THE PREVALENCE OF CASSAVA MOSAIC BEGOMOVIRUSES IN MALAWI

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ABSTRACT

Cassava (*Manihot esculenta)* is an important staple crop for farmers in the tropics due to its drought tolerance, low inputs requirements and flexibility in planting, harvesting and storage. However, sustainable production of the crop is hampered by Cassava Mosaic Disease (CMD) which reduces tuber yield by up to 95%. CMD is caused by several species of circular single-stranded cassava mosaic *begomoviruses* (CMBs). Two comprehensive surveys which employed field leaf incidence evaluation and viral DNA detection methods were done in 2006/7 and 2010 in order to determine the distribution and diversity of CMBs in Malawi. The results revealed wide spread prevalence of CMD and CMBs throughout the cassava growing areas thus threatening cassava production. Although, CMD severity varied significantly among sampled districts (X^2=11.37; $P \leq 0.05$), the mean severity (2006/7; 2.95, 2010; 3.9) for both surveys illustrated that the disease was severe with pronounced leaf mosaic and distortion patterns. Altitude and severity had a weak insignificant correlation suggesting that CMD is likely to be severe regardless of where cassava fields are in the country (2006/7 r= 0.05; P=0.87, 2010

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r=0.12; *P*=0.70). Though significantly variable among districts (X^2=9.87; $p \leq 0.05$), white flies population did not significantly affect percentage leaf incidence and CMD severity indicating that use of clean planting materials was critical for the management of the disease (leaf incidence r=0.14; *P*=0.67, severity r=0.17; *P*=0.61). DNA analysis showed that almost all CMD in the country is caused by EACMV-like viruses which represented 98.5% and 86% of all begomoviruses detected in 2006/7 and 2010 respectively. In view of the continued widespread occurrence of CMD-causing viruses in Malawi, greater emphasis needs to be placed on the development and application of control measures.

Keywords: Cassava, Cassava Mosaic Disease (CMD), Begomoviruses, Surveys, Severity, Leaf incidence

INTRODUCTION

Agriculture is the main driver of the economy in Malawi, contributing 39% to the gross domestic product (GDP) and employing 84% of the national workforce (NS0, 2010). Maize is the main staple crop in Malawi accounting for 54% calories intake of households and covering the largest cultivated land which has remained constant (mean=1,735,000 hectares) over the years though maize yield fluctuates due to various reasons (EAD, 2010; Minot, 2010). Nevertheless Cassava (*Manihot esculenta)* ranks second as the most important food crop and third as a cash crop (Chipeta and Bokosi, 2013). It is staple crop for about 30-40% of Malawi' population contributing 7% of total caloric intake particularly along the lake shore areas of Karonga, Rumphi, Nkhata Bay, Nkhota Kota, Salima and Mangochi (Moyo et al., 1999; Chiwona-Kaltun and Mkumbira, 2000; Chipeta and Bokosi, 2013).

Cassava production in Malawi and generally in Africa is severe hampered by Cassava Mosaic Disease (CMD) which has been known since 1894 (Thresh et al. 1994; Legg et al., 2006). It is estimated that CMD is responsible for 20 to 95% root yield loss (Otim-Nape et al., 19994) and reduced availability of good quality planting material. CMD incidence and severity surveys conducted in Malawi in 2004 and 2007 showed that CMD was widely distributed and incidence and severity of the disease was generally high in the fields (Yajima et al., 2005; IITA/SARRNET, 2007).

In Africa, CMD is caused by seven white fly-transmitted species of circular single-stranded DNA genome *begomoviruses (*Family *Geminiviridae): Africa cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) *and South African Cassava mosaic virus* (SACMV) (Briddon and Markham, 1995; Fauquet and Stanley, 2003; Fauquet et al., 2008). However, Hong et al., (1993) and Zhou et al., (1997) recognized an additional species as the Uganda variant of EACMV (EACMV-Ug) and Harimalala et al., (2012) recently described a new cassava mosaic virus species affecting cassava in Madagascar as *Cassava mosaic Madagascar virus* (CMMV).

Surveys to determine the incidence and severity of CMD and of cassava mosaic begomoviruses (CMBs) have been done elsewhere (Legg and Thresh, 2003) as well as in Malawi. Nyirenda et al., (1993), Sweetmore (1994), Ogbe et al., (1997), Theu and Sseruwagi (2003), Yajima et al., (2005) and IITA/SARRNET, 2007conducted surveys on CMD incidence and severity and whitefly populations in Malawi. Though reported surveys generated significant information on the status of CMD in Malawi, the virus diagnostic methods used were either leaf symptom based (Nyirenda et al., 1993; Yajima et al., 2005; IITA/SARRNET, 2007) or serological diagnostic test (ELISA) based (Sweetmore, 1994; Ogbe et al., 1997; Theu and Sseruwagi, 2003). Both methods have limitations in detecting cassava mosaic begomoviruses (CMBs) in symptomless leaves. In fact ELISA fails to detect EACMV in mixed infection with ACMV or differentiate between ACMV and EACMV-Ug which have similar coat protein (Sseruwagu et al., 2004). Therefore, this paper reports arguably the first DNA based CMD virus detection survey done in a local laboratory in 2007 and another conducted in a Nigerian laboratory in 2010. DNA based diagnostics are known to overcome limitations experienced by leaf symptom and ELISA based methods and have variously provided important information on the status of CMBs (Ogbe et al., 2003).

MATERIAL AND METHODS

Sample collection

A total of 176 (2006/7 survey) and 120 represented by randomly selected 50 (2010 survey) symptomatic stakes were collected from cassava growing districts mainly along the Lake Malawi

shore. Both surveys sampled from the same cassava growing districts except Mzimba, Kasungu and Chiladzulu for the 2006/7 survey and Mangochi, Machinga, Phalombe, Chikwawa, Ntcheu and Dedza for the 2010 survey (Figure 1). The diseased cassava stakes were planted in a screen house at Chancellor College, University of Malawi. In each district, the first cassava field was chosen at random and the rest were sampled approximately every 10 kilometers along major roads across the country. In districts where the disease was minimal, purposive sampling was employed. In each chosen field only predominant variety was sampled according to systematic random sampling procedures described in Sseruwagi et al., (2004).

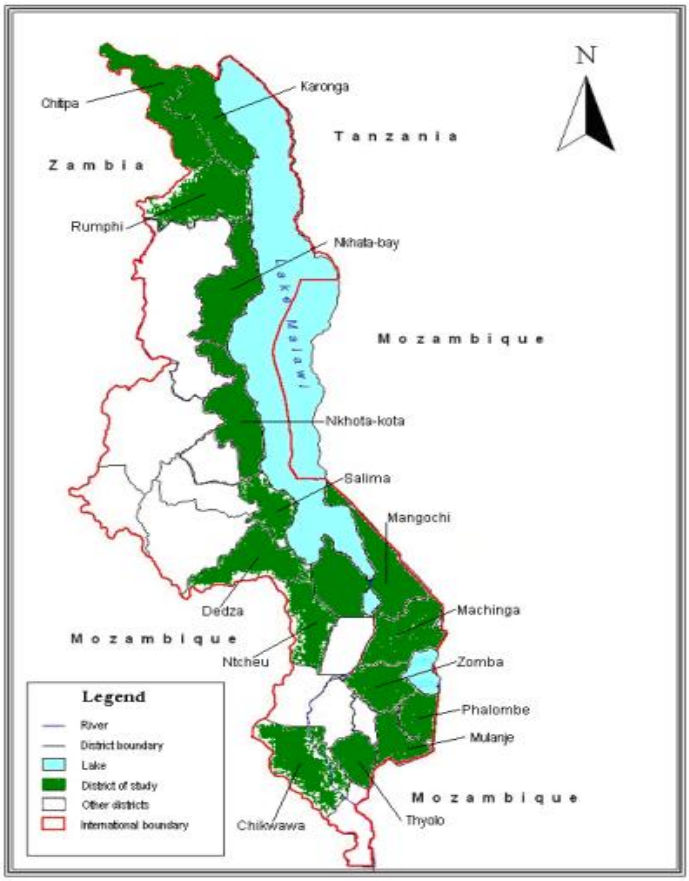

Figure 1 Map of Malawi showing sampled districts during the 2006/7 survey

Field sample assessment

Data collection sheet as described by Sseruwagi et al., (2004) was used for field sample assessment and data collection in both surveys. Data parameters on the data sheet included;

incidence and severity of CMD, white fly abundance and geographical positioning determinants. Incidence was calculated as the number of visibly diseased plants in relation to the total number of assessed plants while severity was determined as the intensity of symptom expression rated on a scale of 1-5 (Sseruwagi et al., 2004).

DNA extraction

Total genomic DNA from 2006/7 and 2010 surveys was extracted from young symptomatic leaves using procedures described by Dellaporta et al., (1983) and Gawal and Jarret (1991) respectively with a few modifications. For the 2010 survey, the sampled leaves were pressed between paper sheets in a herbarium press, allowed to dry and taken to the Virology and Molecular Diagnostics Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, for CMBs detection. The Gawal and Jarret (1991) procedures were carried out in Chancellor College, Biological Sciences department, DNA laboratory of the University of Malawi. It involved punching of two to three leaf discs into 2.0ml microfuge tube and grinding with the aid of Carborundum using a glass micropestle mounted on Industro Power Tools grinder. CTAB DNA extraction buffer (400µl; preheated at 60°C) was added, and the mixture incubated in an Advantec water bath at 60°C for 30 min with mild shaking. Thereafter, 400µl of chloroform:isoamylalcohol (24:1) was added to the homogenate followed by centrifugation at 15000 rpm for 10 min. The supernatant (350µl) was precipitated in 210µl cold Isopropanol in 1.5 ml microfuge tube placed under -20°C. The DNA pellet was separated from the suspension by centrifugation at10000 rpm for 5min and decanting the supernatant. The pellet was rinsed in 500µl 70% ethanol and centrifuged again at 10000 rpm for 1 min. The ethanol was decanted and the DNA air-dried for 15min and rehydrated in 50µl low TE buffer and stored at 4°C.

PCR analysis

Species specific diagnostic primers for EACMV DNA B, EACMKV (2006/7 survey only), EACMZV (2006/7 survey only), SACMV (CP), SACMV, ACMV, EACMMV (2010 survey only), EACMCV (2010 survey only) and EACMV-UG (2006/7 survey only) were employed to detect viruses using PCR and agarose gel electrophoresis (Table 1).

Template DNA (2µl) was placed in 0.2ml PCR tube to which 10.5µl of the PCR master mix was added making a final reaction volume of 12.5µl. The PCR master mix cocktail consisted of 5.7µl

PCR grade water (ddH_2O), 1µl of 10mM dNTP mix, 1.25 µl of 10 x PCR buffer, 1.6µl of 25mM magnesium chloride ($MgCl_2$), 0.2µM (0.75 µl) each of both forward and reverse primers, 0.06µl of 5U *Taq polymerase.*

The amplifications were carried out in a Mastercycler gradient 5331 Eppendorf Version 2.30.31-09 (2006/7 survey) and GeneAmp 9700 PCR® thermal cycler (Applied Biosystems, UK) (2010 survey). The PCR conditions were: one cycle at 95°C for 5 minutes, followed by 10 amplification cycles, each consisting of a 30s denaturing step at 94°C, 15s annealing step at primer specific temperature, and a 30s extension step at 72°C. This was followed by another 25 cycles, each consisting of denaturing step at 89°C for 30s, annealing step at primer specific temperature for 15s and extension step at 72°C for 15s. The final extension was at 65°C for 20min followed by a soaking temperature of 4°C. The Virology and Molecular Diagnostics Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria had slightly different PCR cocktail and amplification conditions though the final reaction volume was the same for both laboratories.

Loading buffer dyed PCR products were separated by electrophoresis in 1.5% agarose gels using TAE buffer for 1 hour at 100 volts. Amplicons were visualized by staining with 10mg/ml ethidium bromide and photographed under ultraviolet light illumination. Samples were scored for presence (CMBs positive) or absence (CMBs negative) of amplified products using 1kb DNA ladder (Promega) as a size marker.

Table 1: Primers used for PCR analysis of cassava mosaic begomoviruses

Virus	Name of Primer	Sequence (5'-3')	Target Region
ACMV	JSP001	ATGTCGAAGCGACCAGGAGAT	5'ACMV/EACMV CP
	JSP002	TGTTTATTAATTGCCAATACT	3'ACMV CP
	ACMV-AL 1/F	GCGGAATCCCTAACATTATC	AC1
	ACMV-ARO/R	GCTCGTATGTATCCTCTAAGGCCTG	AV2
EACMV	UV-AL3F	TACACATGCCTCRAATCCTG	AC3
	UV-AL1/R2	CTCCGCCACAAACTTACGTT	AC1

EACMV	EAB555F	TACATCGGCCTTGAGTCGCATGG	EACMV DNA-B
	EAB555R	CTTATTAACGCCTATATAAACACC	EACMV DNA-B
SACMV	SACMP-CP3	CCTTTATTAATTTGTCACTGC	CP
	SACMV-CP5	GCTGTCCCCATTGTCCARGGN	CP
	SACMV(CP)-F	TGTGATCCGAGCCCGTGN	CP
	SACMV(CP)-R	ATGGGGCTATTGCCATAGN	CP
EACMMV	EACMAL-L3-F	TACGCATGCCTCTAATCCAG	DNA-A
	EACMAL-L1-R	TTCCGCCACAACCTTATGTA	DNA-A
EACMCV	VNF031	GGATACAGATAGGGTTCCCAC	DNA-A
	VNF032	GACGAGGACAAGAATTCCAAT	DNA-A
EACMV-Ug	UV-AL1-F	TGTCTTCTGGGACTTGTGTG	AC1
	ACMV-CP/R3	TGCCTCCTGATGATTATATGTC	CP
EACMZV	SA/EA-KE2/KE3/ZV-F	AGCGGAACCCATCACN	-
	EACMZV-R	CAAGCGTTTTAAAATACN	-
SACMV	SA/EA-KE2/KE3/ZV-F	AGCGGAACCCATCACN	-
	SA/EA-KE3R1	TACGCCAAGGCTCTTAN	-
	SA/EA-KE2/KE3/ZV-F	AGCGGAACCCATCACN	-
	SA/EA-KE3R2	AACCCCAACCAATAAAN	-
EACMKV	SA/EA-KE2/KE3/ZV-F	AGCGGAACCCATCACN	-
	SA/EA-KE3R1	TACGCCAAGGCTCTTAN	-
	SA/EA-	AGCGGAACCCATCACN	-

KE2/KE3/ZV-F		
SA/EA-KE3R2	AACCCCAACCAATAAAN	-

Data analysis

CMD symptom severity data from different cassava growing areas were compared using Chi-square tests while incidence data were analyzed using Kruskal-Wallis test for non-parametric data. Correlations between variables were determined using Spearman' rank correlation coefficient from Genstat for Windows 7.1 and Pearson product moment correlation coefficient from SPSS13.0 for Windows. Arc View GIS was used to prepare CMD distribution map (Figure 2).

RESULTS AND DISCUSSION

Adult White flies count, leaf incidences and severity of CMD

In general, leaf incidence field observations during both surveys revealed prevalence of CMD and CMBs throughout the main cassava growing areas (Figure 2) threatening sustainable cassava production in the country since CMD is responsible for 20 to 95% root yield loss (Otim-Nape et al., 19994). The 2010 survey revealed an average leaf incidence of cassava mosaic disease (CMD) of 26.45% (Table 2) and there were significant variations in CMD severity among districts (X^2=11.37, $p \leq 0.05$). The 2010 survey mean CMD severity (2.95) was lower the that of 2006/7 survey (3.9) but both showed that the disease was severe with pronounced mosaic pattern on most leaves and distortion of at least lower one third of the leaflets according to the CMD symptom scale of 1-5 (Sseruwagi et al., 2004). This result concurs with other Malawi surveys' findings (Yajima et al., 2005; IITA/SARRNET, 2007). The maximum CMD severity score of 4 was observed in Nkhota kota only in 2010 while the 2006/7 survey registered a maximum severity score of 5 in most districts except in Rumphi, Dedza, Ntcheu and Chikwawa suggesting that the disease was more severe in 2006/7 than in 2010.

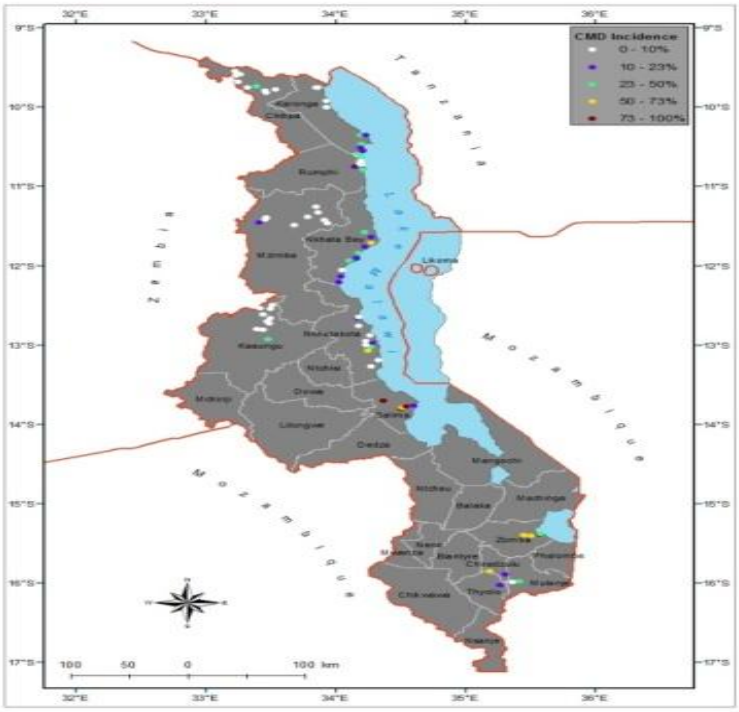

Figure 2: Map of Malawi showing distribution of CMD during the 2010 survey based on leaf incidence

Table 2: Total districts sampled, mean incidences and severity of CMD and adult whiteflies per plant

Sampled District	Mean altitude(m) (2010)	Mean altitude (m) (2006/7)	Mean CMD severity (2010)	Mean CMD severity (2006/7)	Mean CMD leaf incidence (%) (2010)	Mean count of adult whiteflies (2010)
Thyolo	726	980	3.2	3.6	10	1.35
Mulanje	717	660	3	3.3	33.3	0.1
Chiradzulo	807	-	2.8	-	43.3	0.21
Zomba	672	793	2.7	4.4	61.3	0.69
Salima	507	516	3	4	61.3	0.7
Nkhota kota	507	521	3	4.2	15.3	0.23
Nkhata Bay	518	538	3	3.3	29	0.28
Rumphi	589	975	2.9	3.8	32.3	0.71
Karonga	504	486	2.8	4	19.7	0.25
Chitipa	1312	1435	3.1	3.8	5.3	0.28
Mzimba	1214	-	2.6	-	3.3	0.36
Kasungu	1171	-	3.3	-	3.3	0.25
Chikwawa	-	87	-	3.5	-	-
Phalombe	-	722	-	4	-	-
Machinga	-	651	-	4.1	-	-

Mangochi	-	580	-	4	-	-
Ntcheu	-	490	-	4	-	-
Dedza	-	552	-	4	-	-
Mean	**770.33**	**665.7**	**2.95**	**3.9**	**26.45**	**0.45**

CMD severity did not significantly correlate with field altitude in both surveys neither did it significantly correspond with percentage leaf incidence nor adult white flies count in 2010 (Table 3). This result implies that the disease is likely to be severe regardless of altitude of cassava fields contrary to common understanding that cassava disease pressure is high in lower than higher altitudes (Nyirenda et al., 1993; Alicia et al., 2007). This finding is worrisome since the importance of cassava is increasing in Malawi due to drought tolerance, low inputs requirements and flexibility in planting, harvesting and storage and hence its production has extend to traditionally non cassava areas with higher altitude including areas surrounding commercial cities (MoAFS, 2007).

The study has also revealed that adult white flies' population does not vary with field altitude. This is evident by insignificant negative correlation between adult white flies count and field altitude (Table 3). Number of adult white flies was also shown not to significantly affect percentage leaf incidence and CMD severity (Table 3). The result agrees with Theu and Sseruwagi (2003) study which recognized that there was limited CMD spread through white flies' activity and that *Bemisia afer*, a non vector for CMD other than *Bemisia tabaci* the main CMD vector was the predominant species of cassava whiteflies in Malawi. This observation indicates that though white flies were present throughout the sampled cassava growing areas and their populations significantly varied among districts (X^2=9.87; $p \leq 0.05$), CMD severity variations in the sampled areas was not a factor of white flies population only but also of other sources of CMD such as diseased planting materials. Use of diseased cuttings has long been recognized as the main source of CMD infection in Malawi and elsewhere (Theu and Sseruwagi, 2003).

Table 3 Pearson product moment correlation coefficient (r) analysis of mean Cassava Mosaic Disease (CMD) parameters

Parameter 1	**Parameter 2**	**Coefficient (r)**	***P* value**	**Significance**
		2006/7 survey		
Severity	Field altitude	0.05	0.87	ns
		2010 survey		
Severity	Field altitude	0.12	0.70	ns

Leaf incidence (%)	Field altitude	-0.58	0.05	**
Leaf incidence (%)	Severity	-0.35	0.27	ns
Leaf incidence (%)	Adult White fly count	0.14	0.67	ns
Severity	Adult White fly count	0.17	0.61	ns
Field altitude	Adult White fly count	-0.19	0.55	ns

Ns=not significant; **= significant at $P \leq 0.05$ level (2–tailed)

Cassava mosaic begomoviruses detection

Begomoviruses DNA based detection analysis of samples from both surveys showed that East African Cassava Mosaic Virus (EACMV) - like species were most dominant cassava begomoviruses representing 98.5% and 86% of all begomoviruses detected in the 2006/7 and 2010 surveys respectively (Table 4; Figure 3).

Table 4 Cassava begomoviruses detected in Malawi during the surveys

Virus species detected	2006/7 positive in %	2010 positives in %
ACMV	1.0	0
EACMV	79.6	32
SACMV	0.5	14
EACMMV	-	18
EACMCV	-	36
EACMKV	12.4	-
EACMZV	6.5	-
EACMV-Ug	0	-

- = primers for the virus species not available for detection analysis

The 2006/7 survey samples showed that in general East Africa Cassava Mosaic Virus (EACMV) species was wide spread throughout all the cassava growing areas in the country, implying that it was responsible for most of the CMD encountered in the country (Figure 3; Table 5).

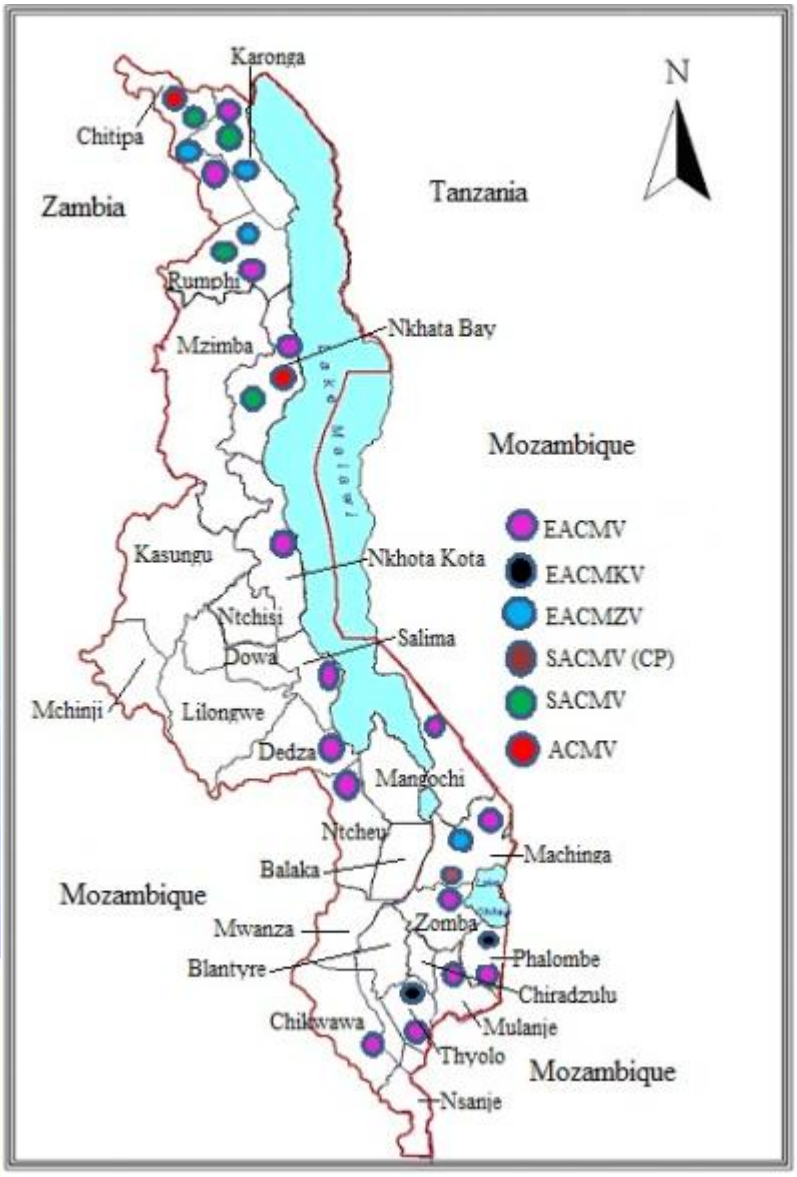

Figure 3: Map of Malawi showing CMD virus species distribution (2006/7 Survey)

The 2010 survey did not detect ACMV in all samples analysed but the 2006/7 survey registered 1% of all detected begomoviruses as ACMV and was restricted to the northern districts of Chitipa and Nkhata Bay while EACMKV was only found in the south (Thyolo and Phalombe) (Figure 3; Table 5). EACMZ, while absent in the centre, was wide spread in the north (Chitipa, Karonga and Rumphi) and was also encountered in Machinga (south) (Figure 3; Table 5). SACMV was only diagnosed in the north (Chitipa, Karonga, Rumphi and Nkhata Bay) while SACMV (CP) existed only in one district in the south (Machinga) (Figure 3; Table 5). The 2006/7 virus detection results also showed that CMD in the centre, represented by the district of

Salima and Nkhota Kota was caused by EACMV only while in the north and south, it was caused by four different viruses (Figure 3). The 2006/7 study did not detect the devastating EACMV-Ug species however the same samples showed EACMV-Ug when analysed using the rolling circle amplification-based PHI29 DNA polymerase method, RFLP, Cloning and Sequencing in the International Laboratory for Tropical Agriculture Biotechnology (ILTAB) of Donald Danforth Plant Science Centre (USA) (data not shown). The finding from ILTAB may strengthen fears that the cassava "pandemic" caused by EACMV-Ug which has more severe symptoms and was known to be moving southwards from its origin in Uganda at 20-30km per year (Legg and Ogwal, 1998), has reached Malawi. The EACMV-Ug, which devastated and halted cassava production for a year in Uganda, has been reported and confirmed in Kenya (Gibson, 1996), Sudan (Harrison et al., 1997), Tanzania (Legg and Okao-Okuja, 1999), DRC (Neuenschwander et al., 2002) and Rwanda (Legg et al., 2001). The 2010 survey confirmed the presence of EACMV, EACMCV, EACMMV, and SACMV in Malawi (Table 4).

Table 5 Cassava begomoviruses detected in various districts in Malawi during the 2006/7 survey

SD	Viruses detected																	
	ACMV			EACMV			EACMKV			EACMZV			SACMV			EACMV-Ug		
	+ve	-ve	%+ve	+ve	-ve	%+ve	+ve	-ve	%+ve	+ve	-ve	%+ve	+ve	-ve	%+ve	+ve	-ve	%+ve
KK	0	7	0	5	2	**71**	0	7	0	0	7	0	0	7	0	0	7	0
NB	1	21	**5**	21	1	**95**	8	22	**36**	0	22	0	0	14	0	0	22	0
RU	0	15	0	15	0	**100**	7	15	**47**	4	11	**27**	0	8	0	0	15	0
CP	1	10	**9**	11	0	**100**	5	11	**45**	1	10	**9**	0	6	0	0	11	0
KA	0	19	0	19	0	**100**	3	19	**16**	5	14	**26**	0	16	0	0	19	0
SA	0	6	0	5	1	**83**	0	6	0	0	6	0	0	6	0	0	6	0
DZ	0	1	0	1	0	**100**	0	1	0	0	1	0	0	1	0	0	1	0
NU	0	1	0	1	0	**100**	0	1	0	0	1	0	0	1	0	0	1	0
MH	0	18	0	15	3	**83**	0	18	0	0	18	0	0	18	0	0	18	0
MHG	0	22	0	20	2	**91**	0	22	0	3	19	**14**	**1**	21	**5**	0	22	0
ZA	0	31	0	29	2	**94**	0	31	0	0	31	0	0	31	0	0	31	0
MJ	0	7	0	5	0	**100**	0	7	0	0	7	0	0	7	0	0	7	0
PE	0	3	0	3	0	**100**	1	2	**33**	0	3	0	0	3	0	0	3	0
TO	0	11	0	9	2	**82**	1	10	**9**	0	11	0	0	11	0	0	11	0
CK	0	2	0	1	1	**50**	0	2	0	0	2	0	0	2	0	0	2	0

KK=Nkhota Kota; NB= Nkhata Bay; RU= Rumphi; CP=Chitipa; KA= Karonga; SA=Salima; DZ=Dedza; NU=Ntcheu; MH=Mangochi; MHG= Machinga; ZA=Zomba; MJ=Mulanje; PE=Phalombe; TO=Thyolo; CK=Chikwawa; SD= Sampling District

CONCLUSIONS

The surveys have documented the presences of ACMV, EACMV, SACMV, EACMMV, EACMCV, EACMKV and EACMZV with the predominance of EACMV-like viruses in Malawi. EACMV-like viruses are distributed through out the country where CMD is prevalent. The wide prevalence of CMD continues to pose a threat to sustainable cassava production in the country.

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