PROTOCOL FOR COST EFFECTIVE DETECTION OF CASSAVA MOSAIC VIRUS

[1]Okereke, N., [2]Tomlinson, J., [2]Adams, I., [2]Laurenson, L., [2]Fairless, N., [2]Boonham, N., [1]Okunade, A.F., [1]Onyeka, J and [2]Smith, J

[1]National Root Crops Research Institute, Umudike, PMB 7006, Umuahia, Abia State, Nigeria
[2] Fera Science Ltd, Sand Hutton, York YO41 1LZ, United Kingdom

Abstract
Early detection of cassava mosaic disease (CMD) is an extremely important step in containing the spread of the disease in Africa. Many nucleic acid based detection tools have been developed for CMD diagnosis but although these methods are specific and sensitive for their target DNA, they are not fast, cost effective, can't be used in poorly resourced laboratories and are not portable enough to be used in field settings. This study makes use of crude, but fast method of DNA extraction, alkaline polyethylene glycol, and an equally fast Loop mediated Isothermal amplification method of detection that doesn't require purified DNA. As a diagnostic method for CMD detection, these protocols take approximately 1 hour to conclude, compared to a normal polymerase chain reaction protocol that can take up to 24 hours to conclude.

Keywords: Polyethylene glycol method, Loop mediated Isothermal Amplification, and Polymerase Chain Reaction

Introduction

Implementation of disease diagnostic methods that are rapid, specific and sensitive for detecting disease causative agents is an imperative for any seed system with the aim of eradicating or containing the spread of diseases, especially those that cause devastating effects on the production of economically viable crops (Legg et al., 2015). Cassava research has revealed that one of the most significant reasons for yield losses by farmers is the cassava mosaic disease (CMD), which is caused by cassava mosaic begomoviruses and spread through infected planting material and to some extent by the whitefly *Bemiscia tabaci* (Legg and Fauquet, 2004).

There are several diagnostic tools ranging from symptom based to nucleic acid based (Abarshi et al., 2012) for CMD detection but studies have shown that nucleic acid or molecular based detection, especially polymerase chain reaction (PCR) methods are the most reliable. PCR is known for its high specificity and sensitivity for a target DNA, but its major draw backs are complexity of use, difficulty in interpreting results and that they are immensely time consuming. PCR in general require the extraction of high quality DNA, and plants produce secondary metabolites (e.g. polyphenols) that inhibit the extraction of quality DNA (Chabi Sika et al., 2015). Many extraction protocols are lengthy and make use of highly hazardous chemicals such as mercaptoethanol and chloroform like in the case of the common cetyltrimethyl ammonium bromide also known as the CTAB method. Asides from the lengthy extraction method, PCRs make use of a thermocycler that is complex, very expensive and bulky to handle making it difficult to use in poorly equipped laboratories or in field settings (Vu et al., 2016b).

In comparison, isothermal methods like the loop mediated isothermal amplification (LAMP) do not require the use of the thermocycler, and for diagnostics, do not require the use of high quality DNA which is an added advantage (Villari et al., 2013). Using two sets of primers at a constant temperature and relying on the strand displacing activity of *Bst* polymerase, LAMP is a rapid amplification method that produces large quantities of looped amplicons which allow the further binding of additional sets of primers (Kazutaka, 2002). With regards to cost effectiveness, specificity, sensitivity, rapidity, and simplicity of use the LAMP method is considered superior to the PCR method (Vu et al., 2016b). LAMP amplicons (amplification products) can be easily

observed by the turbidity formed due to the presence of magnesium pyrophosphate causing a precipitation reaction. LAMP assays have been successfully used in plant diagnostics as well as other organisms (Tomlinson et al., 2010) (Bekele et al., 2011) (Vu et al., 2016a). The aim of this study was to develop a quick and cost effective method for extraction of genomic DNA from dry cassava leaves and testing of these samples for cassava mosaic disease with an equally quick and cost effective isothermal based method that allows for use in field settings.

Materials and Methods

Plant materials

Three months old cassava (*Manihot esculenta* Crantz) field samples, that appeared healthy and CMD infected, were randomly collected from two experimental fields in Abia state and another two fields in Benue state (Table 2). All four fields were managed by the National Root Crops Research Institute. Samples where dried at room temperature and sent to Fera science, United Kingdom for further analysis.

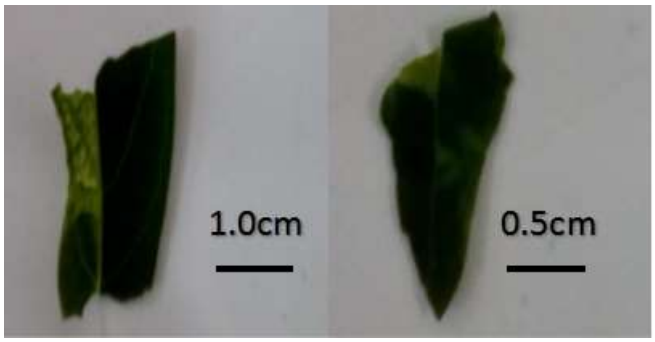

Figure 1: Air-dried cassava leaves, measured for DNA crude extraction used in this study

Simple genomic DNA isolation

The samples were stored at -80^{o}C for 24 hours to aid the homogenisation of the samples during extraction. Genomic DNA was eluted using a modified alkaline polyethylene glycol (PEG; 60 % PEG 200 (Sigma), 20 mM KOH) DNA extraction method. The pH of the buffer was between 13 and 14. Samples were measured (figure 1) and placed in plastic 5ml tubes containing a round steel ball and PEG buffer. After shaking for 2 minutes, the samples were further diluted in nuclease free water to neutralize the PEG buffer before analysis (Tomlinson and Boonham, 2015, Chomczynski and Rymaszewski, 2004) .

LAMP assay and tests

The LAMP primer sets used in this study were based on published sequences (I.e. were retrieved from the National Centre for Bioinformatics Information) targeting the DNA-A segment of the *African cassava mosaic virus*, one of the nine CMD causative begamoviruses. As seen in Table 1 below, degeneracies were included in the primers to cover possible combinations of the coat proteins that might occur. All tests were carried out with 1µl of template DNA in a 25 µl reaction, on a Genie II instrument (OptiGene), with an amplification step at a single 65°C temperature for 30mins. The Genie is a potable, battery operated machine that is suitable for both laboratory tests and tests in field settings (figure 4). Negative controls contained no template DNA and a COX test using previously designed COX primers (Tomlinson et al., 2010) was also carried out to determine how effective the extraction worked. COX or cytochrome oxidase gene is a plant gene that indicates

Table 1: ACMV - LAMP primers used in this study

LAMP Primers	Primer Sequence
ACMV_F3	GCTCGTAATTATGTCGAAGCG
ACMV_B3	TAGGTATGTCTGGGCTTCTGTA
ACMV_FIP_dg	CTGTATGGGCTGTCGAAGTTCAGACCAGGAGATATCAT CATTTMCA
ACMV_BIP_dg	GAACCKTGCTACTGCCCCCACTTTCTGTACATGGGYCT GTT
ACMV_FL_dg	ACGAACCTTGGAKYCTGG
ACMV_BL	TCCACGTCACAAATCGAAAACG

ACMV: African cassava mosaic virus, B & F Backward & Forward; FIP & BIP - Forward & Backward Inner Primer; FL & BL - Forward & Backward Loops and dg indicates presence of degeneracies

Results and Discussion

Optimization of extraction for cassava leaves

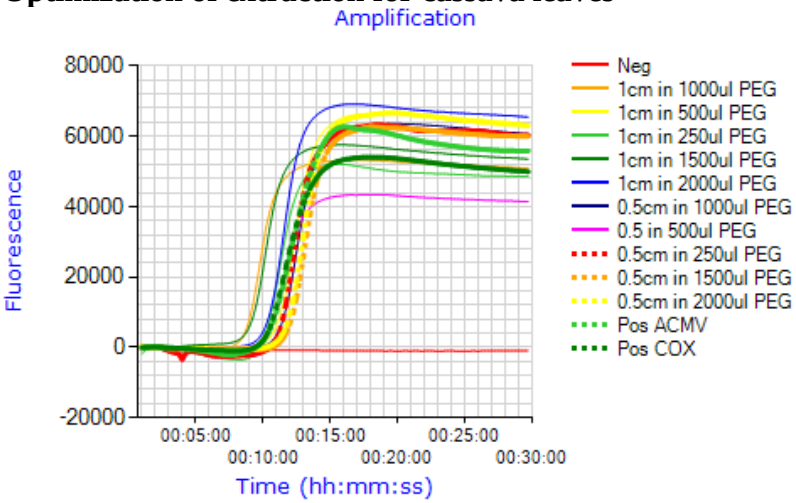

Figure 2: Typical real time plot graph depicting the detection of ACMV after PEG extraction. Pos ACMV and Pos COX are positive controls while Neg contained no template DNA.

This is the first reported use of the alkaline PEG buffer for cassava DNA extraction. 0.5 and 1 cm dried cassava leaf samples where measured with a ruler, into five volumes of the reagent; 250, 500, 1000, 1500 and 2000 µl. Each sample was diluted to 10^{-1} before analysis and as seen in figure 2 below, ACMV was detected in all cases.

This indicates how sensitive LAMP assay is for crude DNA extracts, as other studies have also revealed for different other tissues. The time taken for detection of the virus ranged from 9:45 - 13 minutes, all under the 30 minutes cycle. It is important to note that if a test exceeded 30 minutes the results is considered negative. The annealing temperatures ranged from 86 - 88.7 ℃ (data not shown) in cassava disease diagnostics, LAMP has been used successfully to detect *cassava brown streak virus*, the cassava brown streak disease causative agent and the phytoplasm that cases cassava witches broom disease, which possess credible threat to cassava production in South America. In a yet to be published paper LAMP has also been used to detect ACMV in cassava plant

materials sampled from Nigeria. We also determined the limit of detection of the virus by using 10 fold serial dilutions from 1cm of dried cassava in 500µl of PEG buffer. In figure 3 below we can see that limit of detection was at dilution factor of 10-5. At the dilution factor of 10-6 we can see that there is a negative result.

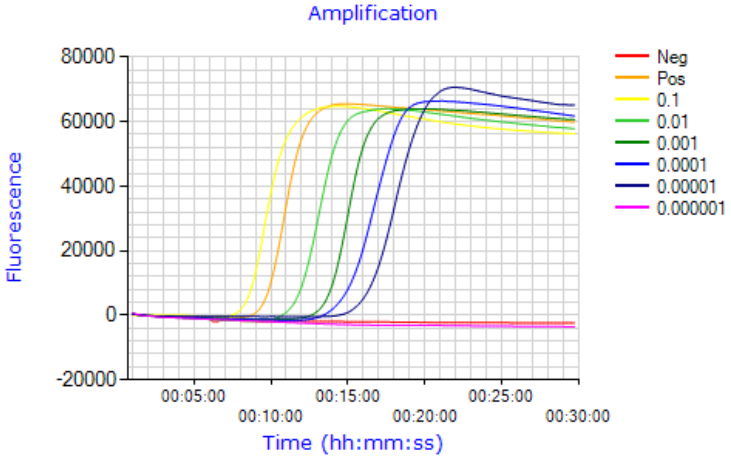

Figure 3: Plot graph showing the limit of detection of ACMV from DNA elucidated from alkaline PEG buffer extraction

Using 24 visually CMD infected and 24 CMD visually healthy cassava leaves, the LAMP assay and DNA extraction method was further tested to determine the efficiency of the protocol as seen in table 3. Each sample was subjected to vigorous shaking, subjecting the plant cell to a mechanical force in the presence of extraction buffer (PEG reagent) and further diluted in nuclease free water. The eluted DNA was tested with LAMP COX assay and as observed in all locations, the samples tested positive. A normal CTAB extraction method would take up to two hours, with multiple steps, while this method takes approximately 3 minutes.

Table 2: Comparison of detection of ACMV visually and by LAMP primers. The COX test indicates the efficiency of the extraction protocol

	Location											
	Western Farm (Umudike)			Eastern farm (Umudike)			Boki			Otobi		
Sample	visual	cox	ACMV LAMP	visual	cox	ACMV LAMP	visual	cox	ACMV LAMP	visual	cox	ACMV LAMP
1	-	+	-	-	+	-	-	+	-	-	+	-
2	-	+	-	-	+	-	-	+	-	-	+	-
3	-	+	-	-	+	-	-	+	-	-	+	-
4	-	+	-	-	+	-	-	+	-	-	+	-
5	-	+	-	-	+	-	-	+	-	-	+	-
6	-	+	-	-	+	-	-	+	-	-	+	-
7	+	+	-	+	+	+	+	+	-	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+

The results of the LAMP assay were in agreement with the visual diagnostic method, with the exception of samples 7 from the eastern farm and Boki. Both samples tested negative even though the samples appeared infected by CMD with the presence of chlorosis. The negative result might mean the extraction of the viral DNA did not occur or the viral titter may not he high enough to be detected by the LAMP assay. Positive results by both COX and ACMV assays indicate the high sensitivity and specificity for their target templates. This corroborates previous studies that LAMP does not require high quality purified DNA, and is able to detect in the presence of secondary metabolites(YaSeeN et al., 2015) .

Figure 4: The Genie II instrument for real-time LAMP. This instrument can be portable and run from a battery. The method is ideally suit for non-research laboratories that need to fulfil a limited number of diagnostic tests on a regular basis

Conclusion

The alkaline PEG buffer used in this study was originally developed by Chomczynski and Rymaszewski 2006 to enable a wide variety of biological samples to be used directly in PCR without further purification steps. The LAMP method was also originally developed by Notomi *et.al,* 2000. This is the first reported use of the reagent in DNA extraction for cassava. In summary, we've established a rapid and cost effective method for both genomic DNA extraction from dry cassava leaves and a sensitive test for cassava mosaic disease. The advantage of this method allows the analysis of large samples faster than the PCR method. It is important to note that for this method to be replicated in other crops, this protocol may need modifications to accommodate the differences in secondary metabolites present in various plant species.

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