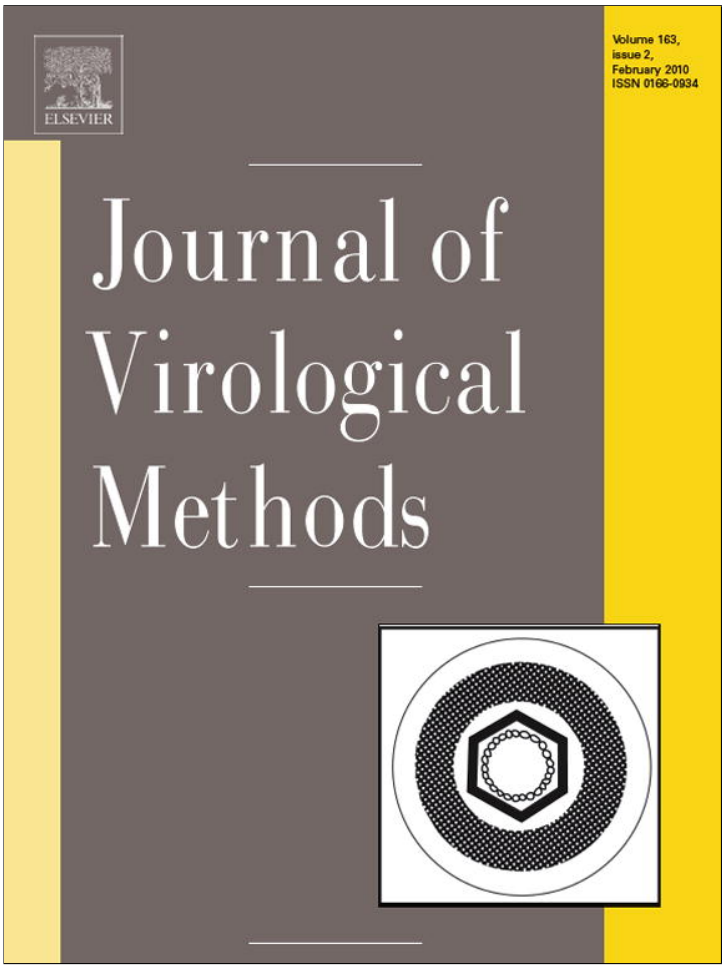

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Optimization of diagnostic RT-PCR protocols and sampling procedures for the reliable and cost-effective detection of *Cassava brown streak virus*

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Sampling procedures and diagnostic protocols were optimized for accurate diagnosis of *Cassava brown streak virus* (CBSV) (genus *Ipomovirus*, family *Potyviridae*). A cetyl trimethyl ammonium bromide (CTAB) method was optimized for sample preparation from infected cassava plants and compared with the RNeasy plant mini kit (Qiagen) for sensitivity, reproducibility and costs. CBSV was detectable readily in total RNAs extracted using either method. The major difference between the two methods was in the cost of consumables, with the CTAB 10× cheaper (£0.53 = US$0.80 per sample) than the RNeasy method (£5.91 = US$8.86 per sample). A two-step RT-PCR (£1.34 = US$2.01 per sample), although less sensitive, was at least 3-times cheaper than a one-step RT-PCR (£4.48 = US$6.72). The two RT-PCR tests revealed consistently the presence of CBSV both in symptomatic and asymptomatic leaves and indicated that asymptomatic leaves can be used reliably for virus diagnosis. Depending on the accuracy required, sampling 100–400 plants per field is an appropriate recommendation for CBSD diagnosis, giving a 99.9% probability of detecting a disease incidence of 6.7–1.7%, respectively. CBSV was detected at 10^{-4}-fold dilutions in composite sampling, indicating that the most efficient way to index many samples for CBSV will be to screen pooled samples. The diagnostic protocols described below are reliable and the most cost-effective methods available currently for detecting CBSV.

1. Introduction

Cassava (*Manihot esculenta* Crantz, family *Euphorbiaceae*), Africa's second most important food crop after maize, provides more than half of dietary calories for over half of both the rural and urban populations in sub-Saharan Africa. Africa produces more cassava than the rest of the world combined; production exceeds 104 million tonnes annually (FAO, 2009). Cassava is particularly popular among the poor for the ease of cultivation, low input requirement, tolerance to low rainfall and poor soils, and ease of propagation through stem cuttings. However, cassava cultivation in sub-Saharan Africa is affected severely by two important viral diseases: cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Thresh et al., 1994; Hillocks and Jennings, 2003; Thresh and Cooter, 2005; Legg et al., 2006).

CMD is distributed throughout the cassava-growing area of sub-Saharan Africa, whereas CBSD was confined until recently to coastal and lake shore areas of Malawi in eastern and southern Africa and at altitudes below 1000 metres above sea level (masl) (Storey, 1936; Nichols, 1950; Hillocks et al., 1999). CBSD is more damaging economically than CMD in the coastal zones from Kenya to the Zambezi River in Mozambique where both diseases occur (Hillocks, 1997; Hillocks et al., 2001, 2002), because in sensitive varieties CBSD causes dry necrotic rotting of tubers which, when severe, makes them unfit for consumption (Storey, 1936; Nichols, 1950; Hillocks et al., 1996). Recently, CBSD was reported at mid-altitude levels (above 1000 masl) in DR Congo (Mahungu et al., 2003), Uganda (Alicai et al., 2007), western Kenya and the Lake zone areas of Tanzania (Legg and Jeremiah, 2008). While the precise reasons for CBSD emergence are yet to be established, the disease has been shown to be highly damaging with 10–100% incidence that can result in up to 70% decrease in root weight of infected plants compared to healthy plants (Hillocks et al., 2001).

CBSD foliar symptoms vary greatly but are characterised mainly by leaf chlorosis in feathery patterns, appearing first along the margins of veins and later developing into chlorotic blotches (Storey, 1936; Nichols, 1950). However, CBSD symptoms are often masked in the field due to plants also being affected by cassava green mite (*Mononychellus tanajoa*), sooty mould (growing on the honeydew excreted by whiteflies) and CMD. Symptoms also vary with the variety, crop age and environmental conditions (Hillocks et al., 1999) and the tendency of cassava to shed older mature symptomatic

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leaves especially during prolonged dry periods further add to the complexity of disease identification.

Serological and/or molecular techniques have been developed in order to provide more reliable diagnosis without having to rely on variable disease symptoms. An antiserum was raised to purified CBSV from cassava, which detected readily the virus in *Nicotiana benthamiana* but failed to detect asymptomatic infections in cassava (Lennon et al., 1985; Sweetmore, 1994). More recently, a CBSV coat protein gene has been expressed and the resulting protein used for antisera production to develop a more reliable enzyme-linked immune-sorbent assay (ELISA) (Winter, 2009). The sensitivity and reliability of this antisera have yet to be reported. A sensitive reverse transcriptase polymerase chain reaction (RT-PCR) protocol was developed by Monger et al. (2001a) and confirmed the association of *Cassava brown streak virus* (CBSV), of the genus *Ipomovirus*, family *Potyviridae* with CBSD (Monger et al., 2001b). An isolate of CBSV (MLB3 from Tanzania) is now fully sequenced (Mbanzibwa et al., 2009a) and based on the comparison of CP gene sequences, two main CBSV strains have been identified (Mbanzibwa et al., 2009b). CBSV has also been shown to be transmitted from infected to healthy cassava plants by whiteflies (*Bemisia tabaci* Gennadius) (Maruthi et al., 2005).

Although a sensitive RT-PCR technique is available for CBSV diagnosis (Monger et al., 2001a), the reports described sample preparation methods only briefly. Parameters such as the selection of plant tissue for virus detection, especially in the absence of CBSD symptoms, and the association of stem and root symptoms with virus infection were not investigated. Commercial kits used commonly for sample preparation and RT-PCR are expensive and alternative cheaper methods are required to reduce the cost of testing cassava, a particularly important consideration for research laboratories in Africa. The main goal of this study was therefore, to optimize cost-effective diagnostic protocols and sampling procedures for the reliable detection of CBSV in cassava plants.

2. Materials and methods

2.1. Virus isolate, detection and characterisation

Cassava plants of an unknown variety infected with CBSV were collected by R.J. Hillocks in farmers' fields in Nampula, Mozambique in 2007 and maintained subsequently in the quarantine facilities of the Natural Resources Institute (NRI), UK. The virus was grafted onto variety Ebwanateraka, which was found to be highly susceptible to CBSV infections. Presence of CBSV was confirmed by observing symptom expression on leaves and by RT-PCR using CBSV10 (5′-ATCAGAATAGTGTGACTGCTG-3′) and CBSV11 (5′-CCACATTATTATCGTCACCAGG-3′) primers (Monger et al., 2001a). Preliminary characterisation of the virus isolate was done by amplifying partial coat protein gene (CP) sequence using the primers CBSV9 (5′-ATGCTGGGGTACAGACAAG-3′) and CBSV11 (Monger et al., 2001a). Amplicons were cloned into the pGEM-T Easy vector (Promega UK Ltd., Southampton, UK), and sequenced at the Geneservice Ltd., Cambridge, UK.

2.2. Sampling of plant tissue

In order to identify appropriate leaf tissue for reliable and consistent detection of CBSV, and to confirm the association of CBSV with root necrosis and streaks/lesions on stems, samples were taken from leaves, stems, tuberous roots and also secondary and tertiary roots of at least five infected Ebwanateraka plants.

Plants were divided into three sections for the purpose of collecting leaf samples: top, middle and bottom. Top leaves consisted of samples from leaf 1 (youngest fully open), 2, 3 and 4, and these leaves were always asymptomatic; middle leaves consisted of samples from leaves 9, 10 and 11 on which symptoms were developing; and bottom leaves consisted of samples from the bottom-most leaves which always exhibited prominent CBSD symptoms (Fig. 1). Twenty samples were tested for each section of the plant.

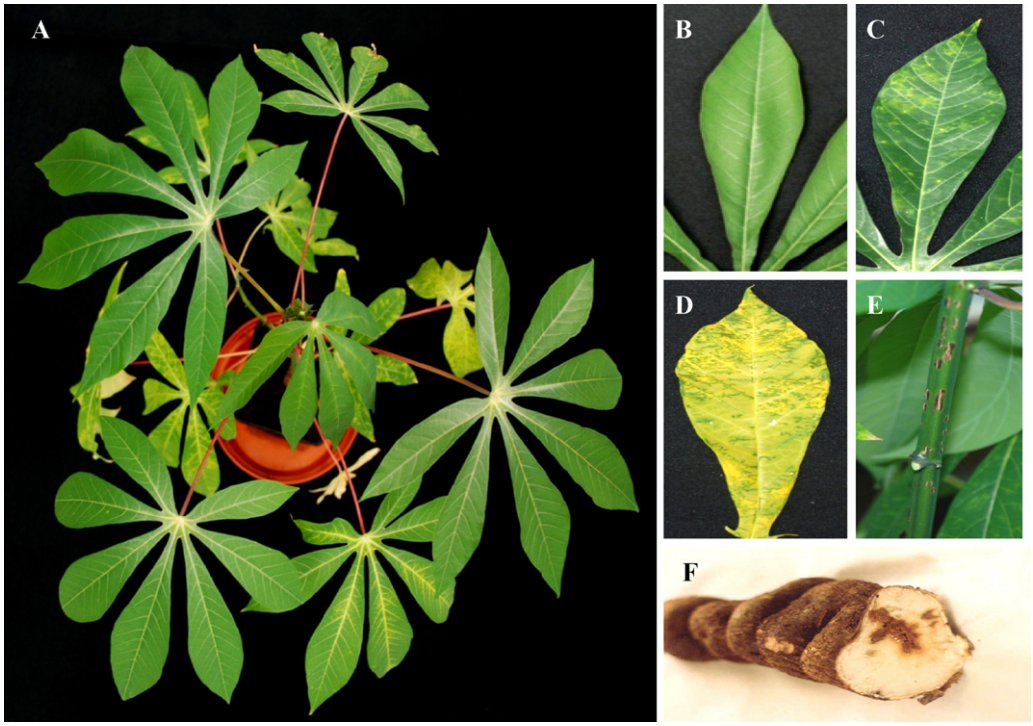

Fig. 1. Cassava plant, variety Ebwanateraka, about four months old, showing typical CBSD symptoms (A), asymptomatic leaves at the top (B), symptoms still developing on middle leaves (C), fully developed symptoms on the bottom leaves (D), necrotic lesions on stems (E), and dry necrosis of tuberous roots (F). Symptoms of root constriction that are occasionally associated with CBSV can also be seen in panel (F). Samples were taken from leaves, stems and roots for the purpose of detecting CBSV and the virus was found in all parts of an infected plant.

Samples from tuberous roots were collected from infected plants from the greenhouse by cutting a cross-section of each root. Samples were collected from three areas based on severity of root rot symptoms: brown necrotic tissue, at the intersection between necrotic and symptom-free tissue and adjoining symptom-free tissue. Samples were also collected from secondary and tertiary non-tuberous roots. Ten to fifteen samples were tested for each category of root and stem tissues.

2.3. Sample preparation

Total nucleic acids were extracted separately from leaves, stems and root tissues collected from cassava plants showing typical CBSD leaf symptoms, using a modified CTAB procedure (Lodhi et al., 1994; Maruthi et al., 2002). About 100 mg of leaf tissue was ground thoroughly in a thick-gauged plastic bag using a hand-held ball bearing sample grinder (Bioreba AG, Reinach, Switzerland) and mixed using a wallpaper seam roller in 10 volumes (1 ml) of CTAB buffer (2% CTAB (w/v), 1.4 M NaCl, 0.2% 2-mercaptoethanol (v/v), 20 mM EDTA, 100 mM Tris–HCl, pH 8.0). About 750 μl of each sample transferred to a 1.5 ml eppendorf tube, mixed and incubated at 60 °C for 10 min. The extract was mixed with an equal volume (750 μl) of phenol:chloroform:isoamyl alcohol (25:24:1), mixed thoroughly and centrifuged at >12,000 × *g* for 10 min. The supernatant was transferred to a new 1.5 ml eppendorf tube and nucleic acids were precipitated by adding 0.6 volumes (300 μl) of ice cold (−20 °C) isopropanol. Samples were then incubated at −20 °C for at least 1 h and centrifuged finally at 12,000 × *g* for 10 min at 4 °C. The pellet was washed in 0.5 ml 70% ethanol, centrifuged for 5 min and vacuum-dried for 5 min in a spin vac. The pellet was dissolved in 1x TE buffer and stored at −20 °C for further analysis. The protocol for extractions of nucleic acids from root and stem samples was similar except that a second phenol:chloroform:isoamyl alcohol extraction was included.

The efficiency and purity of RNA extraction using the CTAB method was compared with RNeasy plant mini kit (Qiagen Ltd., Crawley, UK) following manufacturers' instructions except that the initial grinding of samples was done mechanically without the use of liquid nitrogen. Fifteen leaflets showing typical CBSD symptoms were collected and each was divided into two equal parts for RNA extraction using each of above methods.

2.4. One-step vs two-step RT-PCR

The efficiencies of one-step RT-PCR (Qiagen Ltd., Crawley, UK) vs two-step RT-PCR protocols for CBSV detection were compared by a series of dilution end point experiments up to 10^{-8}. Cassava leaves showing typical CBSD symptoms were collected and total nucleic acids were extracted by the modified CTAB method. Both one-step and two-step RT-PCR were performed on samples from infected plants. In one-step RT-PCR, 2 μl of the sample was used for virus genome amplification, and 20 samples were tested. The RT-PCR cycling conditions included 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and ending with a final extension step of 72 °C for 10 min.

For the two-step RT-PCR, cDNA was synthesized using the OligodT primer and ImProm-II™ Reverse Transcriptase kit (Promega UK Ltd., Southampton, UK), following the manufacturer's guidelines. Subsequent PCR was carried out using Red hot polymerase kit (Thermo Fisher Scientific Ltd., Loughborough, UK) following manufacturer's instructions. The PCR cycling conditions included an initial incubation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and ending with a final extension step of 72 °C for 10 min. For cDNA synthesis, 5 μl of the sample was used in a total of 20 μl reaction, 2 μl of which was used in subsequent PCR. Over 100 samples were tested in two-step RT-PCR as part of various experiments. The choice of kits was largely influenced by price, history and ease of use in our laboratory.

2.5. Composite sampling and determination of virus dilution end points

In order to test the suitability of diagnostic protocols for composite sampling, total nucleic acids were extracted separately from 10 leaves each of CBSV-infected and virus-free cassava cv. Ebwanateraka plants using the modified CTAB method. Virus-infected and virus-free samples were pooled separately to compensate for any within sample variations arising during nucleic acid extractions. Virus-infected samples were then serially diluted with virus-free samples from 10^{-1}-fold to 10^{-5}-fold and tested for CBSV.

Similarly, dilution end points of CBSV from total nucleic acid extractions of infected cassava plants were determined in serial dilutions of up to 10^{-8}. A two-step RT-PCR protocol was followed for virus detection. Samples that failed to produce any PCR products were tested for the presence of cassava host DNA by the amplification of the house keeping gene, the large subunit of ribulose biphosphate carboxylase oxygenase gene (RubiscoL). Primers (RBCL-F535: 5′-CTTTCCAAGGCCCGCCTCA and RBCL-R705: 5′-CATCATCTTTGGTAAAATCAAGTCCA) specific to RubiscoL were used, which amplify DNA fragment of 171 bp (Nassuth et al., 2000; Alabi et al., 2008).

2.6. Estimating the probability of detecting CBSV in field samples

Collection and testing of samples from many plants in the field is both time-consuming and expensive, and so it is pertinent to determine how many plants need to be sampled for reliable virus detection. When using a diagnostic test with high sensitivity, the probability of detecting CBSV depends on two factors: the virus incidence (proportion of infected plants in the sample) and the number of plants sampled. Following an approach used in a different context (Gu and Novak, 2004) the Binomial theorem states that the probability (P) of there being at least one infected plant in the sample is given by

$$P = 1 - (1 - r)^n \quad (1)$$

where r is the virus incidence and n is the number of plants sampled. Eq. (1) is based on the assumption that virus infection is randomly distributed in the sampled area. The incidence of virus is usually unknown, so it is useful to rephrase the problem in terms of the probability of detecting different levels of incidence for a given sample size. By rearranging Eq. (1),

$$n = \frac{\log(1 - P)}{\log(1 - r)} \quad \text{and} \quad r = 1 - (1 - P)^{1/n} \quad (2)$$

One important practical question concerns the risk of releasing infected material, even after a highly efficient diagnostic test has been carried out on a known number of samples per field. This was estimated according to the Bayes Theorem where the joint probability of being infected and undetected equals the conditional probability remaining undetected given infected, which was multiplied by the prior probability of being infected.

2.7. Estimation of consumables costs associated with virus detection

Consumables costs associated at each step of the protocol used were estimated. An estimation of costs for protocols that involve commercial kits was straightforward as the bulk of the cost was for the kit. Protocols involving several reagents and buffers such as CTAB or gel electrophoresis required some assumptions and estimating the amount of each reagent used per sample. For example,

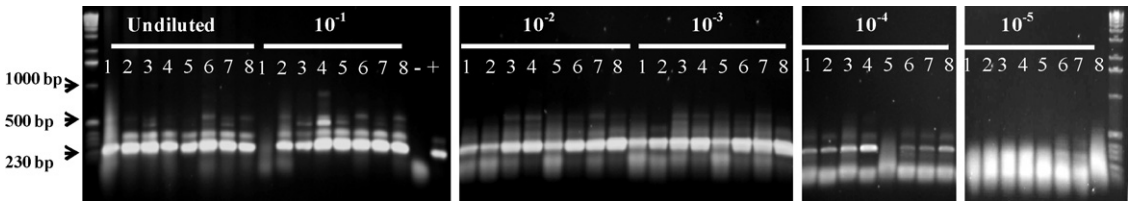

Fig. 2. Detection of CBSV in both symptomatic and asymptomatic leaves of infected cassava plants at various dilutions. Lanes 1, 2, 3 and 4 represent leaf numbers 1 (fully open), 2, 3 and 4, respectively, from top of the plant which are always asymptomatic. Lanes 5 and 6 represent leaf numbers 9 and 10, which are middle leaves and always had developing symptoms (chlorosis). Lanes 7 and 8 represent two bottom-most leaves which always had fully developed CBSD symptoms (see Fig. 1). The size ladder at each border of the gels is the 1 kb molecular weight marker (GibcoBRL, Germany), and '−' denotes a no-RNA water control, and '+' denotes a known CBSV RNA control.

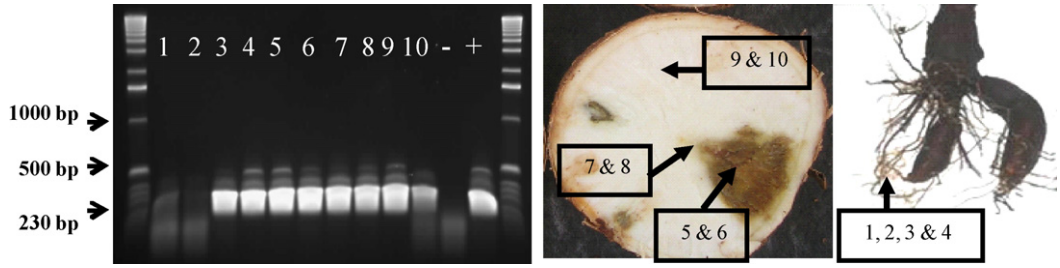

Fig. 3. Detection of CBSV in both symptomatic and asymptomatic root tissues of infected cassava plants. Areas of sample collection from roots and their corresponding lane numbers on the gel picture are shown. Lanes 1 and 2 represent newly developing secondary/tertiary roots which appear whitish cream in colour, lanes 3 and 4 represent old secondary/tertiary roots which appear brown in colour, lanes 5 and 6 represent brown necrotic tissue from tuberous roots, lanes 7 and 8 represent interface between necrotic and symptom-free tissue, and lanes 9 and 10 represent completely symptom-free tissue. The size ladder at each border of the gels is the 1 kb molecular weight marker (GibcoBRL, Germany), and '−' denotes a no-RNA water control, and '+' denotes a known CBSV RNA control.

it was assumed that about 15 ml of TBE buffer was required to analyse each sample. For ease of calculation, items that cost less than £0.001 per sample were rounded up to £0.001. All the plastic ware (various sizes of pipette tips and microfuge tubes) used in the study were from Fisher Scientific Ltd., Loughborough, UK. Labour, equipment and infrastructure costs are not included because they vary greatly from place to place.

3. Results

3.1. Virus characterisation and detection in cassava leaf, stem and root tissues

The partial CP gene of CBSV isolate Nampula consisted of 914 bases, and the sequence of which has been deposited in the EMBL nucleotide database under the accession number FN423417. BLAST analysis of CP gene sequences indicated that the Nampula CBSV shared 93% nucleotide identity to each of five CBSV isolates (accessions each with over 500 nucleotide sequences in the database): Type A (accession number AY008442), Type C (AY008440), KBH1 (FJ821795), KBH2 (FJ821794), and an isolate with accession number AY007597, all of which were from coastal Tanzania.

CBSV was detected in all the leaf samples obtained from top, middle and bottom portions of infected plants including in asymptomatic leaves (Fig. 2). However, there appear to be differences in the efficiency of PCR amplification from different leaves, albeit minor. Samples from leaf positions 3 and 4 consistently produced the brightest bands (Fig. 2).

CBSV was detected both in symptomatic and non-symptomatic tuberous root tissues derived from the same tuber, but only in 100-fold diluted samples. Virus was not detected in undiluted samples possibly because of the high concentrations of polysacharides in tuberous roots and woody tissues. CBSV was also detected in secondary and tertiary roots (non-tuberous) which appeared brown in colour, but not in relatively young roots which appeared white/cream in colour (Fig. 3).

CBSD also produces brown necrotic streaks on stems of sensitive cassava varieties such as Kiroba and Ebwanateraka. Of the 15 stem lesion samples tested, 10 from Kiroba were all positive but the five from Ebwanateraka were all negative.

3.2. Comparison of nucleic acid extraction methods

Sample preparations from infected cassava leaves were compared using the modified CTAB and RNeasy methods for the quality and amounts of total RNA extracted by serial dilutions of samples. CBSV was detected equally in all 20 samples extracted by each method in sample dilutions of up to 1.5×10^{-3}. However,

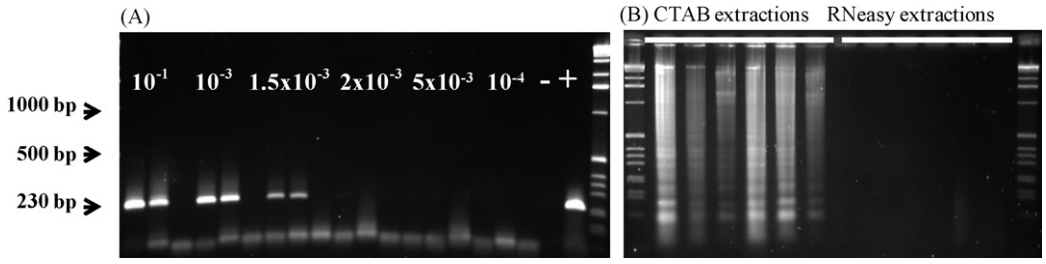

Fig. 4. Detection of CBSV by two-step RT-PCR in samples prepared from the RNeasy plant mini kit (Qiagen) (A). Higher quantities of total nucleic acids obtained by the CTAB method compared to the RNeasy method when 5 μl of the samples were run on a 1% (w/v) agarose gel (B). The size ladder at each border of the gels is the 1 kb molecular weight marker (GibcoBRL, Germany), and '−' denotes a no-RNA water control, and '+' denotes a known CBSV RNA control.

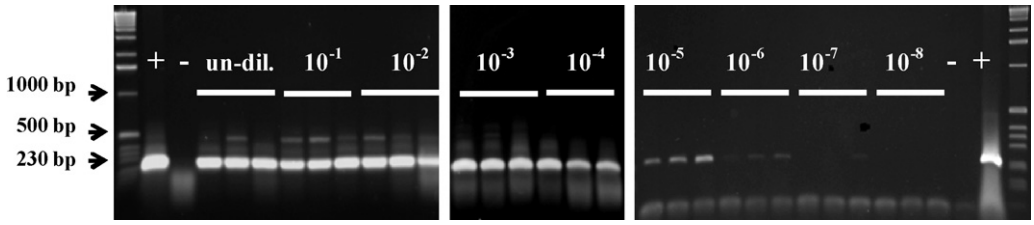

Fig. 5. Detection of CBSV by one-step RT-PCR in CTAB-extracted samples. CBSV was detectable up to 10^{-6} sample dilutions. The size ladder at each border of the gels is the 1 kb molecular weight marker (GibcoBRL, Germany), and '–' denotes a no-RNA water control, and '+' denotes a known CBSV RNA control.

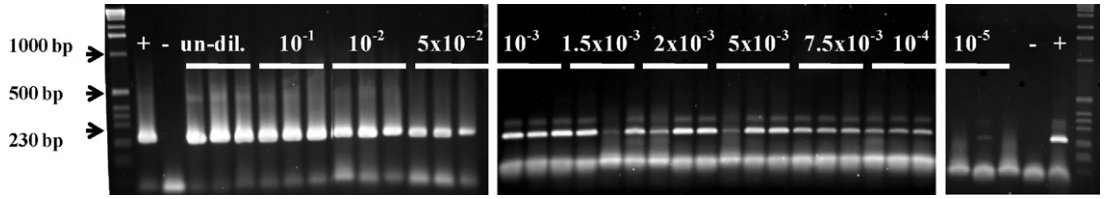

Fig. 6. Detection of CBSV in composite samples by two-step RT-PCR. Proportion of infected: uninfected samples mixed for CBSV detection are shown. CBSV was detectable reliably even when infected samples were diluted 10^{-4}-fold with uninfected samples. The size ladder at each border of the gels is the 1 kb molecular weight marker (GibcoBRL, Germany), and '–' denotes a no-RNA water control, and '+' denotes a known CBSV RNA control.

differences between the two extraction methods were observed at dilutions of 2×10^{-3} where CBSV was still detectable from all the 20 CTAB-extracted samples, but none from the RNeasy method (Fig. 4a). Quantities of total nucleic acids obtained by the CTAB method were also shown to be much higher than the quantities of RNA extracted from the RNeasy method (Fig. 4b).

3.3. Comparison of one-step vs two-step RT-PCR for CBSV detection

CBSV was detected in samples up to 10^{-6} dilutions by one-step RT-PCR (Fig. 5) and only up to 10^{-4} dilutions in two-step RT-PCR (Fig. 2). Samples at 10^{-7} dilutions produced faint bands by one-step RT-PCR, but these were not reproducible, and no visible bands were produced at 10^{-8} dilutions by either method. However, at this dilution expected PCR product size of 171 bp was amplified from RubiscoL (data not shown).

3.4. Composite sampling and virus dilution end points

CBSV was detected in two-step RT-PCR tests in all dilutions of virus-infected samples with those of virus-free plants, except at 1:100,000 (equivalent to 10^{-5} virus dilution) (Fig. 6). As a comparison, virus dilution endpoints were determined by serial dilutions of infected samples with SDW, which is different from diluting with nucleic acid extracts from virus-free cassava leaves. In serial dilution with SDW, CBSV was detectable at dilutions of 10^{-4}.

3.5. Probability of detecting CBSV in field samples

Eqs. (1) and (2) provide a basis to make decisions about sample size based on the probability of detecting CBSV incidence with a given sample size. For example, a 100-plant sample gives a 99.9% chance of detecting 6.7% incidence, but a 400-plant sample gives the same chance of detecting a lower incidence of 1.7%. Various other combinations of sample size and the probability of detecting CBSV at different incidences are given in Table 1.

The joint probability of a plant being infected and of failing to detect this infection is given in Table 2. The values can be interpreted as the expected number of plants being infected in a consignment of 1000 plants given the infection rates and the probability of detection, *P*, in Table 1.

Table 1
The probability *P* (%) of detecting CBSV incidence (% plants infected) is given when a known number of samples were tested per field.

P	Number of plants sampled for CBSV testing[a,b]							
	5	10	20	50	100	200	300	400
99.9	75	50	29	13	6.7	3.4	2.3	1.7
99.5	65	41	23	10	5.2	2.6	1.8	1.3
99	60	37	21	8.8	4.5	2.3	1.5	1.1
98	54	32	18	7.5	3.8	1.9	1.3	1
95	45	26	14	5.8	3	1.5	1	0.7
90	37	21	11	4.5	2.3	1.1	0.8	0.6
80	28	15	7.7	3.2	1.6	0.8	0.5	0.4

[a] Eqs. (1) and (2) rely on random sampling such that infection in a sample is independent of infection in the other samples.
[b] For example, there is a 99.9% chance of detecting a 1.7% infection if 400 samples were tested per field.

3.6. Consumables costs of CBSV detection protocols

Sample preparation using the CTAB method costs £0.53 (equivalent to US$0.80) per sample, which was at least 10-times cheaper than the RNeasy method at £5.91 (US$8.86) per sample, which was the most expensive protocol used in this study (Table 3). For complete virus diagnosis, combinations of sample preparation using the RNeasy method and virus genome amplification using the one-step RT-PCR protocol was most expensive at £10.53 (US$15.79) per sample (Table 4). In comparison, sample preparation using the CTAB method and virus genome amplification by the two-step RT-PCR was at least 5-times cheaper at £2.01 (US$3.02) per sample.

Table 2
The probability of releasing infected material in a consignment of 1000 plants given the infection rates and probability of detection in Table 1 (values rounded to 2 significant figures, and multiplied by 1000 for ease of reading).

P	Number of plants sampled for CBSV testing							
	5	10	20	50	100	200	300	400
99.9	0.75	0.50	0.29	0.13	0.067	0.034	0.023	0.017
99.5	3.3	2.1	1.2	0.50	0.26	0.13	0.09	0.065
99	6.0	3.7	2.1	0.88	0.45	0.23	0.15	0.11
98	11	6.4	3.6	1.5	0.76	0.38	0.26	0.20
95	23	13	7.0	2.9	1.5	0.75	0.50	0.35
90	37	21	11	4.5	2.3	1.1	0.80	0.60
80	56	30	15	6.4	3.2	1.6	1.0	0.80

Table 3
Costs of consumables associated with individual virus diagnostic protocols used in this study.

	Cost per sample	
	UK£	US$[a]
Sample preparation		
CTAB method	0.53	0.80
Buffers and reagents	0.32	0.48
Plastic ware	0.21	0.31
RNeasy method	5.91	8.86
RNeasy plant mini kit[b]	5.87	8.80
Plastic ware	0.04	0.06
PCR amplification		
General PCR	0.47	0.71
Reagents	0.42	0.62
Plastic ware	0.06	0.09
One-step RT-PCR	4.48	6.72
One-step RT-PCR kit[b]	4.42	6.63
Plastic ware	0.06	0.09
Two-step RT-PCR	1.34	2.01
cDNA synthesis kit[c]	0.81	1.22
Plastic ware	0.06	0.09
PCR reagents[d]	0.42	0.62
Plastic ware	0.06	0.09
Gel electrophoresis	0.14	0.21
Reagents	0.13	0.20
Plastic ware[e]	0.01	0.01

[a] Based on an exchange rate of £1 = $1.5.
[b] RNeasy plant mini kit and the One-step RT-PCR kit were from Qiagen Ltd., Crawley, UK.
[c] cDNA synthesis was performed using the ImProm-II™ Reverse Transcriptase from Promega UK Ltd., Southampton, UK.
[d] PCR following cDNA synthesis was using the Red Hot polymerase kit from Thermo Fisher Scientific Ltd., Loughborough, UK.
[e] All the plastic ware (pipette tips and microfuge tubes) used in the study were from Fisher Scientific Ltd., Loughborough, UK.

Table 4
A summary of diagnostic protocols and associated costs of consumables for complete CBSV diagnosis.

Combination extraction and PCR methods	Cost per sample	
	UK£	US$[a]
CTAB method + two-step RT-PCR for CBSV + electrophoresis	2.01	3.02
CTAB method + one-step RT-PCR for CBSV + electrophoresis	5.15	7.72
RNeasy method + two-step RT-PCR for CBSV + electrophoresis	7.39	11.08
RNeasy method + one-step RT-PCR for CBSV + electrophoresis	10.53	15.79

[a] Based on an exchange rate of £1 = $1.5.

4. Discussion

The RT-PCR protocol, first developed by Monger et al. (2001a) for CBSV, is the only means of detecting CBSV reliably in infected cassava plants. However, information is not available on the type of tissue for diagnostic purposes, especially on asymptomatic leaves from the top of the plant which are available readily for collection but do not always express symptoms. In this study we have shown that CBSV was detectable both in symptomatic and asymptomatic leaves from all parts of infected cassava plants. The asymptomatic fully open leaves collected from positions 3 or 4 from the top appear to be the most suitable for diagnosis because they consistently produced the brightest diagnostic bands. CBSV was also detected in extracts from stems and tuberous roots of infected cassava plants, which is the first demonstration of CBSV detection in these plant parts.

Using commercial sample preparation kits such as the Qiagen's RNeasy plant mini kit for sample preparation was expensive, costing around £5.91 (US$8.86) per sample. Such high costs are prohibitively expensive in laboratories with limited resources and so an alternative method of sample preparation was considered by modifying the CTAB method for total nucleic acid extractions from cassava plants (Maruthi et al., 2002). For the purpose of CBSV diagnosis, samples prepared using either the CTAB or RNeasy methods were equally adequate and produced reproducible diagnostic bands. The two methods can only be discriminated at sample dilutions of 1.5×10^{-3}, at which point the virus was still detectable in CTAB extractions but not with the RNeasy method. Perhaps the most important difference between the two methods was cost of consumables. Reagents required for sample preparation by the CTAB method were relatively inexpensive (£0.53 = US$0.80 per sample), which together with the higher quantities of total nucleic acids obtained will make it the preferred method for sample preparations for CBSV diagnosis in cassava.

Both one-step and two-step RT-PCR methods also proved to be highly reliable in detecting CBSV in sample dilutions of up to 10^{-4} which is a great improvement on previously published RT-PCR methods, where CBSV was not detected at sample dilutions exceeding 1/30 (Monger et al., 2001a). The one-step RT-PCR was, however, more sensitive in detecting CBSV at higher dilutions of 10^{-6}. This was probably due to the availability of 10-fold more cDNA for subsequent PCR amplification for one-step PCR than to those available for two-step RT-PCR. Nevertheless, at around US$6.72 per sample, the additional cost of the one-step PCR is not considered justifiable and hence the cheaper two-step RT-PCR protocol (US$2.01) is the preferred method. Two-step PCR also provides flexibility for the simultaneous detection of multiple virus infections such as CBSV together with cassava mosaic viruses in dually infected cassava plants (Abarshi et al., unpublished results). For complete CBSV diagnosis, the two-step RT-PCR combined with sample preparation by the modified CTAB method together cost only £2.01 (US$3.02) per sample and these should be the preferred protocols until more suitable ELISA-based techniques are developed which should reduce costs further.

The development of cost-effective and reliable diagnostic protocols will be particularly useful for laboratories in African countries. Nevertheless, carrying out large field surveys and/or epidemiological studies can be expensive, even following the lowest cost protocols. The high unit costs can be reduced by composite sampling (pooling of samples for testing), which depends on many factors including sample size, virus concentration in infected plants, effects of dilution of infected samples with uninfected samples and the sensitivity of the technique used. Sample preparation using the modified CTAB method and virus genome amplification by the two-step RT-PCR proved to be adequately sensitive for detecting CBSV in composite samples at 10^{-4} dilutions. What remains to be done as part of future studies, however, is to confirm the suitability of CBSV10 and CBSV11 primers (Monger et al., 2001a) to detect CBSV in field-collected samples, which are considered to be difficult to diagnose accurately due to the presence of more than one strain of CBSV (Mbanzibwa et al., 2009b).

Given a test with high sensitivity to the presence of virus, the probability of detecting CBSV infection in the field depends on two factors: the virus incidence and the number of plants sampled in the field. Clearly, as incidence decreases, the probability of detection becomes less and the occurrence of false negatives must be balanced against the practicalities of sample size. All planting material transported between country borders, from regions with CBSD to those without, and for the nucleus material to be used in breeding programmes should be tested for CBSV. For epidemiology

and phytosanitary programmes, testing every plant is logistically impractical, therefore testing a defined number of samples can be considered. Depending on the accuracy required, for example, sampling 100–400 plants per field is an appropriate recommendation giving a 99.9% probability of detecting a virus incidence of 6.7% (100 plants sampled) to 1.7% (400 plants sampled). The chances of releasing infected material are negligible when testing many samples (example 400) using a highly sensitive detection technique (high probability of detection) compared to testing a few samples. The diagnostic protocols used (CTAB extraction combined with two-step RT-PCR) in this study are indeed highly sensitive for the purpose of CBSV diagnosis (virus detection at 10^{-4} dilutions). They are reliable, most cost-effective currently, and provide practical answers for CBSV diagnosis in African countries.

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