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Assessment of the responses of cassava (*Manihot esculenta*) breeder's germplasm to cassava mosaic virus (CMD) infection in Kenya

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Abstract

Cassava is an important cash crop in tropical lands and in Kenya is grown for income and food covering 77,502 ha with an output of 841,196 tons. Most cassava breeding programmes use symptom expression for selection and detection of viruses. Molecular and/or serological techniques to accurately diagnose viruses are required. Information on the responses of varieties to virus infection helps in assessing rate of crop degeneration over time. This study evaluated seven cassava breeders germplasm (08/363, F10-30-R2, Nl, Tajirika, E-Mariakani), a local susceptible (Kibanda meno) and improved (Shibe) to cassava mosaic disease (CMD) under high disease pressure conditions. The populations of whitefly vector, *Bemisia tabaci* (Gennadis), severity and incidence of CMD were recorded during the growth period. Infection was determined through symptom severity, disease incidence, ELISA and PCR assays. The mean numbers of whiteflies was higher in the dry season (3 MAP) compared to the wet season (6 MAP) of crop growth. The CMD disease incidence was highest in Kibanda meno (>70%) and lowest in Shibe and Tajirika. The mean CMD severities in the tested genotypes was highest in Kibanda meno (>1.5) and lowest in Shibe and Tajirika. PCR proved a more sensitive disease diagnostic tool as compared to ELISA in the detection of cassava mosaic virus. However both ELISA and PCR detected CMD in asymptomatic cassava samples. The study confirms that the absence of CMD infection cannot be assumed by only looking for the absence of visual symptoms on the leaves underscoring the need for multiple detection tools.

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Introduction

The importance of cassava in Africa cannot be under estimated as it is considered a resilient crop, urban and rural food staple, livestock feed and an industrial raw material (Nweke *et al.*, 2002). The crop supplies about 500 cal/day of food from the cassava roots to over 70 million people (Chavez *et al.*, 2005). Cassava is a perennial woody herb which is grown as an annual.

Cassava is widely consumed in Sub-Saharan Africa and parts of Asia. Statistics of cassava production indicate that Nigeria is the leading cassava producer in the world generating about 37 million tonnes in 2009 (FAOSTAT, 2009). In Kenya, the crop is grown on 77,502 ha with an output of 841,196 tons (FAO, 2007). A crucial impediment to cassava production in most nations in Africa is the Cassava mosaic disease (CMD) caused by single stranded DNA viruses in the family *Geminiviridae* and genus *begomovirus* (Faquet *et al.*, 2005). According to Thresh *et al.* (1994) worldwide, CMD causes an estimated loss of 1.5 billion dollars annually. The disease is severe on local genotypes causing characteristic severe distortion and leaf stunting almost on the entire plant (Lampte *et al.*, 2000).

CMD has been reported to cause 20-95% losses in various parts of the world (Hahn *et al.*, 1989; Otim-NAPE *et al.*, 1994); Braima *et al.*, 2000). In Kenya, this disease affects production of cassava in all major cassava growing regions namely Eastern, Nyanza, Western and coastal regions. The characterized CMD viruses worldwide are nine with seven of them being reported from sub-Saharan Africa (Alabi *et al.*, 2011). They include EACMV, ACMV, EACMCV (Fondong *et al.*, 2000) EACMKV (Bull *et al.*, 2006), EACMZV (Maruthi *et al.*, 2004), EACMMV (Zhou *et al.*, 1998), and the SACMV (Berrie *et al.*, 1998). Legg *et al.* (2011) reported that cassava mosaic begomoviruses are vectored by whiteflies, *bemisia tabaci* and spread by cuttings that are routinely used by farmers.

There are many methods of controlling disease pathogens which include chemical application, use of biological control, phytosanitation and utilization of resistant varieties. It has been determined that use of resistant varieties is cost effective and has minimum impact to the environment (Hogenboom, 1993). Once resistance has been established to a causal agent then it can be transferred from generation to generation. In Uganda the CMD epidermic has been controlled by use of resistant genotypes (Otim-Nape *et al.*, 2000).

Most cassava breeding programmes use symptom expression for selection and detection of viruses. In this programmes absence of virus infection is shown by absence of visual symptoms. It has been reported that the main significance of diagnosis is to remove sources of viral infection early in the crop cycle (Rozak and zdenka, 2013). Virus indexing schemes can be enhanced through the use of robust, reliable and sensitive detection tools, which include serological and nucleic acid based diagnostic techniques.

In Kenya no studies have been done on the CMD status of cassava breeder's germplasm using sensitive diagnostic tools. The aim of the study was to determine the infection status of the cassava breeder's germplasm using symptoms and sensitive detection tools like ELISA and PCR. These techniques are important for virus indexing to avail to breeders clean materials for further breeding efforts.

Materials and methods

A 12 month trial was established at the Kenya Agricultural Research Institute (KARI)- Mtwapa in Kenya. Mtwapa is located at an altitude of 5m above sea level. The site receives mean annual rainfall of 1000mm, and has a mean annual temperature of 32 C and sandy soil type.

The experiment was laid out as a randomized block design (RCBD) and replicated four times (Fig. 1). Forty two stem cuttings per variety were planted in a plot of 7 rows each measuring 6m long. There was a spacing of 1m between rows and 1m within rows. Five elite genotypes (08/363, F10-30-R2, Nl, Tajirika, E-Mariakani), a local susceptible (Kibanda meno) and a

local improved variety (Shibe) were used in this study. The cassava stock plants from which cuttings were obtained for planting were indexed by PCR and found to be CMD free. Plots were separated by 2m distance and kept weed free without application of neither inorganic fertilizers nor chemical pesticides.

Field symptom assessment

Four plants from each variety were randomly selected within the net plot and tagged from which data was collected. The CMD severity and incidence was recorded after 6, and 9 months after planting (MAP). The severity ratings were based on a visual scale of 1-5; with 1 indicating healthy plants and 5 denoting very severely affected plants (Hahn *et al.* 1989). The scoring scale used was as in Table 1,

Disease incidence

This was calculated by formulae below;

$$\text{Percentage Disease Incidence} = \frac{(N - n)}{N} \times \frac{100}{1}$$

N = Total number of observations

n = Total number of plants with no disease symptoms.

Whitefly survey

Whiteflies are the key vectors for cassava mosaic virus, hence their populations were determined to correlate them with CMD infection. Whiteflies feed on the five top most leaves (Maruthi *et al.*, 2004). Adult Whitefly populations in the tagged plants were counted on the five topmost leaves (Ariyo *et al.*, 2005) of each plant at 3, 6 and 9 months respectively.

Leaf sampling for laboratory analysis

2-3 symptomatic and asymptomatic leaves were collected from tagged plants and placed in small containers lined with silica gel and a layer of cotton wool for molecular analysis. They were then transported to the laboratory and kept at room temperature until analysis.

Serological and molecular virus detection

TAS-ELISA using polyclonal antibody (IgG) and monoclonal antibodies (Mab and RAM) (DSMZ) was done to detect the presence of CMD. Wells were initially coated with igG prepared by dissolving 10 µl IgG in 10 ml buffer. 100 microlitres of this solution were added to each microtitre plate and incubated at 37 C for 3h. The plates were then washed with PBS-T using wash bottle and soaked for a few minutes with washing repeated twice. The plates were then blotted by tapping upside down on paper towel. 100 microlitres of 2% skimmed milk in PBS-T was added to each well and left at 37 C for 30 min. The blocking solution was removed by tap-drying before 100 microliters test sample was added to duplicate wells. This was incubated overnight at 4 C and washed three times using PBS-T. 100 microlitres of MAb in conjugate buffer was added to each well at 37 C for 2 hours before washing thrice using PBS-T. 100 microlitres of RAM-AP in conjugate buffer was added to each well and incubated for 2h at 37 C. Washing was then done three times before addition of 200 microlitres freshly prepared substrate to each well and incubated for 60 min at 37 C. Colour reaction was read at 405 nm using ELISA plate reader Incubation of plates was done or one hour after substrate addition and absorbance values read using a reader at 405nm. Negative and positive controls were included in each microtiter plate to enhance tests validity a value at least two times higher than negative control was considered positive. The experiments were repeated twice and ELISA absorbance values at 405 nm are average of two replicates each. PCR was also done to determine the sensitivities compared to Elisa in detection of low virus level in plants.

For PCR, Extraction of total DNA from cassava leaves was done according to CTAB protocol (Doyle and Doyle, 1990) with modifications. Integrity of DNA was determined by a sample of 2 µl run in a 2% agarose gel in TBE buffer for 30 min stained with 1 µl ethidium bromide. The DNA amplification was done by using a virus specific primer (Table 1) (Fondong *et al.*, 2000) in a 14.6 µl reaction mix containing 2.5 µl 10x reaction buffer, 0.2 mM dNTPs, 0.2 mM MgCl2, 0.2 µl taq polymerase, 0.25 µM forward and reverse primers, 10 µl water and 1 µl of template DNA. Amplification reaction was done in a 96 well Thermal

cycler. The PCR cycle used for the amplification had an initial denaturation step at 95°C for 5 min then 30 cycles of denaturation at 95°C for 0.3 min, primer annealing at 58°C for 0.3 min and elongation at 72°C for 0.45 min. This was then followed by a final elongation step at 72°C for 5 min. 10 µl aliquots of PCR products were mixed with 2 µl of loading dye and electrophoresed on 2% agarose gel at 90 V for 1 h. Bands obtained were viewed in a gel documentation system and photos taken.

Data analysis

Before analysis of variance (ANOVA), severity scores for all plants and whitefly numbers were subjected to square root transformation. Disease incidence values were arcsine transformed before analysis of variance (ANOVA). Data was analyzed by Genstat Statistical software, Version 12 and treatment means compared by Least Significance Difference test. Samples that had A_{405} values twice the value of healthy controls in ELISA were considered positive. Bands from PCR for EACMV that had the expected fragment of 556bp were recorded for each variety.

Results

Incidence of CMD in cassava genotypes

Results on CMD incidence are presented in Table 3. The mean CMD incidence varied significantly ($p<0.001$) among the genotypes in the range of 0-74.97 at 6MAP and 0-82.47 at 9MAP (Table 3). At 6MAP, Kibanda meno recorded the highest mean incidence at 74.97 followed by E-Mariakani with a mean incidence of 26.24. The lowest mean incidence was recorded in varieties Tajirika, Shibe, Nl and F10-30-R2 with 0 each. At 9MAP, highest mean disease incidence was still in Kibanda meno at 82.47. The lowest mean disease incidence was in varieties Nl, Shibe and Tajirika (Table 3).

Table 1. Disease rating and corresponding symptom expression for cassava mosaic disease (CMD).

Rating	Symptom
1	No symptoms observed
2	Mild chlorotic pattern on entire leaflets or mild distortion at base of leaflets
3	Strong mosaic pattern on entire leaf, and narrowing cum distortion of lower one-third of leaflets
4	Severe mosaic distortion of two-thirds of leaflets and general reduction in leaf size
5	Severe mosaic distortion of four fifths or more leaflets, twisted and misshapen leaves.

Table 2. Primer sequences used for amplification cassava mosaic virus in PCR.

Primer name	Sequence (5'→ 3')	Virus species	Target region	Expected size (nt)	Reference
EAB555/F	TACATCGGCCTTTGAGT CGCATGG	CMBs	DNA B	556	Fondong *et. al.,* 2000
EAB555/R	CTTATTAACGCCTATAT AAACACC				

Symptom severity

The results of symptom severity in the varieties are indicated in Table 4. At 6 MAP, there were significant differences (<.001) amongst the tested genotypes. The highest mean severity was in Kibanda meno (1.5) which was different from E-Mariakani (1.18), 08/363 (1.02), Tajirika (1.0), Shibe (1.0), Nl (1.0) and F10-30-R2 (1.0). There were also significant differences (P<.001) at 9 MAP in disease severity amongst the genotypes. The mean severity was highest in Kibanda meno (1.657) followed by E-Mariakani with mean incidence of 1.18. The lowest severity of 1 was recorded in Tajirika, Shibe and Nl (Table 4).

Whitefly Numbers

There were significant differences ($p<0.001$) in whitefly (*Bemisia tabaci)* populations among the tested varieties and also with the season of sampling (Table 5). The mean whitefly numbers in the dry season (September, 2013) were higher compared to

the wet season (December, 2013). In the dry season Nl had the highest mean whitefly numbers (4.5) followed by Kibanda meno (3.9), E-Mariakani (2.7), F10-30-R2 (2.5), Shibe (1.4), Tajirika (0.9) (Fig 3). In the wet season the highest mean whitefly numbers were recorded in Nl (2.5) followed by Kibanda meno (2.3) with the lowest on varieties Shibe (1.1) and E-Mariakani (0.6) (Table 5).

Table 3. Mean CMD incidence at 6 and 9 months after planting (MAP).

Variety	6 MAP	9 MAP
F10-30-R2	0a	29.99a
Nl	0a	0a
Shibe	0a	0a
Tajirika	0a	0a
08/363	7.5ab	26.24a
E-Mariakani	26.24b	26.24a
Kibanda meno	74.97c	82.47a

Means with the same letter in the same column are not significantly different based on Turkey test at P=0.05; Mean incidence values have been arcsine transformed before analysis of variance.

Table 4. Mean severities for Cassava genotypes used in this study over different periods of the crop's development.

Cassava Genotypes	6 MAP	9 MAP
Nl	1^{a}	1^{a}
Shibe	1^{a}	1^{a}
Tajirika	1^{a}	1a
F1O-30-R2	1^{a}	1.10^{a}
08/363	1.03^{a}	1.13^{a}
E-Mariakani	1.18^{a}	1.18^{a}
Kibanda meno	1.59^{b}	1.66^{b}
CV	16.1	16.7
L.S.D	0.1255	0.135

Means with the same letter in the same column are not significantly different based on Turkey test at P<0.05, Mean severity values have been square root transformed before analysis of variance.

There were significant positive correlation (<.001) between whitefly numbers and CMD severity in the tested varieties ranging between (r=0.057-0.663) (Table 6). The highest positive correlation coefficient was in E-Mariakani and weakest in Kibanda meno.

Validation of PCR for the detection of CMD in cassava genotypes from breeder's materials in large scale analyses

Table 7. below indicates the number of samples found to be true positives (Samples testing positive both in ELISA, PCR and exhibiting CMD symptoms) , true negatives (Samples testing negative for both ELISA, PCR and not showing CMD symptoms), false negatives and false positives out of analysis of 112 cassava materials (Fig. 4). 35 samples showed CMD symptoms in the field representing 31% of the total samples. PCR detected CMD virus in 57% of the samples analyzed as compared to 28% in ELISA (Table 7). There was a low frequency of false negatives in PCR (10 out of 112) making the negative results highly reliable (Fig. 4). ELISA had higher numbers of false negatives (23 out of 112). TAS-ELISA correctly detected 75% (58 out of 77) of the asymptomatic samples and the lowest false positives (19 out of 77). The highest frequency of false positives were recorded in PCR (45 out of 112).

Table 5. Mean numbers of *Bemisia tabaci* recorded on the seven cassava accessions during the September 2013, December 2013 and February 2014 periods.

	Mean whitefly numbers		
Genotype	3MAP	6 MAP	9 MAP
E-mariakani	2.74^{ab}	0.68^{a}	0.68^{a}
Shibe	1.49^{a}	1.14^{ab}	1.14a
08/363	1.61^{a}	1.31^{abc}	1.31^{ab}
Tajirika	0.98^{a}	1.59^{abcd}	0.89^{a}
F1O-30-R2	2.51^{ab}	1.81^{bcd}	1.47^{ab}
Kibanda meno	3.98^{bc}	2.39^{cd}	2.25^{bc}
Nl	4.58^{c}	2.57^{d}	2.68^{c}
CV	67.60	63.20	69.40
L.S.D	1.212	0.7256	0.7229

Means with the same letter in the same column are not significantly different based on Turkey test at P=0.05.

Discussion

From this study, it is evident that varieties respond to infection in different ways as indicated by differing incidence and severity levels. There was a general increase in incidence with the age of the crop across all the varieties. The highest incidence of CMD in this study was at 9 MAP when the crop is mature. There has been a record of variation in CMD infection amongst various cassava genotypes at different periods of assessment. This has been due to genotypic levels of susceptibility and resistance to the virus favoring plant transmission agents during crop's growing period (Fokunang *et al.,* 2000).

Table 6. Pearson Correlation Coefficient between whitefly numbers and CMD severity at 3 MAP (September 2013).

Sample	Correlation Coefficient (r)
08/363	0.596
E-Mariakani	0.663
Kibanda meno	0.057
Shibe	0.540
Tajirika	0.59
Nl	0.118
F10-30-R2	0.357

Disease incidence has been linked with fluctuations in whitefly numbers influenced by climatic factors like rainfall, wind and temperature (Fauquet and Fargette, 1990). In most of the genotypes, it took long for the development of severe symptoms like narrowing and distortion of leaves and mosaic pattern. Thresh (1994) reported that CMD severity is severe in susceptible genotypes than in resistant varieties.

Differences in symptom development has been linked to prevailing temperatures during the experiment. In papaya, Papaya ringspot virus symptoms has been affected by temperature in which the viral accumulation and phenotypic symptom expression were higher at (26-31 C) (Mangrauthia *et al.*, 2009). The reason for increased symptom expression at higher temperatures in Papaya was said to be induction of host defensive response against biotic stress (high temperatures) and failure of viral protein HC-Pro to antagonize the effect of host defence gene silencing mechanism (Alviar *et al.*, 2012).

It has been reported that disease incidence is a function of many factors such as vector population, climatic conditions and genetic background of the host plant. Disease severity on the other hand is dependent on the virus strain, age of plant at infection, climatic conditions and type of genotype (Bakker, 1970). In rice yellow mosaic virus, temperature has been linked with expression and intensity of foliar symptoms.

Table 7. Validation of the detection of CMD through PCR and ELISA in cassava genotypes.

Symptomatic	Asymptomatic
True positive	False positive
False negative	True negative

	Asymptomatic samples	
	PCR	ELISA
True Negative	32	58
False positive	45	19
	Symptomatic	
False Negative	10	23
True positive	25	12

Whiteflies *Bemisia Tabaci* are widely distributed and important in agriculture. They can be involved in direct feeding and transmission of plant viruses (Perrings, 2001). This study reported variations in whitefly infestations in varieties and at different times of the year with higher numbers in the dry season (September) as compared to the wet season (December). This is in agreement with Appiah *et al.* (2012) who reported increased numbers of whiteflies on Jatropha accessions during the dry season than in the wet season. This could be due to the death of first instar larvae and suppression of oviposition caused by higher rainfall during the wet season.

Fig. 1. Field layout showing *Manihot esculenta* accessions planted at Kari-Mtwapa at 6 MAP.

A higher number of whiteflies were found in Kibanda meno (Susceptible) compared to other varieties. This coincided with a lower severity in Kibanda meno suggesting that there is no association between virus severity and whitefly populations in this variety. In a study on African cassava mosaic virus in Uganda there was no correlation found between the cassava mosaic virus and whitefly numbers (Otim-Nape *et al.*, 1994). In contrast Asare *et al.* (2014) and Otim-Nape *et al.* (2005) reported higher whitefly populations in cassava mosaic resistant genotypes than the susceptible ones. This was due to broadness and softness of leaves in resistant genotypes as compared to deformed, reduced and severely affected leaves of susceptible genotypes (Ariyo *et al.*, 2005; Omongo, 2003; Sserubombwe *et al.*, 2001).

The use of molecular methods (ELISA and PCR) to detect CMV in cassava proved to be effective in this study. A higher number of infected samples was obtained through PCR and ELISA than through the symptom expression. This concurs with a study done on infection of yams with viruses where it was confirmed that TAS-ELISA is consistent and sensitive compared with use of symptom expression (Toualy *et al.*, 2013). Detection with TAS-ELISA enabled detection of asymptomatic samples that would have been taken resistant if symptom observation was the

only virus indexing method employed.

More samples detected positive through the use of PCR than ELISA. Decreased reliability of ELISA has been linked to regional climatic conditions beside nature of tissue used for analysis (Torrance and Dolby, 1984).The expected 556 bp fragment of CMV coat protein gene was amplified in total DNA from both symptomatic and asymptomatic materials. Through these results it is apparent that PCR is a more sensitive tool to use in the detecting low level CMV virus. In this study PCR analysis detected CMV in samples that had tested negative in TAS-ELISA meaning that it has increased sensitivity (Martin, 1998) than ELISA hence significant in breeding programmes for selection of disease free germplasm. This finding is in agreement with Okorogri *et al.* (2010) who also reported disparities in detection by PCR and ELISA.

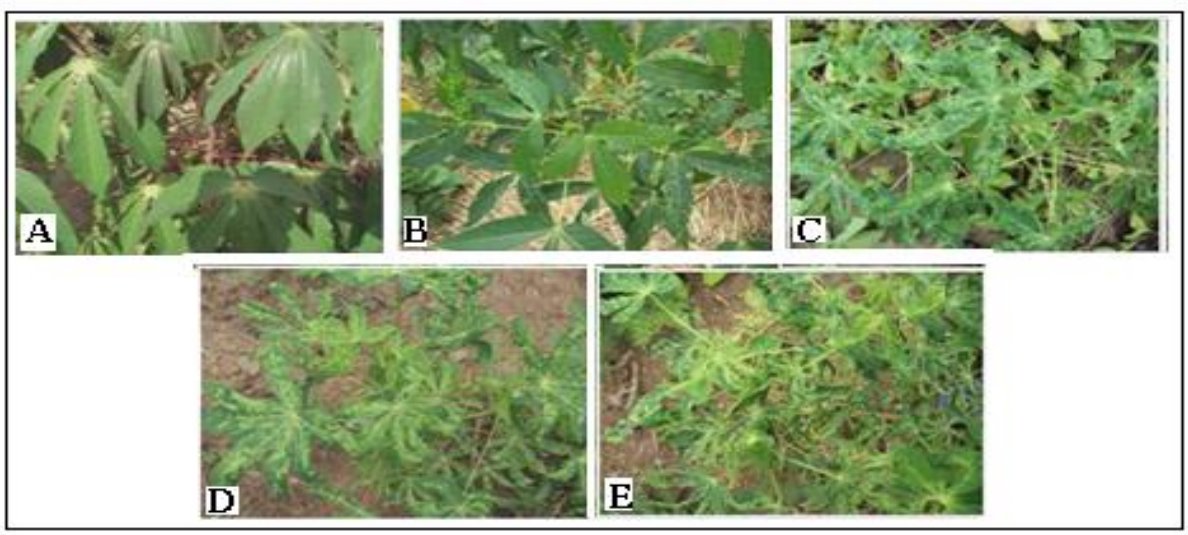

Fig. 2. Field infected cassava showing symptoms of CMD. (A) no symptoms (B) mild mosaic, (C) pronounced mosaic pattern on most leaves (D) severe mosaic, leaf curl, distortion, (E) severe mosaic, severe reduction of leaf area, severe distortion.

Fig. 3. Whitefly *Bemisia tabaci* infested cassava leaves

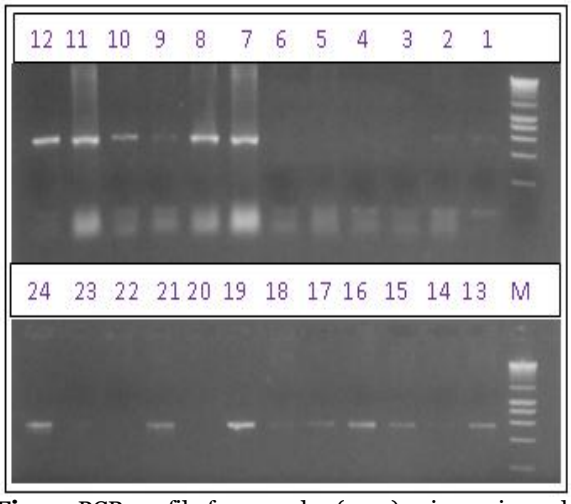

Fig. 4. PCR profile for samples (1-24) using universal primers EABB555-F/EAB555-R (Fondong *et al.*, 2000) for detection of all species of EACMV Lane 3: negative control from healthy plant; lane 12: positive control; lane M for 200bp DNA size marker.

Erroneous detections are common in selection and detection of virus diseased materials especially when

there are low virus titres. The number of false negatives in PCR was extremely low making the negative results highly reliable. This is in agreement with studies done on sharka disease caused by plum pox virus (PPV) where results from PCR reported a lower number of false negatives. On the other hand, there were reduced number of false positives in ELISA than in PCR making the reliability of positive results from ELISA highly reliable (Nieves *et al.*, 2009).

False positives recorded by molecular methods have been linked to virus infections being latent in the plant only detectable by highly sensitive equipment like real-time PCR. Secondly, recent inoculation of plant by CMD/ viluferous whiteflies with the virus titre still very low and restricted to the inoculation site could be the cause of false positives. Contaminants present during sampling, sample preparation and PCR has also been associated with false positives. On the other hand it has been reported that false negatives are linked to uneven distribution of viruses in plants or insufficient sensitivity of the detection methods.

Conclusion

The study confirms that the absence of CMD infection cannot be assumed by only looking for the absence of visual symptoms on the leaves. Serological and molecular methods were able to detect positive CMD samples from the breeder's germplasm where visual field diagnosis failed. This gives breeders better detection tools to enhance selection during breeding.

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