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Relationship between Symptoms Expression and Virus Detection in Cassava Brown Virus Streak-Infected Plants

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Abstract

Diagnosis of Cassava brown streak disease (CBSD) has for long been based on foliar and root symptoms expression on infected plants. Variability in patterns of symptoms expression between varieties and seasons however, has meant that symptom-based diagnostics are unreliable. The current study established the relationship between symptom expression on cassava plants and the infection with *Cassava brown streak virus* (CBSV) using RT-PCR diagnostic tool. It was established that manifestation of CBSD-like symptoms (particularly the foliar chloroses and necrotic blotches) may not be an absolute indication of CBSV-infection. Only 67% of tested samples were both foliarly symptomatic and infected by the virus. About 22% of samples were free from CBSV despite being foliarly symptomatic and 7% were CBSV-infected but foliarly symptom less. Some CBSV-infected plants did not exhibit any foliar symptoms although had root necroses. A few CBSV-free plants were regenerated from infected cuttings in one of the four tested cultivars, Albert. Five out of fifteen (33%) plants cv. Albert were symptom less and two out of the five (40%) were CBSV-free. The findings from this study suggest that symptoms-based diagnosing for CBSV infections is unreliable. As some of CBSV-infected plants tend to be considered CBSV-free due to lack of the disease symptoms, the scenario might have contributed to unlimited spread of CBSD through latently-infected planting materials.

Keywords: Cassava brown streak disease, *Cassava brown streak virus*, RT-PCR, symptomatology, virus distribution in plant parts

1. Introduction

Cassava brown streak disease (CBSD) was reported in the foot-hills of Amani Research Station in coastal areas of (Tanganyika) Tanzania in early 1930s (Storey, 1936). The disease is very serious and has been reported in many countries in the East, Central and Southern Africa, wherever cassava is grown. The disease has been reported in Tanzania (Storey, 1936; Nichols 1950), Uganda (Nichols, 1950), Malawi and Mozambique (Nichols, 1950; Hillocks et al., 1996; Sweetmore, 1994), Kenya (Nichols, 1950; Munga & Thresh, 2002) and Democratic Republic of Congo (Alicai et al., 2007, EARRNET, 2005). Most of locally grown cultivars in Burundi, Rwanda and Zambia are similarly affected by CBSD (James Legg, Personal communication). Up to 70% yield loss has been recorded in CBSD – affected susceptible cassava cultivars. The disease is very serious and has caused enormous devastation among the affected farmers particularly in Tanzania (Rwegasira et al., 2011). Recently, FAO experts have warned that the CBSD was putting cassava, a crucial source of food and income at risk and called for increase in funding, training, research, surveillance and other measures to help farmers and breeders. The disease also appears in a list of top six more serious biological threats to food security in the world published by Science (Pennis, 2010).

Previously, CBSD was reportedly caused by *Cassava brown streak virus* (CBSV) (*Potyviridae*: *Ipomovirus*) alone (ICTV, 2005; Monger et al., 2001). Recent finding has indicated that CBSD is caused by two distinct but closely related *Ipomoviruses*, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (Mbanzibwa et al., 2011). Infection of the susceptible cassava cultivars with either of the viruses would trigger manifestation of CBSD symptoms. In some instances, mixed infections by the two viruses leading to similar symptoms have been reported (Mbanzibwa et al., 2009). The disease symptoms are manifested on leaves, stems and roots of the susceptible cultivars through. Various forms of foliar chloroses, streaks on the stems and necrosis

in the root cortex of affected plants are among the important symptoms of CBSD (Jennings, 1960; Nichols, 1950; Storey, 1936) commonly used in CBSD diagnosis.

Recently, it was increasingly observed that some of the grown cultivars do not exhibit CBSD symptoms despite being infected by the virus (G. Rwegasira, unpublished data). In cultivars where CBSV-infection is not manifested through symptoms, false conclusions could be made considering it healthy although may be infected. Nichols (1950) reported similar confusion in CBSD symptom expression. Recent observation on some cassava cultivars whereby symptom less plants of the cuttings obtained from a previously CBSD-affected mother plants had been recorded, triggered a quest to investigate. The current studies aimed at determining the reliability on symptoms-based diagnosis of CBSD and establish the relationship between the symptom expression and CBSV-infection through molecular diagnostic techniques. The farmers' perceived possibility of regenerating CBSV-free plants from cuttings obtained from CBSV-infected mother plants was also examined.

2. Materials and Methods

2.1 Elucidating the Relationship between Symptoms Expression and Detection of CBSV

Diagnostic surveys were conducted throughout the major cassava growing areas in Tanzania in 2008/2009. Surveyed Regions included: the Coast, Dar es salaam, Morogoro, Lindi, Mtwara, Tanga and the Zanzibar and Mafia archipelagos (all in the Coastal Zone), Kagera, Kigoma, Mara and Mwanza (in the Lake Zone), and Iringa, Mbeya, Rukwa and Ruvuma (in the Southern Zone). Only cassava fields along accessible roads were surveyed by stopping at regular intervals of *ca*. 100 kilometres. Only fields of greater than six month-old crops were assessed and sampled. A total of 91 farmers' fields were covered and 91 leaf samples collected in triplicate from CBSD symptomatic plants (one triplicate leaf sample for each field). The sampled plant was uprooted and the cassava roots cross-sectioned to examine for internal CBSD-root symptom. Root symptoms were not assessed in 10 out of the 91 fields surveyed because the field owner completely denied researchers permission to uproot the plants. Symptom guides for CBSD (Hillocks & Thresh, 1998; Hillocks & Jennings, 2003) were used. In the absence of symptomatic plants, a sample was chosen from any of the available plants. The CBSD related symptoms observed on each sample were recorded. In addition, three cuttings (25 cm long) were obtained from each of the sampled plant and regenerated in pots in the screen house at Mikocheni Agricultural Research Institute (MARI) to be used as sources of backup samples and for repeated testing.

Test samples were collected using gloves and placed into self-sealable plastic bags and kept in the cool box after which, they were sent to MARI laboratory in Dar es Salaam for CBSV detection. The guanidium thiocyanate method was used for isolation of total RNA from the collected samples. Using CBSV coat protein (CP) gene specific primers (CBSV10/11) (Monger et al., 2001), triplicate RT-PCR was run at optimum conditions and the virus-infected samples were delineated. The RT-PCR results were compared to the field CBSD symptoms recorded from the samples.

2.2 RNA Isolation

Total RNA was extracted from 0.1g fresh leaf tissue in 4 M guanidium thiocyanate (Sigma, 59980) buffer mixed with 2-mercaptoethanol (Sigma, M3148) at a ratio of 1:125 (2-mercaptoethanol to guanidium) using a sterile mortar and pestle. Five hundred µl of the lysate was transferred to a 1.5 ml ependorf tube, 500 µl of 2.0 M sodium acetate added and the tube contents were thoroughly mixed. Four hundred µl of 24:1 [chloroform ($CHCl_3$) to isoamyl alcohol (C_5H_2O)] was added to the tube and the contents were mixed and incubated on ice for 10 minutes. Tubes were centrifuged at 13000 rpm for 15 minutes and 450 µl of supernatant transferred to new tubes. Five hundred µl of ice-cold isopropanol was added to precipitate the RNA and the tube was then incubated at -20^0C for 10 minutes. The chilled contents were centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. The RNA pellet was air-dried after washing with 500 µl of 75% ethanol and centrifuging at 13000 rpm for 2 minutes. Thirty five µl of RNase free water was added to dissolve the RNA pellet for analysis in RT-PCR.

2.3 Nucleic Acid Amplification by RT-PCR

Triplicate RT-PCR was performed in a one-step reaction using superscript ^{TM} III RT/Platinum® *Taq* Mix System (Invitrogen Life Technologies) using a GeneAmp PCR system 9700 (Applied Biosystems, UK). The primers, CBSV 10F and 11R (Monger et al., 2001) designed to amplify a 231 bp segment of the CP gene were used. Each 50 µl reaction mixture comprised 25 µl of 2X reaction mix (buffer with 0.4 mM of each of dNTP, 3.2 mM $MgSO_4$ and stabilizers), 1 µl of RNA, 0.4 µl of each of the forward and reverse primers, 1 µl of superscript RT-Taq (Invitrogen 12574-026) and 22.2 µl of sterile water. The PCR conditions were as follows; Initial cDNA synthesis and denaturation; at 55°C for 0.5 min. and 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min (in 35 cycles) followed by final extension at 72°C for 10 min.

2.4 Analysis of RT-PCR Product

RT-PCR products were electrophoretically separated in a 1.2 % agarose gels in 0.5X Tris Acetate EDTA (TAE) buffer, for 1 hour at 92 volts. Amplicons were visualised by staining with ethidium bromide (0.01 µl/ml) under ultraviolet (UV) light and recorded using an image analyser (Syngene).

2.5 Regeneration of Healthy Plants from Diseased Cuttings

This experiment was based on the observation in previous experiments that some cultivars, although obtained from the seemingly CBSD symptomatic mother plants, tended not to exhibit CBSD symptoms when replanted at the same or different locations (G. Rwegasira, unpublished data). During survey, farmers also indicated the occasional regeneration of symptom less plants from symptomatic ones. To clear the doubts, a potted experiment to determine whether CBSV-free plants may be obtained from diseased mother plants, at varying disease severity levels, was conducted in the screen-house at MARI. All planting materials were collected from CBSD symptomatic plants (severity levels 2 through 4), pre-confirmed for CBSV-infection by RT-PCR. Different sections of stem were used as sources of planting materials. The CBSV-infected cultivars: Albert, AR 49/2, Cheupe and Nachinyaya were tested. Stems about 2.5 m in length were cut into three major sections: the mature bottom portion, the middle semi-mature portion and the immature upper green portion. Five cuttings were obtained from each of the three sections in each cultivar. A total of 45 potted plants were tested for each cultivar. The whole experiment comprised 180 plants (pots). CBSD symptoms were recorded monthly, beginning at 2 months for three consecutive months. Detection of CBSV was done in leaf tissues collected from symptom less plants. The experiment was repeated three times at a three months interval.

3. Results

3.1 Types of CBSD Field Symptoms

CBSD foliar symptoms were observed in 81 out of 91 samples and root symptoms recorded in 47 out of 81 assessed (Figure 1. Major types of leaf and root symptoms recorded are shown Table 1). Chlorotic blotch was the major foliar symptom recorded followed by spots and vein chlorosis. At some instances, the chlorotic blotches appeared together with veinal chlorosis although such occurrence was rare. Brown mass or sepia necrosis and chalky necrosis were the most prevalent symptoms diagnosed in roots of CBSV-infected plants, with greater than 34 % and 19 % respectively.

3.2 Foliar CBSD Symptoms and CBSV-detection in Test Samples

CBSD was detected in symptomatic and symptom less samples (Figure 2). Sixty-seven percent of tested samples were both symptomatic and infected with CBSV. Twenty-two percent did not give an RT-PCR amplicon for CBSV despite being symptomatic and seven percent were CBSV infected but symptom less. Only four percent of tested samples were symptom less and CBSV-negative (Figure 3). Some plants without leaf and root symptoms were CBSV-infected (Figure 4, Plate 1a&b), a few CBSV-infected plants did not exhibit any foliar symptoms but had root necroses (Figure 4, Plate 2a&b) while some CBSV-free plants had CBSD-like foliar symptoms but no root symptoms (Figure 4, Plate 3a&b).

Table 1. CBSD leaf and root symptoms recorded during survey

Types of CBSD leaf symptoms (N = 91)				Types of CBSD root symptoms (N=81)			
Symptom less (%)	Chlorotic blotches (%)	Chlorotic spots (%)	Veinal chlorosis (%)	Brown/black necrosis (%)	Chalky necrosis (%)	Necrotic specks (%)	Symptom less (%)
11	46.1	30.8	12.1	34.6	19.7	3.7	42

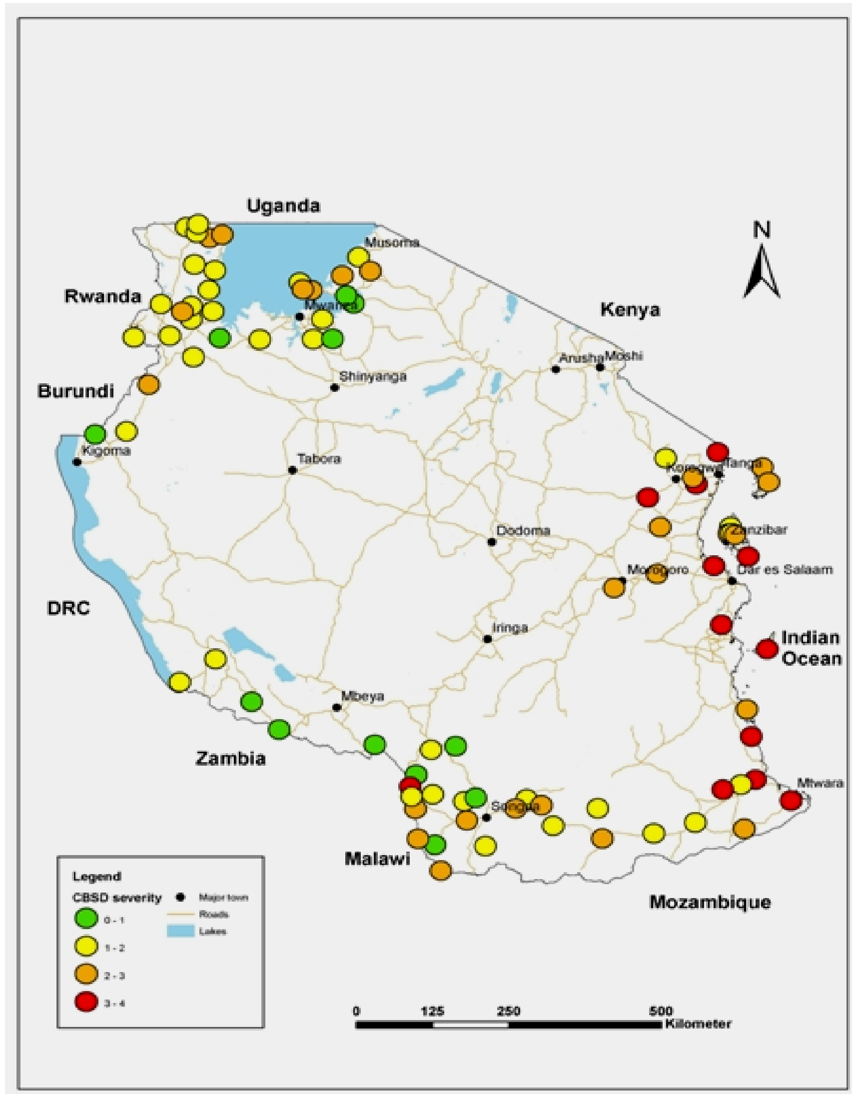

Figure 1. CBSD severity based on foliar symptoms in surveyed field cassava crops in Tanzania

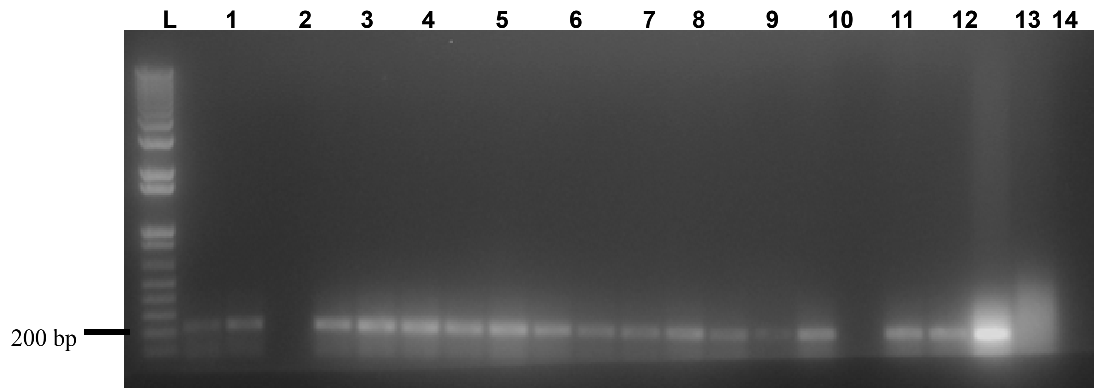

Figure 2. Agarose gel electrophoresis of RT-PCR amplified products (231 bp CBSV-coat protein gene fragment) for selected samples using CBSV-specific primer pair CBSV 10F and CBSV 11R. Lanes **L**; 1kb plus molecular weight marker (Invitrogen, cat.10787-018), **1**; sample from symptom less foliar and roots (plate 1a & 1b), **2;** sample from symptom less foliar with necrotic roots (plate 2a & 2b), **3;** sample from symptomatic foliar but symptom less roots (plate 3a & 3b), **4-18**; other samples collected during survey, +ve; positive control and **d**; primer dimmer

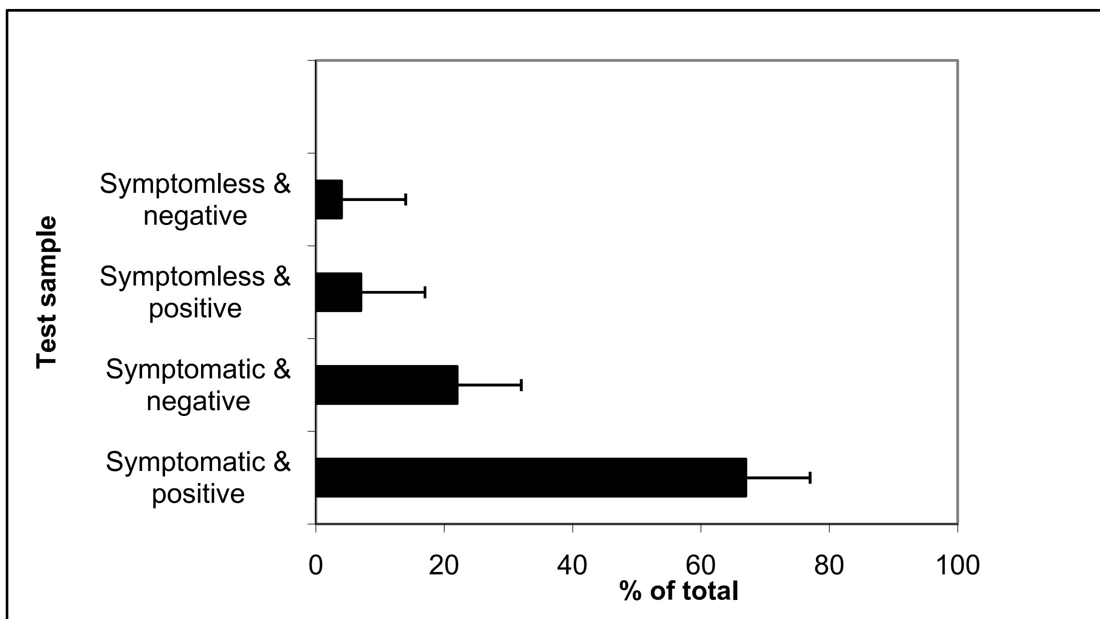

Figure 3. Relationship between foliar symptoms and CBSV detection in samples

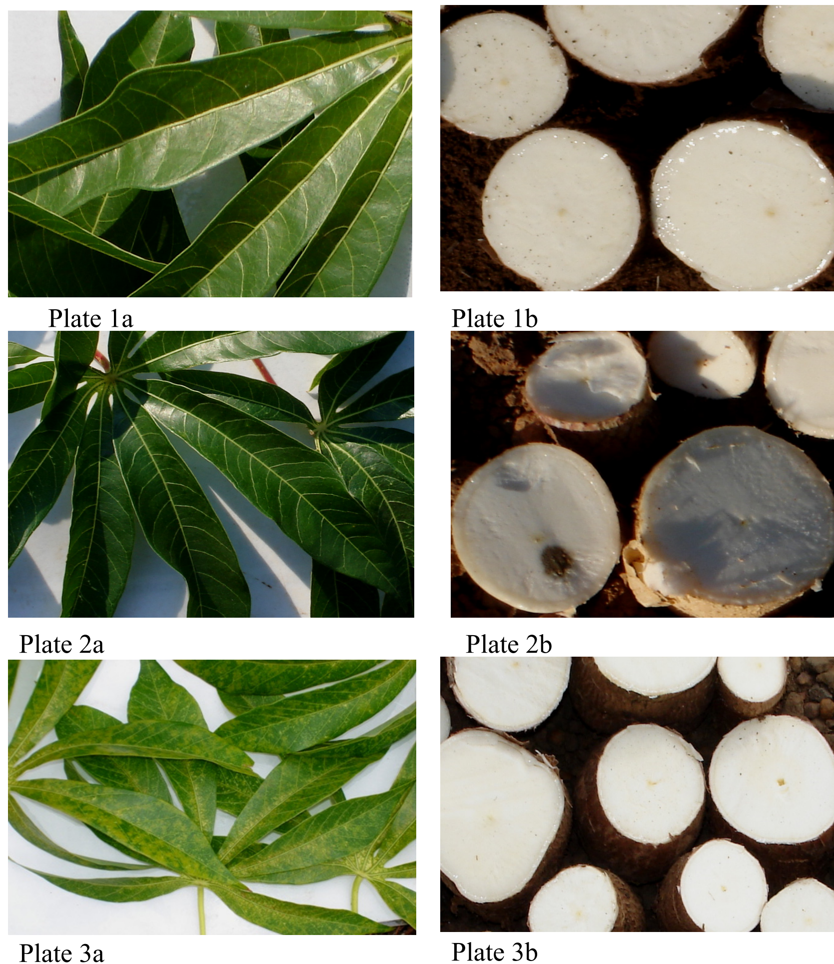

Figure 4. Relationship between foliar and root CBSD symptoms with respect to CBSV-infection. **Plates 1a & 1b;** Plant without foliar and root symptoms but CBSV-infected, **Plates 2a & 2b**; Plants without foliar symptoms but with necrotic roots and CBSV-infected, **Plates 3a & 3b**; Plant with CBSD-like foliar symptoms but symptom less roots and CBSV-free

3.3 Regeneration of Healthy Plants from CBSD Affected Cuttings

With the exception of Albert, none of the potted cuttings from the four CBSV-infected cultivars' stems sprouted disease free (Table 2). Foliar chlorotic blotches and vein chloroses were the major symptoms observed. Five out of 15 (33%) cuttings from cv. Albert sprouted symptom less despite being obtained from CBSV-infected mother plants with severity score 2 and confirmed to contain CBSV by RT-PCR. The symptom less plants emerged irrespective of the stem portion from which the cuttings were obtained. Detection of CBSV by RT-PCR indicated the presence of the virus in two of the five (40%) symptom less plants.

Table 2. Regeneration of cassava plants from infected cuttings of cultivars Albert, Cheupe and Nachinyaya

				Mean severity score and % of symptomatic plants						
				2MAP		3MAP		4MAP		RT-PCR
Variety	Section of mother plant	CBSD-score at planting*	Test plants (mean)	Score	Symptomatic	Score	Symptomatic	Score	Symptomatic	CBSV-free (%)
	B	2	15	2.0	10 (67%)	3.6	10 (67%)	3.7	10 (67%)	4.4
Albert	M	3	15	2.8	15 (100%)	3.2	15 (100%)	3.6	15 (100%)	0
	T	4	15	3.6	15(100%)	3.8	15(100%)	4.2	15(100%)	0
	B	3	15	3.8	15 (100%)	3.8	15 (100%)	4.3	15 (100%)	0
AR 49/2	M	4	15	4.0	15 (100%)	4.2	15 (100%)	4.8	15 (100%)	0
	T	4	15	4.0	15 (100%)	4.5	15 (100%)	5	15 (100%)	
	B	2	15	2.9	14 (93.3%)	3.1	15 (100%)	3.4	15 (100%)	0
Cheupe	M	3	15	3.6	15 (100%)	4.0	15 (100%)	4.8	15 (100%)	0
	T	4	15	3.6	15(100%)	4.0	15(100%)	4.8	15(100%)	0
	B	2	15	2.4	11.3 (75%)	2.6	13.8 (92%)	2.9	15 (100%)	0
Nachinyaya	M	3	15	2.4	11 (73%)	2.9	13 (87%)	3.7	15 (100%)	0
	T	4	15	3.0	13 (87%)	3.3	15 (100%)	3.3	15 (100%)	0

Abbreviations used; B=cutting from bottom stem section, M=cuttings from the middle stem section, and T=cutting from the top stem section. All the cuttings tested in table 2 were obtained from CBSV-infected mother plants as confirmed by RT-PCR tests. Five cuttings were collected from each of the top green portion, middle part and bottom woody portion of the stem. Cuttings from each stem section were planted in separate pots. A total of 45 cuttings for each variety were established in pots in the screenhouse and the rate of symptom development monitored for four months. * Indicates severity score of the mother plant from which the cuttings were derived. The disease free percentage is calculated as the proportion of the total number of the assed plants per cultivar.

4. Discussion

Expression of foliar symptoms of CBSD was found to be suggestive of CBSV infection but not conclusive of the presence of the virus. Some plants with apparent CBSD-like symptoms (22%) were diagnosed virus-free while 7% of symptom less plants tested positive for CBSV. Most of foliar symptomatic but CBSV-negative samples were recorded in the Lake Zone. Failure to detect CBSV in apparently symptomatic plants indicates that symptoms observed were caused by either another pathogen or some other abiotic stress such as mineral deficiency. Deficiency of nutrients elements such as Phosphorus, Magnesium, Potassium, Iron and Zinc have been reported to cause abnormal colouration, discolouration and or death of leaf tissues which may sometimes be confused for viral disease symptoms (Matthews, 2002). Similar observations have been reported by other workers on viral diseases. According to Jaggard et al. (1998) deficiencies of Magnesium and Iron caused yellowing and necrosis in sugar beet similar to that of *Beet yellows virus* (BYV). In potato, Potassium and Magnesium deficiencies causes marginal and interveinal necrosis similar to the viral symptoms (Matthews, 2002).

On the other hand, the high environmental temperature could have induced the virus-like symptoms including vein clearing and chloroses that were observed in some plants suspected to be CBSV-infected. This suspicion is based on the fact that the survey was conducted during the dry season (August to October) when temperatures in Tanzania are excessively high. Most cuttings obtained from the CBSD-symptomatic but CBSV-free plants did not exhibit the disease symptoms upon sprouting in pots in the screen house. Similar findings were reported (John & Weintraub, 1966) in *Nicotiana glutinosa* plants. When held at 37.8 ^{0}C for 4-8 days the new leaves displayed a pattern of mosaic, vein clearing, chloroses and other abnormalities which resembles virus infections.

Non-uniform distribution of CBSV in sampled leaf tissue may also have caused non-detection of the virus in symptomatic tissues. Atkins and Matthews (1970), reported on the variation in virus distribution based on mosaic

pattern. According to Maule (1991), working on *Tobacco mosaic virus* (TMV), the final distribution of virus (after successful infection) through tissue and organs may be very uneven and it may take long time to attain evenness. In an experiment on the distribution of TMV in a tobacco leaf, it was established that the dark green areas contain very little virus titre compared with the yellow or yellow green areas. Similar phenomenon could be happening in plants that exhibited CBSD symptoms but whose tested leaf samples were negative to CBSV.

Latent infection of the virus could explain the detection of CBSV in symptom less plants. According to Fargette et al. (1996) working on *Cassava mosaic virus* (CMV), some tolerant cultivars may co-exist with the virus without showing symptoms. Similar scenario could be happening with CBSV. However, adequate researches would be required to confirm this particularly on CBSV. Plants co-existence with infective pathogens was described as a mechanism of adaptation (Lerner, 1999). Despite these discrepancies, the majority of CBSV positive samples (67%) were symptomatic.

The non-uniform distribution of CBSV in the mother plants from which the cuttings were obtained may have influenced the occurrence of a few CBSV-free plants. Some symptom less plants sprouted from cuttings obtained from the bottom and middle stem sections CBSV-infected plants with mild foliar symptoms (score level 2). Historically, the mother plants from which the cuttings were obtained were originally CBSV-free, developed through tissue culture but became infected in the field. Due to the mild CBSD severity, the plants were suspected to have been recently infected before the cuttings were derived from them. According to Maule (1991) the final distribution of virus (after successful infection) through tissue and organs may be very uneven and it may take long time to attain evenness. It is suspected that CBSV in the mother plants had not spread throughout the plant systems.

5. Conclusion

The findings from this study suggest that CBSD symptoms are diverse and highlight the difficulty of using leaf symptoms to diagnose CBSV-infection. Expression of foliar symptoms similar to that of CBSD may not necessarily indicate CBSV-infections. Symptom-based diagnosis of CBSD is not solely reliable and robust molecular diagnostic techniques should always supplement the symptom-based CBSD diagnosis. The need for affordable diagnostic techniques cannot be overemphasized. Detection of the virus in symptom less plants complicates its diagnosis and the possible implementation of the known disease management techniques. Further studies are recommended to authenticate symptoms fidelity for harmonized CBSD diagnosis.

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