bunchy top virus* by polymerase chain reaction using the primer pair BBT1 and BBT2 (amplicon size 349 bp), based on Thomson and Dietzgen (1995): Lane 1: 100 bp plus DNA ladder (Vivantis); Lane 2-13: bunchy top infected abaca samples from Bicol Region; Lane 14: bunchy top infected abaca sample as positive control; Lane 15: bunchy top infected banana as positive control; Lane 16: healthy abaca; Lane 17: PCR negative control.

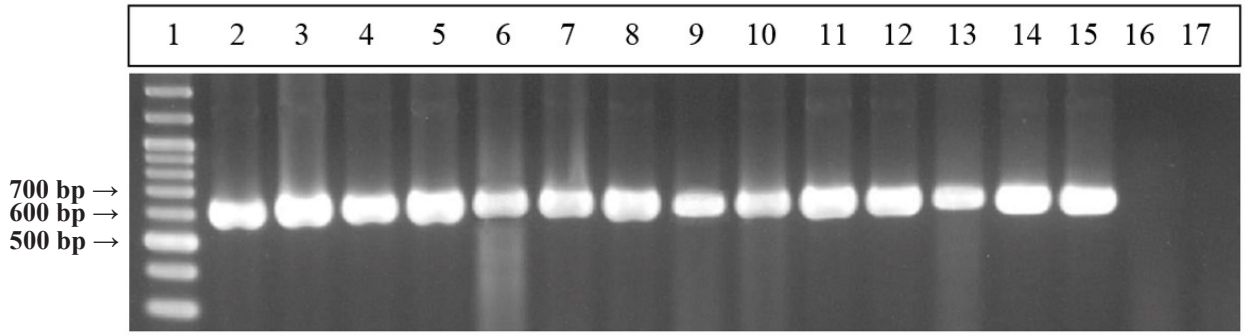

Fig. 6. Detection of *Banana bract mosaic virus* by reverse transcription-polymerase chain reaction using the primer pair bract 1 and bract 2 (amplicon size = 604 bp) based on Sharman et al., 2000: Lane 1: 100 bp plus DNA ladder (Vivantis); Lane 2-14: bract mosaic infected abaca samples from Bicol Region; Lane 15: bract mosaic infected abaca as positive control; Lane 16: healthy abaca; Lane 17: PCR negative control.

Table 3. Viruses detected by enzyme-linked immunosorbent assay in asymptomatic plants.

Place of Sample Location	No. of Samples Tested	Virus-Positive Plants (%)			
		BBTV	BBrMV	ScMV	CMV
Abuyog, Leyte	18	100	67	78	72
Baybay, Leyte	7	100	71	86	29
Tanauan, Leyte	8	100	63	100	100
Mean		100	67	88	67

BBrMV – *Banana bract mosaic virus*, BBTV – *Banana bunchy top virus*, CMV – *Cucumber mosaic virus*, SCMV – *Sugarcane mosaic virus*

Table 4. Detection of *Banana bunchy top virus* by polymerase chain reaction (PCR) using different primers.

Place of Sample Collection	No. of Samples Tested	No. of Virus-Positive Samples[1]			
		BBT1/BBT2	F3/ FPCR4	J02/G01	CR-SL (F) /CR-SL
Bicol	40	33	0	0	5
Eastern Visayas	50	33	16	14	24

[1]The number was the total of PCR positive samples using undiluted and 1:10-1:20 diluted template DNA

was also obtained using the primer pair U341 and D341, a potyvirus degenerate primer (Langeveld et al. 1991), which may detect BBrMV as well as SCMV. The same samples did not react positively using the Abaca mosaic virus (SCMV-abaca strain) specific primers, ABAMVUF1 and ABAMVUR1 designed to amplify the 3' untranslated region of the SCMV genome (Gambley et al. 2004). Among the primers used, bract 1 and bract 2 can be used to specifically detect BBrMV.

Comparison between ELISA and PCR Detection

Virus detection by ELISA and PCR were not always consistent for BBTV detection. Some BBTV-positive samples in ELISA were not detected by PCR, while few ELISA-negative samples were positive in PCR (Table 5). For BBTV, efficiency of PCR detection increased with dilution of the template DNA. For the batch 1 samples, BBTV was detected from 1:10 diluted DNA by PCR using the BBT1 and BBT2 primer pair in 2 out of 2 ELISA-positive samples with high absorbance values (1.000–1.4000), in most samples (14/16) with low absorbance values (0.395–0.999) and in samples (2/5) with absorbance (0.295–0.394) just above the threshold value (Table 5). Similar result for batch 2 samples was observed, although lesser samples were positive in PCR than ELISA. When the negative samples were retested using 1:20 diluted DNA, the result was positive amplification for some samples. For instance, one of the two PCR negative samples (batch 1 sample with low ELISA absorbance value), and three of the five (batch 2 sample with low ELISA absorbance value) that were tested became positive when the DNA template used was diluted to 1:20 (Table 5).

Detection of BBrMV by ELISA was consistent with RT-PCR depending on the primer that was used in the amplification. All the ELISA-positive samples, except those with absorbance value slightly higher than the threshold value, were also positive in RT-PCR using the BBrMV-specific primer pair bract 1 and bract 2, and the potyvirus degenerate primer P1 and P2 (Table 6). However, some BBrMV-negative samples were positive in RT-PCR using P1 and P2. Since the samples were negative using bract 1 and bract 2, the positive reaction using the P1 and P2 primers may not be due to BBrMV but to other potyvirus, possibly SCMV. Thus, only the samples that were positive using the primer pair bract 1 and bract 2 can be considered as positive to BBrMV.

Table 5. Comparison of enzyme-linked immunosorbent assay (ELISA) and polymease chain reaction (PCR) for detection of *Banana bunchy top virus* (BBTV).

Level of Absorbance Values[1]	Range of Absorbance Values	No. of Samples Tested	No. of Virus-Positive Samples		
			ELISA	PCR (BBT1 and BBT2 primers)	
				1:10 [2]	1:20[2,3]
Batch 1 samples					
High absorbance	1.000 -1.400	2	2	2	not tested
Low absorbance	0.395-0.999	16	16	14	1(2)
Absorbance slightly higher (+ 0.001- 0.1) than the threshold value of 0.294	0.295-0.394	5	5	2	1(3)
Absorbance below the threshold value of 0.294	≤0.294	2	0	1	not tested
Batch 2 samples					
High absorbance	1.000 -1.400	1	1	0	not tested
Low absorbance	0.337-0.999	12	12	7	3(5)
Absorbance slightly higher (+ 0.001- 0.1) than the threshold value of 0.236	0.237-0.336	1	1	1	not tested
Absorbance below the threshold value of 0.236	≤0.236	1	0	1	not tested

[1]Samples with high and low absorbance values, and those with absorbance slightly higher than the threshold value were positive to BBTV in ELISA; samples with absorbance lower than the threshold value were negative; threshold value computed as 2× the average absorbance of three healthy control samples.
[2]DNA template dilution
[3]Samples that were negative at 1:10 DNA dilution were retested using 1:20 diluted DNA. The number in parenthesis represents the number of negative samples at 1:10 dilution that were retested using 1:20 diluted DNA.

Table 6. Comparison of enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) for detection of *Banana bract mosaic virus* (BBrMV).

Level of Absorbance Values [1]	Range of Absorbance Values	No. of Samples Tested	No. of Virus-Positive Samples		
			ELISA	RT-PCR	
				Bract1/ Bract2	P1/P2
Batch 1 samples					
High absorbance	1.000 -3.400	5	5	5	5
Low absorbance	0.309-0.999	6	6	6	6
Absorbance slightly higher (+ 0.001- 0.1) than the threshold value (0.208)	0.209-0.308	4	4	3	3
Absorbance below the threshold value of 0.208	≤0.208	3	0	0	3
Batch 2 samples					
High absorbance	1.000 -3.400	4	4	4	4
Low absorbance	0.375-0.999	3	3	3	3
Absorbance slightly higher (+ 0.001- 0.1) than the threshold value of 0.274	0.275-0.374	3	3	3	3
Absorbance below the threshold value of 0.274	≤0.274	4	0	0	4

[1]Samples with high and low absorbance values, and those with absorbance slightly higher than the threshold value were positive to BBrMV in ELISA; samples with absorbance lower than the threshold value were negative; threshold value computed as 2× the average absorbance of three healthy control samples

CONCLUSION

ELISA and PCR showed and confirmed the occurrence of mixed infections with abaca bunchy top, bract mosaic, abaca mosaic and cucumber mosaic viruses in the areas surveyed whereby BBTV, BBrMV, SCMV and CMV were detected in abaca samples collected from three and four locations in the Bicol region and Eastern Visayas, respectively. Our results also provide proof of more reliable abaca virus disease identification with the use of more sensitive and discriminating methods such as ELISA and PCR/RT-PCR. Diseased plants showed various kinds of symptoms including those that have not been previously described such as hardened light green areas or stripes, leaf distortion and leaf rolling. Asymptomatic plants were also found to be virus-infected. The occurrence of mixed infection suggests that all four viruses must be considered in any virus disease resistance breeding program, and that serological or molecular detection must be employed for accurate and reliable disease diagnosis. This result must also be considered in the abaca rehabilitation program wherein the tissue-cultured plating materials being used for replanting must be tested and assured free of mixture of viruses.

For PCR/RT-PCR detection, degenerate and virus-specific primers were used to detect bunchy top and mosaic diseases in the Philippines. BBTV was detected from abaca samples with BBT1 and BBT2 primer pair being the most consistent. The quality of the template DNA including the presence of inhibitors may also effect the sensitivity of PCR detection. The common PCR

inhibitors are plant polysaccharides, excess salts such as KCl and NaCl, and ionic detergents such as sarkosyl, SDS, ethanol, isopropanol and phenol (Weyant et al. 1990; Demeki and Adams 1992; Katcher and Schwartz 1994; Loffert et al. 1997; Radstrom et al.2004; Bessetti 2007). In this study, dilution of the template DNA has increased the number of BBTV-positive samples in PCR. Thus, template DNA dilution of 1:10 up to 1:20 can increase the efficiency of detection. In future studies, the template dilution or the amount of DNA needs to be optimized for BBTV detection by PCR.

BBrMV in abaca can be efficiently detected by ELISA and by RT-PCR using the virus-specific primers, bract1 and bract2. Since cDNA was used in the detection of BBrMV, the amount of inhibitors had been eliminated, and that did not affect the sensitivity of RT-PCR. On the other hand, RT-PCR detection of SCMV infection in abaca requires a virus-specific primer to be able to distinguish any amplicon attributed to or from the BBrMV amplicon.

In the Philippines, reliable diagnosis of abaca virus diseases by PCR has not been well established. Our results and those by others provide proof that molecular methods are applicable or useful. However, the consistency of PCR detection particularly for BBTV or ABTV needs to be resolved. One of the factors that may affect the consistency of PCR detection is the presence of inhibitors in the DNA extract, and this can be overcome by diluting the template DNA or probably using lesser amount of template DNA. An improved method which can minimize the amount of inhibitors in the abaca DNA extract may also increase the sensitivity of BBTV detection by PCR.

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