Molecular Detection and Diagnosis of Phytoplasma Infection in Cassava (*Manihot esculenta* Crantz) in Eastern Visayas, Philippines

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ABTRACT

Phytoplasma disease is a threat to the cassava industry but little is known about the disease in the country. This study was conducted to develop and optimize a protocol for the detection of phytoplasma and determine the existence of phytoplasma strains in the different cassava growing areas in Eastern Visayas, Philippines.

PCR reaction using primer pair PA2F/R and nested primer pair NPA2F/R as well as the use of primer pair P1/P7 followed by nested primer pair R16F2n/R2 with slight modifications in the thermocycling conditions detected phytoplasma infecting cassava.

Ten out of the eleven areas in Eastern Visayas, a scale insect and a cassava grown from tissue culture tested positive for phytoplasma using nested primer pairs NPA2F/R and R16F2n/R16R2. Six band patterns were detected when NPA2F/R primer amplicons were digested with *Rsa*I and *Msp*I restriction enzymes suggesting that more than one strain of phytoplasma infect cassava.

1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important source of food, feed and starch in the Philippines. The cassava production areas and volume of production has increased since mid-2000s. In fact, Eastern Visayas is one of the top producing regions (BAS, 2013). But like any crop, it is vulnerable to pests and diseases. One particular disease that has gained economic importance is “witches broom”. Symptoms of the disease include shoot proliferation, shortening of internodes, reduced leaf size, yellowing and dieback (CIAT, 2010). This disease is reported to be caused by phytoplasma, a wall-less bacteria that is an obligate symbiont of plants and insects. It spreads throughout the plant by moving through the phloem sieve tubes and can be

transmitted by sap-feeding insects (Hogenhout et al., 2008; Lozano et al., 1976). Currently, the spread of the disease in the country is attributed to the distribution and use of diseased planting materials.

No phytoplasma detection and diagnosis protocol has been established yet on any phytoplasma disease in the Philippines owing to its novelty, ultramicroscopic size and difficulty to culture *in vitro*. Hence, this study covered the optimization of different DNA extraction methods and employed molecular-based methods of pathogen detection such as Polymerase Chain Reaction (PCR) assays using universal primers based on cloned phytoplasma DNA sequences and Restriction Fragment Length Polymorphism (RFLP) analyses to provide a simple and quick method in the differentiation of phytoplasma strains associated with cassava.

2 MATERIALS AND METHODS

2.1 Optimization of DNA Extraction Protocol

Symptomatic plants were collected from the PhilRootcrops Germplasm, Visayas State University (VSU), Baybay, Leyte. Three procedures replicated three times were compared in terms of reagents and materials used, extraction time, and DNA quantity/quality. Quantity was measured by comparing 2 µL of DNA to 1 µL λDNA. Quality was assessed by the color of extract and presence of degraded nucleic acids. Extracted DNAs were suspended in 10-40 µL Tris-EDTA (pH 8.0) with 2 µL RNAse and incubated at 37°C for 1 hr then stored at –20°C.

Procedure A follows the International Rice Research Institute (IRRI) extraction procedure. Half a gram (0.5 g) of cassava tissue was pulverized in liquid nitrogen using a mortar and pestle added with 665 µL extraction buffer (1 M Tris-HCl pH 8.0, 5 M NaCl, 0.5 M EDTA pH 8.0) then placed in a clean microtube. About 35 µL of 20%

SDS was added and then incubation at 65°C for 10 min. Another 65°C for 10 min was set after addition of 115 µL of 5 M NaCl and 90 µL 10x pre-heated CTAB. Nucleic acid was then extracted with 900 µL chloroform:isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 3 min. Precipitation with 600 µL cold isopropanol followed before storing overnight at –20°C. Tubes were centrifuged at 12000 rpm for 5 min. Pellet was washed 2x with 300 µL 70% ethanol before air-drying.

Procedure B follows the procedure from the Philippine Rice Research Institute (PhilRice). Pulverized tissues (0.5 g) added with 750 µL 2x pre-warmed CTAB and 35 µL 20% SDS was incubated at 65°C for 30 min to 1 hr. Nucleic acids were extracted with 750 µL chloroform:isoamyl (24:1) alcohol and centrifuged at 10000 rpm for 30 min. The aqueous phase was added with 600 µL cold isopropanol and stored at -20°C overnight. Samples were further centrifuged at 10000 rpm for 3 min and pellet washed with 300 µL 70% ethanol.

Procedure C follows the method of Montano et al. (2000) based on the phytoplasma DNA extraction method developed by Ahrens and Seemüller (1992) with few modifications. The 0.5 g tissues were soaked in 1.75 mL Phytoplasma Grinding Buffer (100 mM K_2HPO_4, 31 mM KH_2PO_4, 10% sucrose, 2% polyvinylpyrrolidone-10 (PVP-10), 10mM EDTA pH 8.0) at –4°C for 10 min and pulverised. The aqueous phase was centrifuged at 13000 rpm for 30 min. Pellet was dissolved in 750 µL warm 2% CTAB and incubated at 60°C for 30 min. Samples were purified with 900 µL chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 5 min then precipitated with 600 µL isopropanol before incubating at –20°C overnight. Samples were again centrifuged at 13000 rpm for 30 min and washed with 70% ethanol, air-dried and re-suspended in 100 µL TE. Two extractions of 100 µL phenol:chloroform:isoamyl alcohol (25:24:1) and 100 µL chloroform followed. About

300 µL of cold absolute ethanol was added to the aqueous layer to precipitate the nucleic acids. DNAs were centrifuged at 13000 rpm for 30 min, washed with cold 300 µL 70% ethanol and air-dried.

2.2 PCR Optimization and Amplification

Five universal primers pairs including R16mF2/R16mR1 (Gundersen and Lee, 1996) and P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) were used to initially amplify 16S rDNA and 16S-23S rDNA sequences, respectively. Initial PCR products were then subjected to nested PCR using R16F2n/R16R2 (Lee et al., 1995; 1993). Another universal primer pair PA2F/PA2R (Heinrich et al. 2001) was used in initial PCR of the 16S-23S rDNA sequences followed by NPA2F/NPA2R (Heinrich et al., 2001) for nested assays.

For the initial PCR using R16mF2/R1, the procedure by Gundersen and Lee (1996) was carried out in a volume of 25 µL: 2 µL DNA template, 1 µL dNTP mix (10 mM, Vivantis, Oceanside CA, USA), 1.5 µL of each primer pair (0.5 µM, Vivantis), 2.5 µL PCR buffer A (10X, Vivantis), 0.75 µL $MgCl_2$ (50 mM, Vivantis), 0.7 µL Taq polymerase (5u/µL, Vivantis), and sterile deionized water (diH_2O). The following thermocycling parameters was used: initial denaturation at 1 min for 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and primer extension at 72°C for 3 min, with a final extension of 7 min at 72°C. This same protocol was also used for P1/P7. Another protocol for P1/P7 based on Sharmila et al. (2004) used a 20 µL reaction mixture containing 1µl of DNA template, 1 µL of each primer pair, 0.8 µL of dNTP mix, 2 µL PCR buffer A, 0.2 µL $MgCl_2$, 0.1 µL of Taq DNA polymerase, and sterile diH_2O. The thermocycling parameters used were: initial denaturation at 95°C for 2 min followed by 35 cycles of 94°C for 30 sec, 53°C for 60

sec, 72°C for 90 sec, and a final extension of 72°C for 10 min. Using PA2F/R, a modified protocol of Heinrich et al. (2001) was used with initial denaturation set at 94°C for 2 min then 35 cycles of 94°C for 30 sec, 60°C for 75 sec and 72°C for 90 sec, and final extension of 72°C for 10 min. PCR mix with 20 µL volume contain: 3 µL DNA template, 0.8 µL dNTP mix, 1 µL of each primer pair, 2 µL PCR buffer (5X, Green GoTaq Reaction Buffer, Promega, Madison, WI, USA), 0.4 µL $MgCl_2$, 0.2 µL Taq polymerase (5u/µL), and sterile diH_2O.

In nested assays, products were diluted to 1:10 (R16mF2/R1), 1:20 (P1/P7), and 1:40 (PA2F/2R). R16F2n/R2 assays used the same reaction mixture and thermocycling parameters of R16mF2/R1 except annealing temperature was at 55°C. NPA2F/NPA2R also used the same reaction mix of PA2F/R except DNA template was reduced to 1 uL. The same thermocycling conditions was also used except annealing and extension times were reduced to 30 and 45 sec, respectively. Without prior report of phytoplasma detection in the Philippines, optimization required changes in the reaction mixture components and thermocycling conditions.

Water was used as negative control and PCR products were analyzed by electrophoresis through 1% agarose gel (Vivantis) stained with GelRed™ (Biotium, Hayward, CA, USA). DNA fragment size standards/markers used 1 Kb Kapa Universal Ladder (KapaBiosystems, Massachusetts, USA).

2.3 Detection of Phytoplasma from Cassava Samples in Eastern Visayas

Cassava samples were collected from eleven (11) areas including – 5 localities in the province of Leyte (VSU-Baybay, Bato, Capoocan, Leyte and Kananga); 1 in Biliran (Cabucgayan); 2 in Southern Leyte (Maasin City and Bontoc); and 3 in Samar (Calbiga, San Jorge and Mondragon). Another four (4) samples were extracted

including cassava grown from tissue culture from PhilRootcrops; a scale insect, *Pseudaulascaspis pentagona* Targioni-Tozzetti, from a field in Capoocan, Leyte; and two (2) weed species, *Ageratum conyzoides* L. from Maasin City and *Centella asiatica* (L.) Urb. from Capoocan, showing yellowing. Collected samples were extracted using the best extraction method and stored at -20°C.

Products from nested PCR were analyzed by single restriction endonuclease digestion reaction with *Msp*I, and *Rsa*I, (New England Biolabs, Beverly, MA, USA) following the manufacturer's instructions. The digestion products were electrophoresed in a 1% agarose gel.

3 RESULTS

3.1 Total DNA Extraction

Of the three methods used in DNA extraction, Procedure C required more time to process and chemicals compared to A and B which makes it time consuming and expensive. But Procedure C is able to provide a clearer DNA solution while procedures A and B produced off white to brown extracts. Gel electrophoresis (Fig. 1) also shows smears indicating that some of the DNAs had been degraded and plenty of RNA and non-DNA materials were evident at the bottom of the gel especially for procedure A and B.

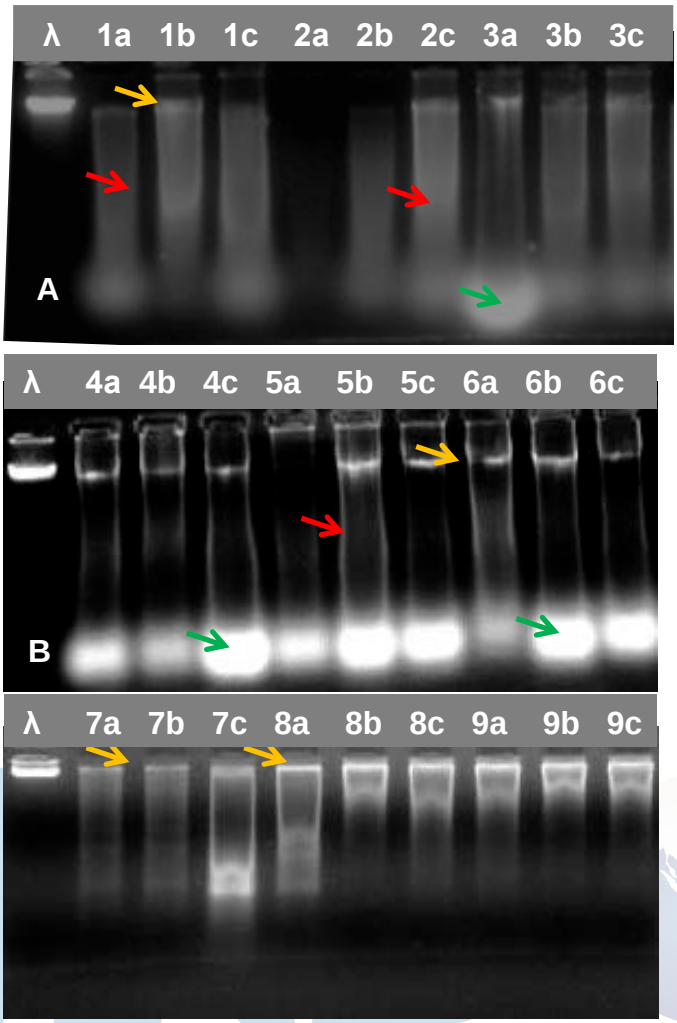

Fig. 1 DNA check of samples extracted using Procedure A (1-3), B (4-6) and C (7-9). 2 µL of DNA samples were run by electrophoresis through 0.5% agarose gel using 90V. λDNA (100ng/µL) served as check (leftmost lane). Red arrow shows smears of degraded DNA while green arrow shows RNA and non-DNA materials. Yellow arrows points to non-degraded total DNA. Small letters indicate replicates.

3.2 PCR Optimization for Phytoplasma Detection

3.2.1 *R16mF2/R16mR1 and R16F2n/R16R2*

R16mF2/R1 was first used in PCR optimization using cassava samples extracted with procedure A. Initial PCR amplification yielded weak bands (~1.4 Kb) similar to the expected band size in 2 samples. After nested PCR, a 1.2 Kb band was expected but

only one sample showed amplification of weak bands between 500 bp and 1.5 Kb. These results prompted to make changes in the nest reaction mixture; first, the amount of *Taq* polymerase was reduced from 0.7 to 0.5 µL, second, the amount of DNA was reduced from 2 µL to 1 µL, and third, both *Taq* polymerase and DNA template were reduced to 0.5 µL and 1 µL, respectively. These changes yielded very faint bands (near 1 Kb). Increasing the dilution from 1:10 to 1:20, 1:30, and 1:40, however, did not yield any amplification while using 1 µL and 2 µL undiluted initial PCR products resulted in the amplification of several bands of different molecular weights.

DNA extracted using Procedure B was also used in PCR assays, however, there was no amplification of bands thus Procedure B was not used further. On the other hand, DNA extracts from Procedure C, amplified bands of different molecular weights from all samples.

3.2.2 P1/P7 and R16F2n/R16R2

Nested PCR products using protocols of Gundersen and Lee (1996) and Sharmila et al. (2004) showed amplification of several bands in different molecular weights. A series of nested PCR assays that increased and decreased the annealing temperatures to 58°C and 48°C, respectively, yielded the same results.

Alterations made in the Sharmila et al. (2004) protocol included changing the PCR buffer to 4 µL Gotaq buffer (a dyed buffer containing $MgCl_2$) that eliminates the use of loading dyes which were observed to obscure bands during UV viewing. The nested PCR protocol, on the other hand, underwent several changes including 3 µL Gotaq buffer, 5 min final elongation time, and 1:40 dilution of the initial product. This revised protocol amplified a band around 1200 bp from samples extracted using Procedure A. On the other hand, a similar band was amplified in a sample extracted

with Procedure C using an undiluted initial product, 3.50 µL Gotaq buffer, 1 µL of dNTP, and a final elongation time of 8 min. Other samples amplified a similar band size using 4 µL Gotaq buffer and 1µL dNTP. These amplifications, however, included other weak bands of different sizes.

3.2.3 PA2F/PA2R and NPA2F/NPA2R

Initial PCR assays showed no amplification of the expected band, ~1187 bp, while a couple of samples extracted using Procedure C produced very weak bands near the expected band size, ~ 485 bp, as well as other different band sizes after nested PCR. Upon changing the PCR reaction mix around these ranges: 3 – 3.5 µL Gotaq buffer, 0.5 – 1 µL of each primer pair, 0.5 – 1 µL of dNTP mix, and 0.1 – 0.2 µL *Taq* polymerase, gel electrophoresis of nested PCR products revealed amplification of a single band that is approximately 600 bp.

3.3 Optimum Protocol for Phytoplasma Detection

Based on optimization tests, two (2) PCR protocols were used in detecting pytoplasma in cassava extracted with either Procedure A or C. Using PA2F/2R, a 20 µL mix comprised of 1 µL of each primer (0.5 µM), 0.8 µL dNTP mix (10 mM), 2 µL Gotaq buffer, 0.4 $MgCl_2$ (50 mM), 0.2 µL *Taq* polymerase (5u/µL), 3 µL of DNA template and sterile diH_2O. This is followed by 20 µL volume nested PCR with 0.5 - 1 µL of each primer (0.5 µM), 0.5 - 1 µL dNTP mix (10 mM), 3 – 3.5 µL Gotaq buffer, 0.1 - 0.2 µL *Taq* polymerase (5u/µL), 1 µL (1:40) of initial PCR product, and sterile diH_2O. Thermocycling parameters for initial PCR started with 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 60°C for 75 sec and 72°C for 90 sec, with final extension of 72°C for 10 min. Nested conditions are the same, except annealing and

extension times were at 30 and 45 sec, respectively.

Another protocol used was for P1/P7 followed R16F2n/R2. Initial PCR was carried out in a volume of 20 µL: 1 µL of each primer (0.5 µM), 0.8 µL dNTP mix (10 mM), 4 µL Gotaq buffer, 0.1 µL *Taq* polymerase (5u/µL), 1 µL of DNA template, and sterile diH_2O. Nested PCR was also carried out in a 20 µL volume containing 1 µL of each primer (0.5 µM), 1 µL dNTP mix (10 mM), 3.5 - 4 µL Gotaq buffer, 0.1 µL *Taq* polymerase (5u/µL), 1 µL of initial PCR product and sterile diH_2O. Dilution of the initial product ranges from undiluted to 1:40 depending on titer and quality. Ideal thermocycling parameters follows an initial denaturation at 95°C for 2 min followed by 35 cycles 94°C for 30 sec, 53°C (55°C for R16F2n/R2) for 60 sec, 72°C for 90 sec, and a final extension step of 72°C for 10 min (8-10 min for R16F2n/R2).

3.4 Phytoplasma Detection in Cassava in Eastern Visayas

Using PA2F/R and NPA2F/R, samples from San Jorge (S2) and Mondragon (S3) Samar, VSU (L2S1, L2S3), Bato (L3S1-S3), Leyte (L4S1-3), Capoocan (L5S1-2), Maasin (SL1S2), Bontoc (SL2S3) and Cabucgayan (Bi1S2-3) including the tissue-cultured cassava (TC1) and *P. pentagona* (L5I) produced the ~600 bp band while Calbiga (S1) produced a ~800 bp band (Fig. 2). Additional PCR assays using primer pairs P1/P7 and R16F2n/R16R2 amplified bands (~1200 bp) in samples from Calbiga, San Jorge, Mondragon, VSU (L2S1), Bato (L3S1-3), Leyte (L4S1-2), Capoocan (L5S2), Maasin (SL1S2), Bontoc (SL2S3), Cabucgayan (Bi1S2), tissue-cultured cassava (TC1), and *P. pentagona* (L5I) (Fig. 3).

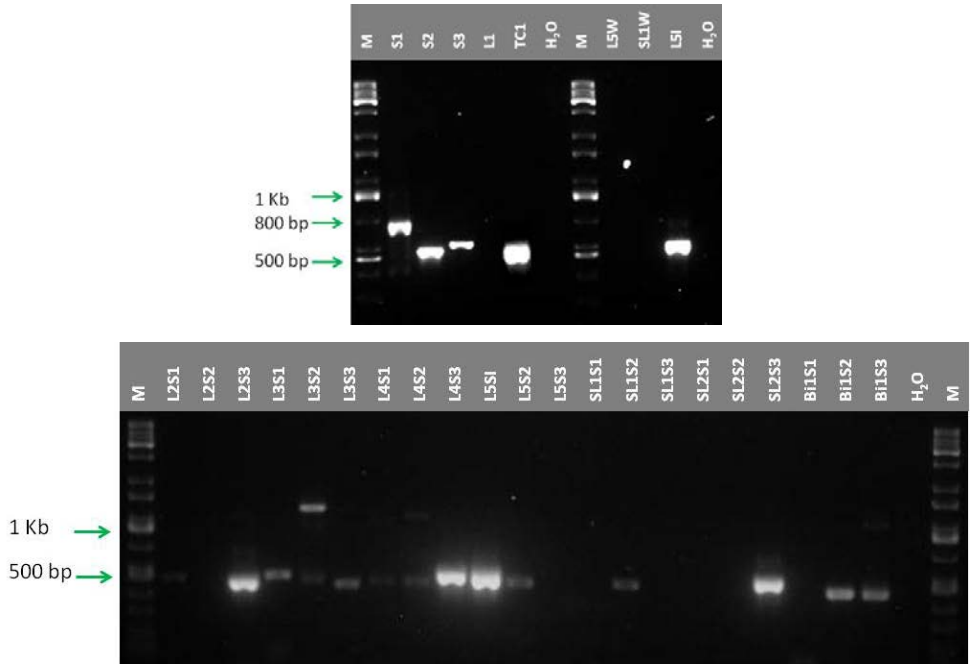

Fig. 2 Gel electrophoresis showing nested PCR detection of phytoplasma in cassava using initial primer pair PA2F/R followed by nested primer pair NPA2F/R (M = DNA marker and H_2O = negative control)

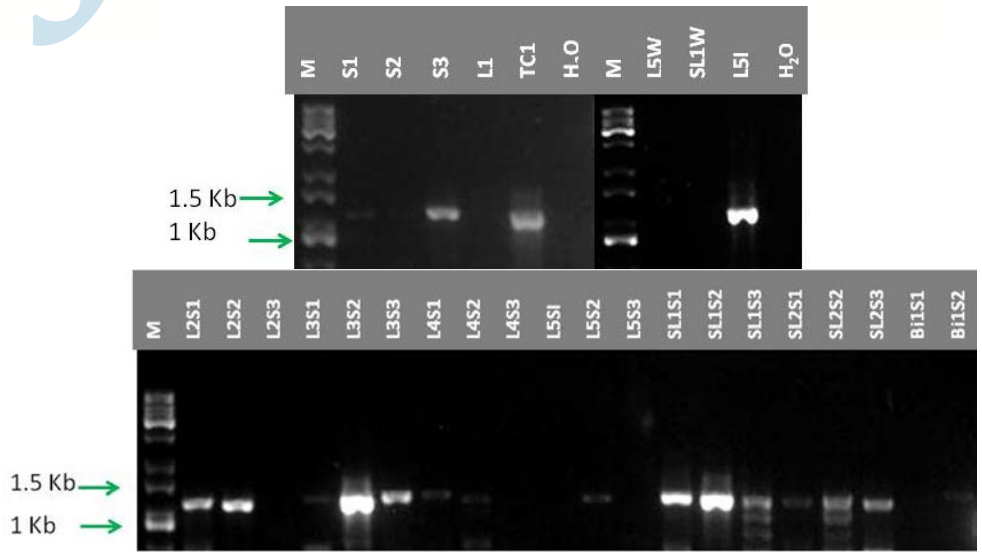

Fig. 3 Gel electrophoresis showing nested PCR detection of phytoplasma in cassava using initial primer pair P1/P7 followed by nested primer pair R16F2n/R16R2 (M = DNA marker and H_2O = negative control)

3.5 Detection of Phytoplasma Strains Using RFLP Analyses

The amplified products of nested primer pair NPA2F/R showed six (6) restriction band patterns when digested with *Rsa*I and *Msp*I (Fig. 4). Amplicons from San Jorge (S2), Bontoc (SL2S3), tissue-cultured cassava (TC1) and *P. pentagona* (L5I) showed a similar restriction pattern while the other five samples exhibited different digestion patterns.

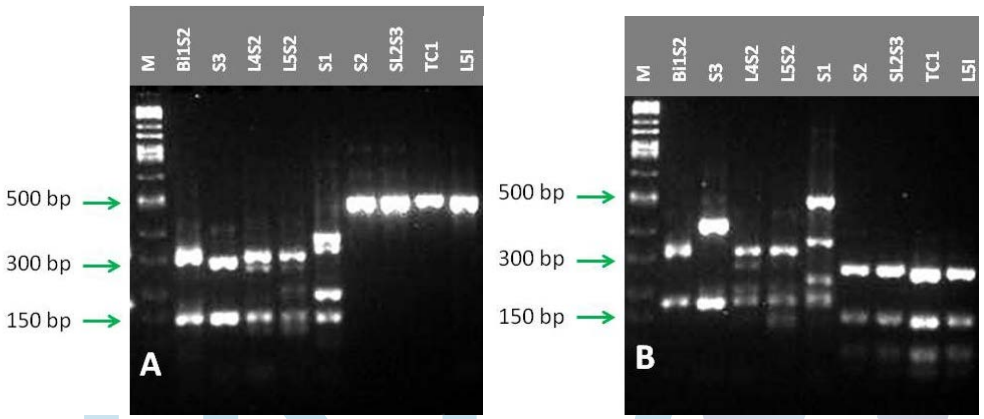

Fig. 4 *Rsa*I (a) and *Msp*I (b) restriction patterns of amplified DNA using nested primer pair NPA2F/R (M = DNA marker)

4 DISCUSSION

DNA extraction Procedure C used more chemicals and performed more steps making it expensive and requiring a longer time to carry out, however, a clean DNA is a necessity in the detection of phytoplasma through PCR. This is highlighted by Daire (1994) who reported the importance of eliminating cell debris and reaction inhibitors such as tannins, enzymes, and phenolic compounds that prevents specific targeting of the desired DNA fragment that may lead to non-amplification. Although non-amplification can be caused by very low amounts of extracted DNA. It has been reported that phytoplasma titer in diseased plants varies depending on the stage of the

disease development and time of sampling (Berges et al., 2000; Seemüller et al., 1998). Phytoplasma is also low in woody hosts (Daire, 1994; Kartte and Seemüller, 1991; Lederer and Seemüller, 1991). Likewise, collected samples with symptoms similar to phytoplasmal infection may be affected by other biotic and abiotic factors such as virus. Viral symptoms have similarities with phytoplama and even present in phytoplasma-affected plants (Calvert et al., 2004; Chaparro-Martínez and Trujillo-Pinto, 2001).

Other than non-amplification, bands of different molecular weights were also produced regardless of the DNA extraction method used. This indicates unspecific binding of the primers which agrees with some papers reporting amplifications of bands in different sizes together with the expected band during assays (Green et al., 1999; Siddique et al., 2001). Thus these sets of primers may not be the best primers to use for phytoplasma detection in cassava. These non-specific amplifications could indicate homology of the primers to plant or other microorganism sequences (Heinrich et al., 2001; Nejat and Vadamalai, 2010). But in the case of PA2F/R and nested primer pair NPA2F/R, strong and clear bands were detected albeit quite a longer band size than the expected. The slightly longer band size may be due to the primers being initially developed from the European Stone Fruit Yellows (EFSY) for phytoplasma detection in woody species like fruit trees in a different geographical location (Heinrich et al., 2001; Zirak et al., 2009). Nonetheless, they were able to detect 19 phytoplasma strains from different phylogenetic groups. Moreover, sequence analysis showed no significant homology between primers PA2F/R and NPA2F/R to any published plant sequences, instead the primers showed homology to 16 phytoplasma sequences (Heinrich et al., 2001). The results instead suggest of a different phytoplasma strain infecting cassava.

A positive amplification of phytoplasma DNA from a tissue-cultured cassava also shows that tissue culture alone may not be effective in eliminating phytoplasma

from infected plants. Although tissue culture techniques have produced sanitized shoots, the end result, it seems, would depend on the explants and additional treatments used (Chalak et. al, 2013). Moreover, the amplification of phytoplasma DNA in *P. pentagona*, a sedentary sap-sucking insect presents a challenge in the lookout for a vector. Although it has been established that phytoplasma is spread mainly by insect vectors (Sugio, 2011; Hogenhout, et al. 2008; Lee et al., 2000), however, they are primarily very mobile phloem-sucking insects such as leafhoppers.

The restriction enzyme assays showing different digestion patterns of the DNA also suggests of the possibility of different phytoplasma strains affecting cassava. Several literatures have already reported that a single crop can be infected with different phytoplasma groups (Del Serrone et al., 1998; Lee et al., 1995; 1994) or different strains of a phytoplasma (Paltrinieri et al., 2010; Seemüller et al., 2010). This can be confirmed further through RFLP analyses using other restriction enzymes and/or sequence analyses.

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6 ACKNOWLEDGEMENT

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