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Detection of cassava brown streak viruses in costal Tanzania

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Abstract

Cassava brown streak disease (CBSD) is a factor which can decrease cassava production up to 70%. Cassava is an important crop, especially in sub-Saharan Africa for small-scale farms, as it is a staple food with tolerance to local conditions such as draught. The disease is caused by two viruses: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Symptoms of the disease can be seen as chlorosis, especially along secondary veins in leaves, and as brown streaks along the stem. A severe symptom is root necrosis, which makes the starchy tissue unsuitable for human consumption. The CBSD is not always visible as symptoms on leaves or stems and can be very hard to detect and it is difficult to know the extent of losses before the plants are harvested. In this study two field trips for collecting samples were made, one along the coast towards Tanga and the other one to Kibaha, also located in Tanzania's coastal region. Leaf samples were collected in a plant press as dry samples. Cassava was sampled to compare virus composition between the two areas and also other species of plants were sampled to search possibilities of them as alternative hosts for the viruses. An alternative hosts can function as a reservoir for the viruses. Knowing them would limit spreading of CBSD. RT-PCR was used to detect the viruses in non-cassava plants, and seven of the tested plants gave positive results. The RT-PCR products of the seven samples were sent for sequencing, but the sequencing results were of poor quality with high background. The determined sequences were compared to the sequences in GenBank through BLAST and no CBSV was found. Thirty extracted cassava samples were tested with both RT-PCR and Real-Time RT-PCR and some differences in virus composition were found in the two different areas. In this study two main findings were made: i) more mixed infections of CBSV and UCBSV in Kibaha than in Tanga ii) UCBSV was found in Mwamkongo, Muheza close to the border of Tanga.

Keywords: Cassava brown streak virus, CBSV, Ugandan cassava brown streak virus, UCBSV, Cassava, Tanzania, *Ipomovirus*

Sammanfattning

Cassava brown streak disease (CBSD) är en faktor som kan minska skörden av kassava med upp till 70 %. Kassava är en väldigt viktig gröda, speciellt i Afrika söder om Sahara för bönder som odlar i liten skala, eftersom kassava är väldigt tolerant mot torka. Sjukdomen orsakas av två virus: *Cassava brown streak virus* (CBSV) och *Ugandan cassava brown streak virus* (UCBSV). Symptomen av sjukdomen syns som kloros längs bladnerverna och bruna strimmor längs stammen. Det allvarligaste symptomet är dock när roten visar nekros och den stärkelserika vävnaden är då olämplig för mänsklig konsumtion. CBSD visar sig inte alltid i form av symptom på blad och stammar och kan därför vara väldigt svårt att upptäcka och det är svårt att veta omfattningen av förlusterna innan plantorna är skördade. I den här studien har två fältresor gjorts för att samla prover, längsmed kusten till Tanga och en till Kibaha, som också ligger utmed Tanzanias kust. Bladproverna samlades i en växtpress som torkade prover. Kassava och även andra växter skulle testas i sökandet efter alternativa värdar och för att jämföra viruskompositionen mellan de två områdena. Alternativa värdar kan fungera som en virusreservoar. Det skulle vara värdefullt att känna till det i arbetet med att försöka stoppa virusspridningen. För att detektera virus i de extraherade proven från andra växter än kassava så användes RT-PCR, och sju av proven gav positivt resultat. De proven testades även med Real-Time RT-PCR, men de resultaten var negativa. För att kunna dra någon slutsats så skickades RT-PCR produkterna för de sju proven iväg för sekvensbestämning men sekvenseringsresultaten var av dålig kvalité med hög bakgrund. De bestämda sekvenserna jämfördes med sekvenser i GenBank genom BLAST-analys men inget CBSV hittades. Trettio extraherade kassavaprover testades med både RT-PCR och Real-Time RT-PCR och skillnader i viruskomposition hittades för de olika områdena. Denna studies främsta resultat var: i) fler blandade infektioner av CBSV och UCBSV i Kibaha än i Tanga ii) UCBSV hittades i Mwamkongo, Muheza nära Tanga.

Nyckelord: Cassava brown streak virus, CBSV, Ugandan cassava brown streak virus, UCBSV, Kassava, Tanzania, *Ipomovirus*

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1 Introduction

Cassava (*Manihot esculenta*) is an important crop for the people of sub-Saharan Africa. Studies have shown that approximately 37% of their dietary calories come from cassava (IITA, 2012). Cassava was brought to Africa from South America around year 1550. Eight hundred million people in the world have cassava as their primary staple crop (Lebot, 2008). In Tanzania, seven million tonnes of cassava are harvested each year (FAO, 2012). Why it is so important is due to its many uses and that it is a relatively easy crop to grow. Apart from food, cassava many industrial uses such as for production of glue, plywood and textile. Cassava requires less labour than other staple crops and it can be harvested between six months and three years after planting and has good resistance to draught (IITA, 2012).

However, there are some diseases and pests affecting cassava production. One of the most important diseases is called Cassava brown streak disease (CBSD) which can decrease production up to 70% (Hillocks et al., 2001). The disease is caused by two viruses, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). The viruses belong to the genus *Ipomovirus* and the family *Potyviridae*, and they have a genome of positive sense single-stranded RNA (Rwegasira et al., 2011). To refer to them collectively the term CBSVs will be used. The disease causes necrotic tubers and can sometimes be detected also as brown streaks along stems and chlorosis in leaves, especially along secondary veins. However, the disease can be really hard to detect because young leaves are often symptomless and the older leaves can be masked by senescence, or the symptoms of attack by mites or other pathogens. Repeated samplings of cassava indicate that among common varieties the infection rate approaches 85% (McSween et al., 2006) for CBSVs. The viruses are spread through stem cuttings and by the whitefly *Bemisia tabaci*. CBSD was first described in 1936 in Tanga at the coast of Tanzania (Storey, 1936). During most of the 1900s the spread of the disease was under control, but now there has been an increase of whiteflies which has led to a more critical

scenario. Today, the disease has spread to Uganda, Kenya, Mozambique, Malawi and the Democratic Republic of Congo (Legg et al., 2011).

In this study, the main objective was to detect the two cassava brown streak viruses (CBSVs) in cassava to see how the virus composition differed between two areas. One area was along the coast towards Tanga, where CBSD first was described and the other area was Kibaha, where the lab is located. Earlier studies have shown that a large part of the samples from Kibaha had mixed infections of UCBSV and CBSV and some with single infections of CBSV. Studies in Tanga have shown several single infections of CBSV, but no UCBSV (Mbanzibwa et al., 2011; Abarshi et al., 2011).

During the field trips other plants than cassava, which were potential virus hosts, were sampled and screened for virus. Because cassava was originally imported from South America to Africa and CBSVs never have been found there, it is suspected that CBSV originated in Tanzania and maybe has other plants as reservoirs, which do not become as affected by virus infection as cassava. So far, CBSV is only known to infect tree cassava (*Manihot glazovii*) (Mbanzibwa et al., 2011). If a reservoir for the CBSVs would be identified it would hopefully make the spread of the disease easier to control and that would be a great improvement for many East-African farmers.

2 Materials & Methods

2.1 Sampling

Two field trips were made in Eastern Tanzania to collect samples: one in Kibaha and the other one along the north costal region towards Tanga (Fig. 1). GPS-coordinates for each village can be found in the appendix.

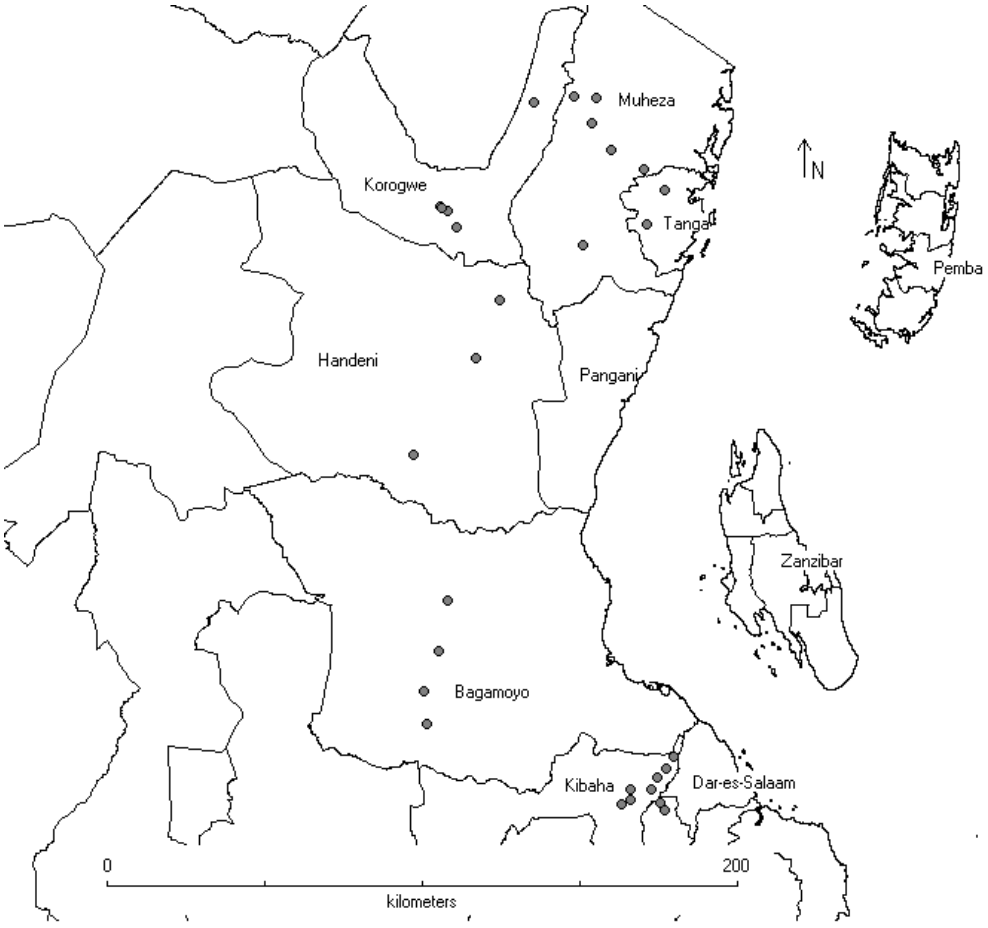

Figure 1. A map indicating sampling sites in Eastern Tanzania (grey dots).

At least one sample of cassava was collected at each field, with symptoms if it was found and otherwise without any symptoms. Symptoms include chlorosis along secondary veins or brown streaks along the stem. Other plants which were suspected to host CBSVs were also collected (Table 1). Those samples were collected according to three criteria i) species from the order Cucurbitales that are previously known

to be affected by other ipomoviruses ii) weeds which had symptoms similar to brown streak disease iii) plants that were visited by whiteflies. During sampling, the leaf from the third position from the top was chosen. The leaves were collected as dry samples in a plant press.

Table 1. Plant samples collected during the survey.

Village	Cassava samples	Alternative hosts		Date
		Unidentified	Identified	
Msoga	2	-	*Ricinus communis* (2), *Comellina sp.*	11-04-2012
Tonga	2	3	*Solanum melongena, Citrullus lanatus, Ipomoea batatas*	11-04-2012
Mkwazu	1	3	*Cucurbita sp.* (2), *Ipomoea batatas, Euphorbia sp.*	11-04-2012
Mandera	1	-	*Manihot glazovii, Vigna unguiculata, Abelmoschus esculentus*	11-04-2012
Mkata	1	-	*Solanum sp.*	11-04-2012
Kunga clones	1	-	-	12-04-2012
Nazareth	1	3	-	12-04-2012
Welei	1	2	*Cucurbita sp.*	12-04-2012
Mbzambiazi	3	2	-	12-04-2012
Magati	3	8	-	13-04.2012
Maramba	4	3	-	13-04.2012
Kwetonga	1	3	*Ipomoea batatas*	13-04.2012
Kwanganga	4	2	-	14-04-2012
Mapambano	1	3	-	14-04-2012
Mwamkongo	1	3	*Cajanus cajan, Rammelina sp.*	14-04-2012
Mbleni	1	1	-	14-04-2012
Kwamdakeo	1	2	-	15-04-2012
Michangwani	1	2	*Cucumis melo*	15-04-2012
Kabuku	2	3	*Cucurbita sp., Ricinus communis*	15-04-2012
?	2	1	-	08-05-2012
Pangani	1	4	-	08-05-2012
Kibamgini	2	4	-	08-05-2012
Wikawe	2	1	-	08-05-2012
Bungo	2	1	-	08-05-2012
Mikongani	2	2	-	08-05-2012
Sagale	2	-	-	08-05-2012
Kiluvya	2	1	-	08-05-2012
Tondoloni	2	1	-	08-05-2012

2.2 RNA extraction

A CTAB (cetyl trimethyl ammonium bromide) method was used (modified from Lodhi et al., 1994; Xu et al., 2010) to extract RNA from 66 plant samples and two positive controls of infected cassava (TME4 and Mba 195 Beatrice). The first step was to grind approximately 45 g of dry leaves in a mortar with 1 ml of 2% CTAB extraction buffer (2.0 M NaCl, 2.0% PVP, 25 mM EDTA, 100 mM Tris-HCl pH 8.0 and 5 mM TCEP). The homogenized samples were as a paste, which was transferred to Eppendorf tubes and incubated at 65°C for 15 min. Then 750 µl of a 24:1 mix of chloroform and isoamyl alcohol was added to each sample and mixed before centrifugation. The tubes were centrifuged at 4°C for 10 min at 12,000 rpm. The upper phase was transferred to new tubes and 300 µl of ice-cold isopropanol was added. The extracts were incubated for at least 10 min at -20°C. After centrifugation at 4°C for 10 min at 13,000 rpm, the supernatant was removed. To purify the pellet, 700 µl ethanol was added and tubes were again left to incubate at -20°C for at least 10 min and centrifuged at 4°C for 5 min at 13,000 rpm. The ethanol was removed from the tubes and the pellets were left to dry for approximately 30 min at room temperature. When the pellets were dry they were resuspended in 100 µl of SDW. The quality of the extraction was controlled with nanodrop. It was an efficient method although some RNA extracts, especially from weeds could fail. Fail means that when checking the extract by absorbance measurements at 230, 260 and 280 nm using a nanodrop a very low RNA concentration was shown and the RNA had probably been degraded along the process. However, sometimes the RNA concentration was high, but the extract was not pure showing a high ratio of the absorbance values at 260/280 or 260/230 and then the samples just needed some extra purification. The extra purification started with 100 µl 5 M NaCl and 300 µl of chloroform:isomayl alcohol (24:1) being added to each RNA extract. Then the RNA samples were centrifuged again at 4°C for 10 minutes at 13,000 rpm and 300 µl of isopropanol and 250 µl of 0.8 M trisodium citrate dehydrate mixed with 1.2 M NaCl were added to the supernatant. The final step was to wash, dry and resuspend the pellet as described before.

2.3 RT-PCR

Successful RNA extractions were used as template for reverse transcription polymerase chain reaction (RT-PCR) to detect CBSVs. Two master mixes were made

with two different primer pairs: CBSV10 and CBSV11 with an expected amplification product of 250 bp (Monger et al., 2001); CBSVF3 and CBSVR3 with an expected product of 300 bp (Shirima et al., unpublished). CBSV10 and CBSV11 are designed to amplify within the coding region of the viral coat protein (Table 2). Master mixes had a final concentration of 1 x buffer, 10 mM DTT, 60 µM dNTPs, 0.2 mM primer, 0.04 U/µl Taq DNA polymerase, 0.6 U/µl MMLV-reverse transcriptase and 1 µl of RNA template was added. The RNA template solutions were not of a certain concentration because it was detection that was wanted. However the concentration was controlled for not being too low, which could prevent detection. The concentrations were often in the lower range since it was such difficult, starchy tissues, and probably some RNA was degraded during the purification steps. The total volume for the RT-PCR was 10 µl. The RT-PCR program was set to 30 min at 42°C, a denaturation step of 1 min at 94°C, an annealing step at 52°C, elongation 3 min at 72°C and then looping with 30 cycles of 94°C for 30 s, 52°C 30 s, 72°C 40 s and a final step at 72°C for 10 min. To visualize the RT-PCR products, a 2% agarose gel was prepared with ethidium bromide in 1 x TAE buffer. Five µl of One Step ladder (50 bp) or 1 kb plus DNA ladder was used to determine the size of the products. Three positive controls were used and they were all infected cassava plants called TME4, TME4R and Mba 195 Beatrice. One of the negative controls consisted of RNA from a non-infected cassava plant (TC) and the other one was sterile water (SDW) being added instead of template. From the start both primer pairs were used but CBSV10 and CBSV11 gave the most prominent bands and therefore it was decided to continue using only that pair for the rest of the study. To test the positive results for alternative hosts further, two more runs with RT-PCR were made with other annealing temperatures. The first run was made with an annealing temperature of 53 °C and the other one with an annealing temperature of 55 °C.

Table 2. Sequences of primers used for detecting CBSV and UCBSV with RT-PCR.

Primer	Sequence
CBSV10	ATCAGAATAGTGTGACTGCTGG
CBSV11	CCACATTATTATCGTCACCAGG

2.4 Real-Time RT-PCR

Twenty-nine cassava samples, 15 from Kibaha and 14 samples collected along the way during the field trip to Tanga, were analyzed with Real-Time RT-PCR to detect the two viruses which cause CBSD. Also 6 weed samples which gave positive results with the RT-PCR were analyzed for the two viruses. As a control, a COX assay was carried out with four DNase-treated RNA extracts of cassava samples. The DNase treatment was performed using 8 µl of extracted RNA and a mix with 1 µl of Amplification Grade DNase I (1 unit/µl; Sigma) and 1 µl of 10 x Reaction Buffer (Sigma). The RNA solutions were treated with DNase I for 15 minutes at room temperature and DNase I was then inactivated by adding 1 µl of Stop Solution (Sigma) and incubating at 70°C for 10 minutes. The COX assay was performed to see if there was RNA in the samples. It amplifies products of the housekeeping gene cytochrome c oxidase. Three master mixes were made (CBSV, UCBSV and COX) with the final concentrations of 1 x PCR buffer, 5.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.3 mM primer (Shirima et al., unpublished), 0.1 µM probe, 30 nM reference dye (Stratagene), 0.025 U/µl Taq DNA polymerase and 0.4 U/µl MMLV-reverse transcriptase. One negative control with sterile distilled water was used. RNA extracts from CBSV- and UCBSV-infected plants were used as positive controls. TME4 was used as a positive control for both CBSV and UCBSV and 1pool was used as a positive control for only UCBSV. The Real-Time PCR program started with 30 min at 48°C for the reverse transcription and then a 10 min denaturation step at 95°C. The cDNA synthesis was followed by 40 cycles of 15 s at 95°C and a 1 min long combined annealing/elongation step at 60°C (Shirima et al., unpublished).

2.5 Sequencing

For sequence analysis the RT-PCR procedure was repeated but with a larger total volume of 50 µl. The primers CBSVF3 and CBSVR3 were used for sequencing of the amplification products of the cassava samples with an expected product size of 300 bp. The amplification products of alternative host samples were sequenced with the primers CBSV10 and CBSV11 with an expected product size of 250 bp. The amplification products for five cassava samples were sequenced as well as for seven of the alternative host samples. They were sequenced through Sanger-sequencing at Macrogen, USA. The sequences were analyzed with BLAST at the webpage of NCBI to compare them with the sequences in GenBank.

3 Results

3.1 Field sampling

CBSD is not always easy to detect in the field by symptoms since mostly the old leaves show symptoms, and they are usually masked by other factors such as senescence. It was planned that at least one cassava sample was to be collected in each field but it was found to be more difficult in the northern area towards Tanga where they looked more affected by draught and mites than in Kibaha where the symptoms of CBSD were clearer. When collecting alternative host samples a good way to sample was to look for whiteflies (Table 3). It seemed like whiteflies were more abundant during our field trip in Kibaha than in the field trip towards Tanga.

3.2 RNA extraction

RNA was extracted from 66 plant samples and 30 of those were *M. esculenta*. The RNA extraction failed for one cassava sample, but otherwise CTAB was a very efficient method for extracting RNA from cassava. RNA was also extracted successfully from one sample of *M. glazovii*. The other plants were more difficult. Thirty-six samples of non-cassava plants were used for RNA extraction and from those there were 11 RNA extracts which were not pure enough for continued analyses. The 54 successful RNA extractions were analyzed for infection with CBSVs using RT-PCR.

3.3 Alternative hosts

Eight of the alternative host samples were positive for CBSVs using RT-PCR, although their amplification products were a bit smaller than of the positive cassava controls (Table 3). For both the cassava and alternative hosts the primer pair CBSV10 and CBSV11 was used and it was expected to give a product of 250 bp. Several RT-PCRs were run for these samples, both with lower RNA concentrations and with different annealing temperatures and all the results were positive for CBSV (Fig. 2). Unfortunately not all tested plants could be identified. Photograph records of the unidentified plants which showed bands with RT-PCR are shown in Figure 3.

Table 3. RT-PCR results for the tested non-cassava samples.[a]

Species	Village	Selection criteria	RT-PCR
Ricinus communis	Msonga	Chlorosis	-
?[b]	Tonga	Whiteflies	+
Citrullus lanatus	Tonga	Cucurbitales	-
Solanum melongena	Tonga	Cucurbitales	-
?[c]	Mkwazu	Whiteflies	+
Cucurbita sp.	Mkwazu	Cucurbitales	-
Euphorbia sp.[d]	Mkwazu	Whiteflies	+
Manihot glazovii	Mandera	Relative to cassava	+
Abelmoschus esculentus[e]	Mandera	Whiteflies	+
Cucurbita sp.	Welei	Cucurbitales	+
?	Mbzambiazi	Chlorosis	-
?	Magati	Chlorosis	-
?	Kwetonga	Chlorosis	-
?	Kwetonga	Chlorosis	-
Ipomoea batatas	Kwetonga	Chlorosis	-
?	Mapambano	Chlorosis	-
?[f]	Mwamkongo	Whitefly nymphs	+
?	Mwamkongo	Chlorosis	-
Cajanus cajan	Mwamkongo	Chlorosis	-
?	Mbleni	Chlorosis	-
?	Kwamdakeo	Chlorosis	-
?	Michangwani	Chlorosis	-
?	Michangwani	Chlorosis	-
Cucumis melo	Michangwani	Cucurbitales	-
?[g]	Kabuku	Whiteflies	+
?	Kabuku	Chlorosis	-
Cucurbita sp.	Kabuku	Cucurbitales	-
Ricinus communis	Kabuku	Chlorosis	-

[a] Not all plant species could be identified, but the plants which gave positive bands are shown in Fig. 3.
[b] The same sample as no. 1 in Fig. 2 and Fig. 3
[c] The same sample as no. 2 in Fig. 2 and Fig. 3
[d] The same sample as no. 3 in Fig. 2 and Fig. 3
[e] Visible as no. 2 in Fig. 3
[f] The same sample as no. 6 in Fig. 2 and Fig. 3
[g] The same sample as no. 7 in Fig. 2 and Fig. 3

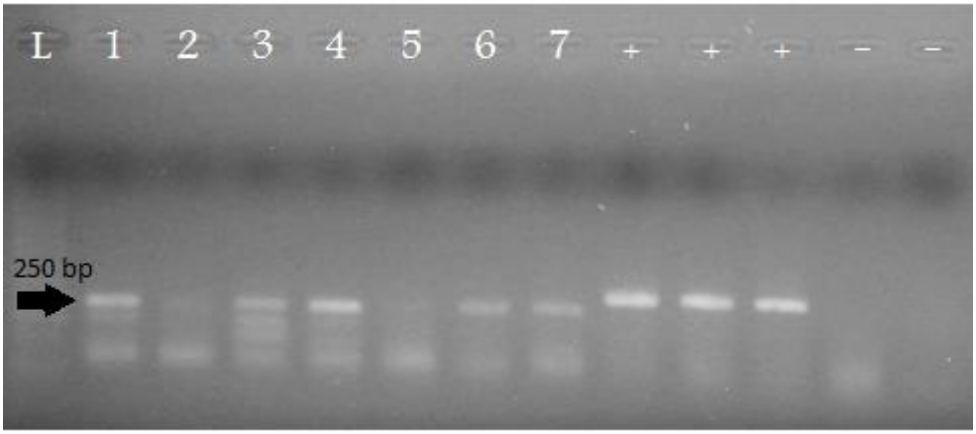

Figure 2. Gel electrophoresis of amplification products. Detection of CBSVs using primers: CBSV10 and CBSV 11. L: One Step ladder (50 bp); 1: Unidentified species (Fig. 3, no. 1); 2: Unidentified species (Fig. 3, no. 2); 3: Euphorbia sp. (Fig. 3, no. 3); 4: *Abelmoschus esculentus*; 5: Unidentified species; 6: Unidentified species (Fig. 3, no. 6); 7: Unidentified species (Fig. 3, no. 7); Positive controls: infected cassava plants TME4, TME4R and Mba 195 Beatrice; Negative controls: non-infected cassava plants TC and SDW

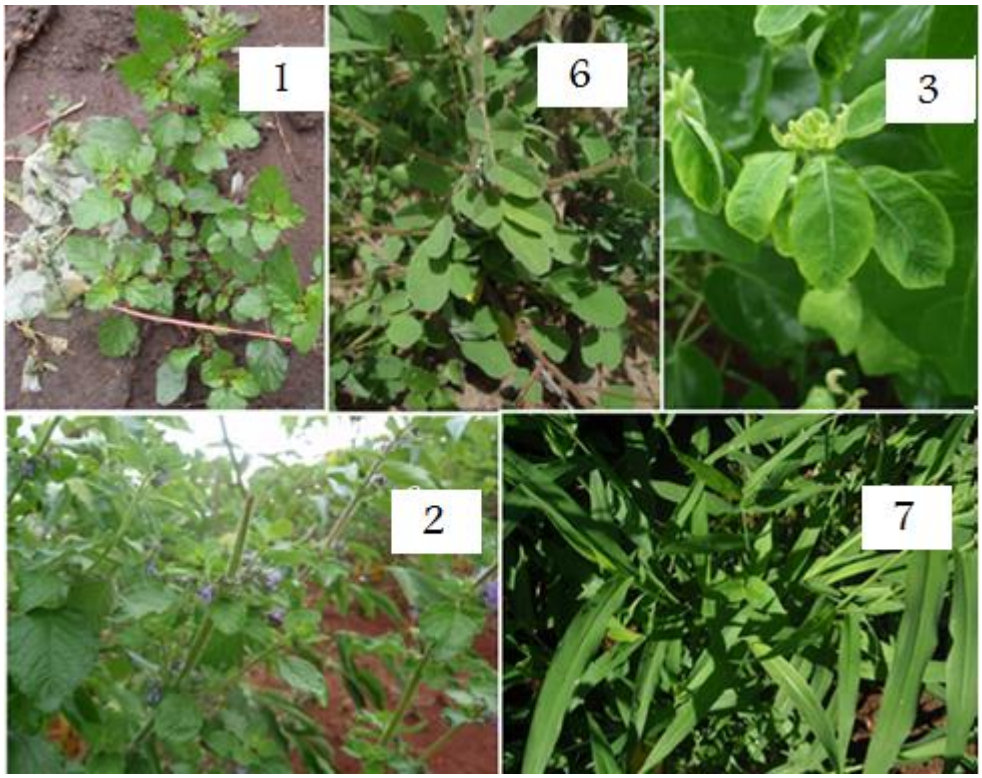

Figure 3. Photographs of the unidentified plant species which were sampled and gave tentative positive results with RT-PCR analysis for cassava brown streak viruses. The numbers indicate the plants for which RT-PCR bands of the expected size were obtained as shown in Fig. 2.

Table 4. Sequence comparison of amplification products from non-cassava samples and data in GenBank

Sample and Primer	Accession no.	Description	Identity	E-value	Query coverage
W1-CBSV10	HQ664613.1	Caryocar glabrum ribosomal protein L2 (rpl2) and ribosomal protein L23 (rpl23) genes…	99%	1e-27	80%
W1-CBSV11	HQ664648.1	Mirabilis jalapa rpl23 pseudogene and tRNA-Ile (trnI-CAU) gene…	95%	2e-30	94%
W2-CBSV10	AK107856.1	Oryza sativa Japonica Group cDNA…	97%	1e-22	89%
W2-CBSV11	EU117376.1	Manihot esculenta cultivar TME3 chloroplast, complete genome	84%	6e-15	93%
W3-CBSV10	HQ664613.1	Caryocar glabrum ribosomal protein L2 (rpl2) and ribosomal protein L23 (rpl23) genes…	96%	2e-20	81%
W3-CBSV11	HQ664565.1	Neurada procumbens ribosomal protein L2 (rpl2) and ribosomal protein L23 (rpl23) genes…	92%	5e-33	97%
W4-CBSV10	AK107856.1	Oryza sativa Japonica Group cDNA…	96%	2e-15	76%
W4-CBSV11	JN637765.1	Eleutherococcus senticosus chloroplast, complete genome	92%	3e-24	97%
W5-CBSV10	JN861110.1	Oryza sativa Indica Group chloroplast, complete genome	100%	2e-30	100%
W5-CBSV11	JN861110.1	Oryza sativa Indica Group chloroplast, complete genome	99%	2e-36	100%
W6-CBSV10	EF380354.1	Illicium oligandrum chloroplast, complete genome	97%	9e-35	94%
W6-CBSV11	JF746994.1	Erycina pusilla chloroplast, complete genome	96%	3e-28	98%
W7-CBSV10	-	-	-	-	-
W7-CBSV11	-	-	-	-	-

The alternative host samples were as well examined through Real-Time RT-PCR but those results were negative. The amplification products from RT-PCR were sent for sequencing for confirmation of the results.

When looking at the sequencing results many of the base peaks seemed to be a mix of different genetic material and the seventh sample was impossible to use for BLAST analysis. The other ones were compared to sequences in GenBank through BLAST although the quality was poor. According to the BLAST results, the amplified gene products were of plant origin (Table 4).

3.4 Cassava

Thirty cassava samples were tested both with RT-PCR and Real-Time RT-PCR. A compilation of the results can be found in Table 5. The Real-Time RT-PCR gave high CT values for some samples, and the results were then regarded as inconclusive. This can be due to background amplification leading to a false positive result. Real-Time RT-PCR was the most trusted method due to the specificity of the primers and the ability to check the CT-values. With the Real-Time RT-PCR it was possible to differentiate between the two viruses causing brown streak disease (Table 5). The COX assay for the DNase treated samples was successful and gave a positive result indicating that the samples contained RNA.

UCBSV was found in one sample collected in the village Mwamkongo in the region Muheza close to Tanga which is interesting because its presence has not been detected there before. Differences in virus composition could be detected between the different areas with a higher proportion of mixed infections with CBSV and UCBSV in Kibaha, which coincides to earlier studies. An incidence of infected plants of 80% (12 out of 15) was found in Kibaha compared to 50% (7 out of 14) in the northern regions towards Tanga (Table 6). Virus incidence might be higher in Kibaha than in regions towards Tanga, but that is impossible to conclude with such a small sample size and also due to the way of sampling, plants with symptoms were searched for and that could bias the observation. Amplification products for five cassava samples were sent for sequencing to confirm the presence of the viruses and they confirmed that it was CBSV and/or UCBSV that had been found. The result from the sequencing corresponds with the Real-Time RT-PCR results (Table 7).

Table 5. RT-PCR and Real-Time RT-PCR results for cassava samples.

Village	Field Trip	Real-Time RT-PCR		RT-PCR
		CBSV	UCBSV	
Mkata	Tanga	+	-	+
Tonga	Tanga	+	-	+
Kunga clones	Tanga	-	-	+
Kwamdakeo	Tanga	-	-	+
Michangwani	Tanga	+	-	+
Kabuku	Tanga	+	-	-
Mbleni	Tanga	-	-	-
Mwamkongo	Tanga	-	+	-
?	Kibaha	+	+	-
Pangani	Kibaha	-[a]	-	-
Kibamgini	Kibaha	+	+	+
Wikawe	Kibaha	+	+	-
Bungo	Kibaha	+	+	+
Mikongani	Kibaha	-	-[a]	-
Sagale	Kibaha	+	+	+
Kiluvya	Kibaha	-[a]	-[a]	-
Tondoloni	Kibaha	+	-[a]	+
Tondoloni	Kibaha	+	+	+
Mikongani	Kibaha	+	+	+
Bungo	Kibaha	+	-	+
Wikawe	Kibaha	+	+	+
Kibamgini	Kibaha	+	+	+
?	Kibaha	+	+	+
Kabuku	Tanga	+	-	-
Kwanganga	Tanga	+	-	-
Mapambano	Tanga	-	-	-
Kwetonga	Tanga	-	-	-
Maramba	Tanga	-	-	-
Mbzambiazi	Tanga	-[a]	-[a]	-

[a] Inconclusive results due to large deviation from other CT-values and therefore referred to as negative results

Table 6. Summarized results of the two field trips from the Real-Time RT-PCR analysis of cassava samples for CBSV and UCBSV

Field Trip	CBSV	UCBSV	Single	Mixed	No infection
Kibaha[a]	12	10	2	10	3
Tanga[b]	6	1	7	0	7

[a] 15 samples were tested

[b] 14 samples were tested

Table 7. Sequence comparison of amplification products from cassava samples and data in GenBank

Sample and Primer	Accession no.	Description	Identity	E-value	Query coverage
C1-CBSVF3	HM346954.1	Cassava brown streak virus isolate Naliendele3-1 poly-protein gene, partial cds	99%	6e-101	99%
C1-CBSVR3	HM346954.1	Cassava brown streak virus isolate Naliendele3-1 poly-protein gene, partial cds	98%	8e-84	100%
C2-CBSVF3	FN423416.1	Cassava brown streak virus partial gene for polyprotein, coat protein region, strain Naliendele-1, genomic RNA	97%	1e-87	100%
C2-CBSVR3	FN423416.1	Cassava brown streak virus partial gene for polyprotein, coat protein region, strain Naliendele-1, genomic RNA	99%	2e-74	100%
C3-CBSVF3	HM171303.1	Cassava brown streak Uganda virus-[Malawi:Nkhata 29:2009] coat protein gene, partial cds	96%	4e-82	100%
C3-CBSVR3	HM171303.1	Cassava brown streak Uganda virus-[Malawi:Nkhata 29:2009] coat protein gene, partial cds	95%	2e-85	100%
C4-CBSVF3	HM346946.1	Cassava brown streak virus isolate Kikonde11-5 polypro-tein gene, partial cds	94%	3e-73	100%
C4-CBSVR3	JN817417.1	Ugandan cassava brown streak virus isolate Bur_21 coat protein gene, partial cds	97%	9e-73	99%
C5-CBSVF3	HM171300.1	Cassava brown streak Uganda virus-[Malawi:Zomba 1:2009] coat protein gene, partial cds	92%	5e-66	100%
C5-CBSVR3	JN817411.1	Ugandan cassava brown streak virus isolate Buj_3 coat protein gene, partial cds	95%	4e-66	99%

4 Discussion

To extract RNA a CTAB method was used which was effective for cassava but unfortunately it was not optimal for the extraction from other plants. There is probably a more efficient extraction method for some of these plants. Successful RNA extracts were tested with RT-PCR and in the beginning two primer pairs were used: CBSV10/CBSV11 and CBSVF3/CBSVR3. The primer pair CBSV10/CBSV11 was chosen for continued analyses because it produced the most prominent bands. However, CBSV10/CBSV11 might have produced such bands due to a lower specificity compared to CBSVF3/CBSVR3, which gave weaker bands even for cassava extractions and a slightly larger amplification product.

All cassava samples and the positive samples from alternative hosts were also tested with Real-Time RT-PCR, which gave a slightly different outcome. Some samples which were negative for CBSVs with RT-PCR were positive for CBSVs when using Real-Time RT-PCR and also the other way around. The Real-Time RT-PCR method was considered to be the most reliable; it is more sensitive with more specific primers. Still some results were not conclusive, probably due to some background amplification and have been labelled as "inconclusive" because they had higher CT-values than positive samples.

When comparing the virus composition in the samples of the different areas two observations were made i) more mixed infections of CBSV and UCBSV were found in Kibaha than along the coast towards Tanga ii) UCBSV was found in Mwamkongo, in the region Muheza close to the border to Tanga.

Of 15 samples from Kibaha, 10 samples had a mixed infection of CBSV and UCBSV and when comparing that to 0 out of 14 from the Tanga field trip, a difference of viral composition is apparent. Similar findings about Kibaha have been presented previously where 65% of the affected plants had mixed infections, no single infections of UCBSV and 32.5% with single infections of CBSV (Abarshi et al., 2011). Why there were no mixed infections in regions towards Tanga could simply be due to the lower frequency of UCBSV. In the present study, UCBSV was found in a single infection close to the border of Tanga in Mwamkongo, Muheza. That could indicate a new path of spread which could be due to imported stem cuttings from another area affected by UCBSV. Two surveys have been made where samples from the Tanga area were analyzed without finding UCBSV (Mbanzibwa et al., 2011; Abarshi et al., 2011).

It is possible to believe that the disease should be more abundant in Tanga because it was first described there and therefore Tanga is often regarded as the centre

of origin. However it was much harder to identify CBSD in areas towards Tanga and this study indicates a slightly lower incidence of CBSVs in Tanga than in Kibaha, although to conclude this more samples would be needed, preferably randomly picked. However, the presence or ability to identify CBSVs in the different areas could be discussed. Differences could be due to different cassava varieties used in the two areas. The new varieties introduced in Tanga could be more tolerant to infections of CBSVs or they may show less leaf and stem symptoms which makes it more difficult when trying to collect infected samples. Earlier studies have had similar results and conclusion about Tanga with a limited incidence of CBSD due to other varieties of cassava (Legg & Raya, 1998)

A sample of tree cassava (*M. glazovii*) with symptoms was according to RT-PCR positive for CBSV and the band was of the correct size. It would have been preferable also to test the sample with Real-Time RT-PCR and sequence it to assure the finding. However, an article has already been published where they have detected and sequenced CBSV from *M. glazovii* (Mbanzibwa et al., 2011). Seven other non-cassava samples showed positive results according to the RT-PCR, but they had bands of slightly lower molecular size compared to the positive cassava samples. All the alternative hosts which gave bands, except *Cucurbita sp.,* were the ones known to be visited by whiteflies. When analyzed with Real-Time RT-PCR the outcome was negative. RT-PCR products of non-cassava samples were then sent for sequencing to be able to conclude the presence of CBSVs. Unfortunately, the sequencing results were of poor quality with a mix of genetic material and sequence searches using Blast showed highest identity to plant genes. When analyzing the results it can be concluded that when analyzing such a difficult tissue it would have been useful to purify the amplification products before sequencing to improve the sequencing result.

In this study, differences of virus composition in cassava were detected when comparing two different areas and UCBSV was found in Mwamkongo, Muheza close to the border of Tanga. Unfortunately, no alternative hosts were detected, but an infected *M. glazovii* plant was found which strengthens the findings of Mbanzibwa et al. (2011).

References

ABARSHI, M. M., MOHAMMED, I. U., JEREMIAH, S. C., LEGG, J. P., KUMAR, P. L., HILLOCKS, R. J. & MARUTHI, M. N. 2012. Multiplex RT-PCR assays for the simultaneous detection of both RNA and DNA viruses infecting cassava and the common occurrence of mixed infections by two cassava brown streak viruses in East Africa. *Journal of Virological Methods,* 179, 176–184

FOOD AND AGRICULTURAL ORGANIZATION OF THE UNITED NATIONS (FAO) HOMEPAGE (2013) http://www.fao.org/docrep/012/ak341e/ak341e06.htm

HILLOCKS, R. J., THRESH, J. M., TOMAS, J., BOTAO, M., MACIA, R. & ZAVIER, R. 2002. Cassava brown streak disease in northern Mozambique. *International Journal of Pest Management,* 48**,** 178-181.

INTERNATIONAL INSTITUTE OF TROPICAL AGRICULTURE (IITA) HOMEPAGE (2012) http://www.iita.org/cassava;jsessionid=D9445197434F78EBAF1D783C2118A8FC

LEBOT, V. 2008. Section I. Cassava: origin and history. In: *Tropical root and tuber crops: cassava, sweet potato, yams and aroids***,** 3-11. CABI Publishing.

LEGG, J. P., JEREMIAH, S. C., OBIERO, H. M., MARUTHI, M. N., NDYETABULA, I., OKAOOKUJA, G., BOUWMEESTER, H., BIGIRIMANA, S., TATA-HANGY, W., GASHAKA, G., MKAMILO, G., ALICAI T. & LAVA KUMAR P. 2011. Comparing the regional epidemiology of the cassava mosaic and cassava brown streak virus pandemics in Africa. *Virus Research,* 159, 161–170

LEGG, J. P. & RAYA, M. D. 1998. Survey of cassava virus diseases in Tanzania. *International Journal of Pest Management,* 44**,** 17-23.

LODHI, M. A., YE, G. N., WEEDEN, N. F. & REISCH, B. I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. *Plant Molecular Biology Reporter,* 12, 6-13.

MBANZIBWA, D. R., TIAN, Y. P., TUGUME, A. K., MUKASA, S. B., TAIRO, F., KYAMANYWA, S., KULLAYA, A. & VALKONEN, J. P. T. 2011. Simultaneous virus-specific detection of the two cassava brown streak-associated viruses by RT-PCR reveals wide distribution in East Africa, mixed infections, and infections in *Manihot glaziovii*. *Journal of Virological Methods,* 171**,** 394-400.

McSWEEN, S., WALKER, T. S., SALEGUA, V. A. & PITORO, R., 2006. Economic Impact on Food Security of Varietal Tolerance to Cassava Brown Streak Disease in Coastal Mozambique. Food Security Collaborative Working Papers 55863, Michigan State University, Department of Agricultural, Food, and Resource Economics.

MONGER, W. A., SEAL, S., COTTON, S. & FOSTER, G. D., 2001. Identification of different isolates of Cassava brown streak virus and development of a diagnostic test. *Plant Pathology*, 50, 768-775

RWEGASIRA, G. M., MOMANYI, G., REY, M. E. C., KAHWA, G. & LEGG, J. P., 2011. Widespread occurrence and diversity of *Cassava brown streak virus* (*Potyviridae*: *Ipomovirus*) in Tanzania. *Phytopathology*, 101, 1159-1167

STOREY, HH, 1936. Virus diseases of East African plants: VI a progress report on studies of the diseases of cassava. *East African Agricultural Journal*, 2, 34–9.

XU, J., AILENI, M., ABBAGANI, S. & ZHANG, P. 2010. A reliable and efficient method for total RNA isolation from various members of spurge family (Euphorbiaceae). *Phytochemical Analysis*, 21, 395-398.

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Appendix

Table 1. GPS coordinates of the sampling sites

Field	Village	S (La)	E (Lo)	A (m)
1	Msoga	06.57762	038.33138	209
2	Tonga	06.48465	038.32483	298
3	Mkwazu	06.36566	038.36704	281
4	Mandera	06.21948	038.39190	201
5	Mkata	05.79730	038.29193	418
6	Kunga clones	05.05581	038.37666	1100
7	Nazareth	05.07759	038.37178	1100
8	Welei	05.09086	038.39210	520
9	Mbzambiazi	05.13569	038.41916	326
10	Welei	05.09061	038.39294	550
11	Magati	04.83325	038.81137	234
12	Maramba	04.75971	038.76021	388
13	Kwetonga	04.77684	038.64320	382
14	Kwanganga	04.76199	038.82389	332
15	Mapambano	04.91485	038.86444	204
16	Mwamkongo	04.97077	038.96144	110
17	Mbleni	05.02846	039.02024	56
18	Kwamdakeo	05.19035	038.78623	202
19	Michangwani	05.34621	038.54328	299
20	Kabuku	05.51785	038.47300	372
21	?	06.76813	038.98257	132
22	Pangani	06.73100	038.99974	146
23	Kibamgini	06.70863	039.02519	148
24	Wikawe	06.67367	039.04746	119
25	Bungo	06.76667	038.92277	172
26	Mikongani	06.79638	038.92115	141
27	Sagale	06.80883	038.89536	165
28	Kiluvya	06.80650	039.00867	126
29	Tondoloni	06.82730	039.02216	143

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