VALIDATION OF MONOCLONAL ANTIBODIES IN DETECTION OF CASSAVA BROWN STREAK VIRUSES USING TRIPLE ANTIBODY SANDWICH ENZYME-LINKED IMMUNOASSAY TECHNIQUE

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DECLARATION

This is my original work and it has not been submitted for award of a degree in any other University

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DEDICATION

To my lovely wife, Aneth and my daughter, Adventina who endured loneliness during my long absence from home, but showed patience and love. Also to my late Grandmother Ms. Cecilia Mboyelwa who took care of my early school life.

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ABBREVIATIONS

Ab	Antibody
ANOVA	Analysis of Variance
bp	Base pair
CBSD	Cassava brown streak disease
CBSV	*Cassava brown streak virus*
cDNA	Complementary deoxyribonucleic acid
CEA	Cost Effectiveness Analysis
CP	Coat protein
CTAB	Cetyl tri-methyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
FAO	Food and Agriculture Organization of the United Nations
GAR	Goat Anti-Rabbit
HCl	Hydrochloric acid
IFAD	International Fund for Agricultural Development
Kb	Kilo base
Kg/ha	Kilogram per hectare
L.S.D	Least Significant difference
MAb	Monoclonal antibody
m.a.s.l	Metres above sea level
ml	Millilitre
MT	Metric Tonnes
NCM-ELISA	Nitrocellulose membrane- Enzyme Linked Immunosorbent Assay

NPK	Nitrogen Phosphorus and Potassium
PBST	Phosphate Buffered Saline with Tween
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
r.p.m	Rotation per minute
RT-PCR	Reverse transcription- Polymerase Chain Reaction
SSA	Sub- Saharan Africa
TAE-buffer	Tris- base, acetic acid and EDTA-buffer
TBS	Tris-buffered saline
TAS-ELISA	Triple Antibody sandwich- Enzyme Linked Immunosorbent Assay
µg	Micrograms
µl	Microlitre
μM^{+}	Micromoles monovalent
µM	Micromoles
UCBSV	*Ugandan cassava brown streak virus*
USD	United States Dollar
UTR	Untranslated region
W/V	Weight per volume

ABSTRACT

Cassava is an important food crop in tropical and semi-tropical regions. Its production in East and Central African regions has been devastated by Cassava brown streak disease (CBSD), caused by two distinct virus species, *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), both being referred to as CBSVs. Different control methods has been adopted, with the use of clean planting materials being the most common since there are no resistant varieties available in the market. Use of clean planting materials requires availability of effective virus detection methods. Several molecular detection techniques with different costs and efficacy are used to detect CBSD-causative viruses including reverse transcription-polymerase chain reaction (RT-PCR), real time-polymerase chain reaction (qRT-PCR), loop-mediated isothermal amplification (LAMP). However, these methods are expensive; require specialized laboratories and highly skilled manpower. A highly efficient and cost effective detection method is needed for daily screening of CBSVs in cassava planting materials. In this study, TAS-ELISA technique using monoclonal antibodies (MAbs) and mixed CBSVs-antibodies was validated for detection and discrimination of CBSVs by determining their specificity and sensitivity in comparison to RT-PCR, which was used as a gold standard technique. Validation also involved determination of the cost and cost effectiveness ratios of both techniques for routine detection of CBSV and UCBSV in cassava. Screenhouse samples were used in determining the sensitivity of MAbs in ten-fold dilutions, best leaf position for reliable detection of CBSVs and specificity. Cost effectiveness of the two diagnostic techniques and the specificity of monoclonal and mixed antibodies were also determined using field samples.

RT-PCR was more sensitive in less diluted samples detecting the CBSV viruses than TAS-ELISA technique which detected 100% positive samples from 1:20 to $1{:}1{:}10^{-4}$ w/v. The specificity of the antibodies showed that both monoclonal antibodies (CBSV and UCBSV-

MAbs) had the highest virus mean absorbance of 1. 634 nM and 1.1173 nM, respectively, in co-infected samples. The combined antibodies had a specificity of 100% in both virus species. The sensitivity of antibodies in different plant positions was higher in low plant leaves in both MAbs but most in UCBSV-MAbs with 64.7%. Cost-effectiveness of both CBSD diagnostic techniques had 452.06 and 558.98 US$ for TAS-ELISA and RT-PCR respectively, while analyzing 100 cassava leaf samples. This means that TAS-ELISA was cheaper and can analyze 24 samples more than RT-PCR. However, RT-PCR assay was 100% effective compared to TAS-ELISA with 60.8% and 59.09% true positive detection for CBSV and UCBSV-MAb, respectively. TAS-ELISA took more time than RT-PCR to analyze the same number by a difference of 10 hours and 30 minutes. Moreover, TAS-ELISA had least cost-effectiveness ratio of 7.53 US$/% effectiveness than 5.58 US$/% effectiveness for RT-PCR.

This study found that CBSVs MAbs in TAS-ELISA was more sensitive below the manufacturer recommended ratio. The monoclonal antibodies were less specific compared to CBSVs mixed monoclonal antibodies and RT- PCR technique. The later technique was more specific in discriminating the two virus species than TAS-ELISA using MAbs. The lower cassava plant leaves are the best positions sampling for CBSVs detection when using CBSVs-MAbs in TAS-ELISA. RT-PCR technique is most cost-effective than CBSVs- MAbs for reliable detection of the viruses on routine basis, CBSVs-MAbs using TAS-ELISA can be used where there is no access to RT-PCR.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Importance of cassava and its production in the world

Cassava (*Manihot esculenta* Crantz) is an important dicotyledonous food crop in the tropics. The crop is rich in carbohydrate, vitamins C and several minerals (Mabasa, 2007) and is mostly consumed in sub-Saharan Africa (SSA), Asia and Latin America where it has been cultivated since 2000-4000 BC (Howeler, 2007). The crop was brought to East Africa by Portuguese traders in the 16th Century (Olsen *et al.*, 2001).

Cassava production has been fluctuating globally while in African region it has increased from 2008 to 2012 (**Table 1.1**). Annual production in Africa was estimated to be 149,479,840 tonnes, with Tanzania contributing 5,462,454 tonnes in year 2012 (FAOSTAT, 2013) due to its tolerance to low moisture conditions and less fertile soils and its vast uses compared with other crops where it feeds around 700 million people around the world (Legg *et al.*, 2014). In Tanzania, it is the second staple food after maize and is commonly produced and consumed in the coastal belt, Lake Victoria zone, Zanzibar Island, part of central zone and southern highlands of the country.

1.2 Cassava production constraints

Cassava, like many crops, is affected by both biotic and abiotic factors which lower the quality and quantity of the produce. These constraints include diseases and pests, poor agronomic practices, and use of low yielding varieties (Thresh *et al.*, 1994).

1.2.1 Abiotic factors

Among the key abiotic factors are poor soil fertility and drought. Poor soil fertility hinders maximum cassava root production especially in alfisols deficient in potassium (Wargiono *et al.,* 2001). Although most African farmers do not supplement soil nutrients with inorganic fertilizers, except for mulches (Ezekiel *et al*., 2009 and Ojeniyi *et al.,* 2009) indicated that application of NPK 15:15:15 at 100kg/ha and palm residues at 2 tons/ha increased root growth and yield. Soil texture also affects cassava yield whereby light textured soils contribute to higher yields than heavy textured soils.

Table 1. 1: World leading cassava producers 2008-2012 (Metric tonnes)

Countries/ Year	**2008**	**2009**	**2010**	**2011**	**2012**
World	233,083,324	237,985,098	243,489,480	262,753,309	262,585,741
Africa	122,246,224	123,080,801	134,406,803	147,597,851	149,479,840
Angola	10,057,375	12,827,580	13,858,681	14,333,509	10,636,400
Benin	3,144,551	3,787,918	3,444,950	3,645,924	3,295,785
Cameroon	2,882,734	3,340,562	3,808,239	4,082,903	4,200,000
Congo	1,196,300	1,231,000	1,148,500	1,150,000	1,200,000
Côte d Ivoire	2,531,241	2,262,170	2,306,839	2,359,015	2,412,371
DRC	15,013,490	15,054,450	15,013,710	15,024,172	16,000,000
Ghana	11,351,100	12,230,600	13,504,086	14,240,867	14,547,279
Kenya	750,964	819,967	323,389	679,167	930,922
Malawi	3,491,183	3,823,236	4,000,986	4,259,301	4,692,202
Mozambique	4,054,590	5,670,000	9,738,066	10,093,619	10,051,364
Nigeria	44,582,000	36,822,250	42,533,180	52,403,455	54,000,000
Sierra Leone	1,988,561	2,814,576	3,250,044	3,412,546	3,520,000
Tanzania	**5,392,358**	**5,916,440**	**4,547,940**	**4,646,523**	**5,462,454**
Uganda	5,072,000	5,179,000	5,282,000	4,757,800	4,924,560
Zambia	1,185,600	1,160,853	1,151,700	1,266,295	1,300,000

*FAOSTAT (2013)

1.2.2 Biotic constraints

Insect pests, weeds and pathogens including fungi, bacteria, viruses and mycoplasmas affect physiological activities in cassava resulting in yield loss (Lozano and Booth, 1974; Kizito, 2006).

Cassava green mites, mealybug, whiteflies, variegated grasshopper, some termite species, and cassava root scale are among destructive insect pests of cassava (http://www.fao.org/ag/save-and-grow/cassava/en/6/index.html, last visit 24th May 2016). For instance, the cassava mealybug (*Phenacoccus manihoti)* and striped mealybug distort cassava leaves by causing curling and chlorosis which leads to lowering photosynthesis and thus starch synthesis (James *et al.,* 2000). The parasitic wasp *Epidinocarsis lopezii* was used to successfully control cassava mealybug in different African countries (Herren *et al.,* 1991). Management strategies of insect pests include quarantine approach where suspected materials that host such pest are not allowed to cross the border of a specified zone to pest free areas (Zeddies *et al.,* 2001).

The whitefly (*Bemisia tabaci*) is the most devastating insect pest in east African region. It is a polyphagous pest that transmits two major cassava viruses and has been increasing its population possibly due to global warming (Legg *et al.,* 2014). Gerling *et al.* (2001) reported three parasitic wasps as potential biological control agents against *B. tabaci* from *Encarsia* spp., *Eretmocerus* spp. and *Amitus* spp. However, Integrated Pest Management (IPM) practices can be employed by small-scale farmers to reduce *B. tabaci* damage (Legg *et al.,* 2014).

Among the important weeds include wild cassava weed (*Euphorbia hirta*) and *Talinum triangulate*, sedges (*Cyperus rotundus*), *Rottboellia exaltata*, *Sorghum halepense*, *Ipomea spp, Pteridium aquilinum, Imperata cylindrica, Melius minutiflora, Commelina diffusa, Argeraum conyzoides* and *Portulaca oleraceae* (Melifonwu, 1994). Weeds can be controlled using suitable herbicides or by mechanical weeding (Melifonwu, 1994).

The main bacterial disease of cassava is cassava bacterial blight caused by *Xanthomonas campestris pv manihotis.* The disease infects leaves and stems and may lead to shortage of planting materials. The disease was reported to cause 100% loss in the Democratic Republic of Congo and Nigeria in the 1970s. It is transmitted between plants through stomata lenticels, wounds and other openings on leaves and stems. Contaminated equipment can also be a source of disease spread (Hillocks and Wydra, 2002). Disease symptoms include water soaked leaf spots, which may coalesce to form large brown patches or blight, which may kill leaves. Control strategies for the disease include use of clean cassava planting materials, use of uncontaminated farm techniques, and use of resistant varieties and early planting (Wydra *et al.,* 2004).

According to International Committee of Taxonomy for Viruses, there are eleven virus types reported to be infecting cassava (Fauquet and Stanley, 2003). However, cassava mosaic begomoviruses (CMBs) and cassava brown streak viruses (CBSVs) which cause cassava mosaic disease (CMD) and cassava brown streak disease (CBSD), respectively, have been reported to cause more than 50% yield loss (Perring, 2001; Legg, 2014).

1.2.2.1 Cassava brown streak disease

Cassava brown streak disease (CBSD) is the most devastating disease in the east and southern region of Africa where it causes about $100 million loss per year (Ndunguru *et al.,* 2015; Abarshi *et al.,* 2010; Alicai *et al*., 2007). Since it was first observed in 1936 at Amani in Tanzania (Storey, 1936), the disease has spread to different zones of the country encompassing Lake Victoria zone, Coastal, northwest and southern zones. Moreover, the disease has been reported in Kenya, Uganda, Malawi, Mozambique, Burundi, Rwanda and the Democratic Republic of Congo-DRC (Mohammed *et al.,* 2012).

The CBSD causing viruses belong to the genus Ipomovirus, *Potyviridae* family, and comprise two distinct virus species; *Cassava brown streak virus (*CBSV*) and Ugandan cassava brown*

streak virus (UCBSV) (Mbanzibwa *et al.,* 2011), they are collectively referred as Cassava brown streak viruses (CBSVs) in this study. CBSD is transmitted mainly using infected cassava cuttings and through whitefly vector (Ndunguru *et al.,* 2015). Infection causes disease symptoms in cassava leaves, stems and roots, and as the name suggests, the infected stem and roots exhibit brownish streaks and patches and yellowing of the leaves (Rwegasira *et al.,* 2011). Susceptible cassava varieties develop severe necrotic symptoms, reducing both the quality and quantity available for consumption (Ogwok *et al.,* 2014).

1.3 Problem statement

Loss to cassava production due to CBSD was estimated at 100 million US$ a year in East, Central and Southern part of Africa (Ndunguru *et al.,* 2015). The necrosis in the roots results in direct losses which cause food shortage to cassava dependent families. Since the first report, CBSD incidence has been recorded from different places in Tanzania and some neighbouring countries where it was not initially found (Nichols, 1950; Hillocks *et al.,* 1999; Hillocks *et al.,* 2001; Mahungu *et al.,* 2003; Alicia. *et al.,* 2007; Legg *et al.,* 2011). The main cause of disease spread is the use and transfer of CBSD- infected cassava cuttings (Alicai *et al.,* 2007; Tomlison *et al.,* 2012; Ndunguru *et al.,* 2015).

The disease may be managed in different ways including use of symptomless cuttings and isolation of multiplication sites from production fields (Rwegasira *et al.,* 2015). These disease management approaches minimize the possibilities of using the infected cuttings but need to be supplemented with disease diagnosis to confirm their health status especially when disease is in latent period (Rwegasira *et al.,* 2011). Use of resistant genotypes is the most appropriate and economical method of management, but development of CBSVs resistant/tolerant cassava varieties is still at research stage (Vanderschuren *et al.,* 2012).

There has been development of several diagnostic techniques including reverse transcription-polymerase chain reaction (RT-PCR), quantitative Real time polymerase chain reaction

(qRT-PRC), Loop-mediated isothermal amplification (LAMP) (Webster *et al.,* 2004, Tomlinson *et al.,* 2012), as well as use of serological techniques (Osogo *et al.,* 2014). Molecular diagnostic methods such as RT-PCR, qRT-PCR and LAMP are expensive for use in screening of planting materials on daily basis. They also require highly trained personnel to run the tests with risk of handling harmful reagents like Ethidum bromide (Lamour and Finley *et al.,* 2006). These techniques can only be carried out in highly advanced laboratories and not at the farm level (Dreo *et al.,* 2007). Other diagnostic techniques that are cheap, sensitive and easy to use are therefore needed for use in the region.

1.4 Justification

Cassava brown streak disease (CBSD) diagnosis in cassava planting materials is an important part of disease management since it is necessary for phytosanitary purposes. The disease is mainly spread through use of already infected cassava cuttings. Therefore, planting materials need to be screened for CBSVs before releasing them to the farmers. Several molecular diagnostic techniques have been developed and used in the detection of CBSVs such as RT-PCR, qRT-PCR and Loop-mediated isothermal amplification (LAMP). These techniques are sensitive and specific to detect both CBSV and UCBSV. However, wide use of these molecular techniques in routine screening of CBSVs is still low as the methods are expensive, and require specialized equipments and skilled personnel.

This study was undertaken to determine the sensitivity and specificity of MAbs in the detection of CBSVs using TAS-ELISA, and to determined the cost effectiveness of TAS-ELISA using MAbs. Since RT-PCR is sensitive and specific and has been widely used in CBSD diagnosis, it was in this study used as a gold standard to validate the newly developed CBSVs-MAbs in detecting CBSV, UCBSV and co-infected cassava leaf samples. This will eventually contribute to improved management of CBSD using CBSVs-free planting materials at low cost.

1.5 Objectives

1.5.1 General objective

The main objective of the study was to contribute to reduction of losses associated with cassava brown streak disease (CBSD) through use of improved serology-based diagnostic technique using monoclonal antisera that is more specific, sensitive and cost-effective in the detection of CBSV and UCBSV for sustainable cassava productivity in Tanzania.

1.5.2 Specific objectives

The specific objectives were

1. To determine the efficacy of commercially available monoclonal and mixed antibodies in detection of cassava brown streak viruses using Triple antibody sandwich enzyme- linked immunosorbent assay technique.
2. To determine the cost-effectiveness of Triple antibody sandwich enzyme-linked immunosorbent assay and reverse transcription- polymerase chain reaction in detection of cassava brown streak viruses.

1.6 Hypothesis

1. The CBSV and/or UCBSV-antibodies for Triple antibody sandwich-enzyme linked immunosorbent assay are effective in detection and discrimination of cassava brown streak viruses.
2. The Triple antibody sandwich-enzyme linked immunosorbent assay has most cost-effective ratio compared to RT-PCR assay in the detection and discrimination of cassava brown streak viruses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botany of cassava

Cassava (*Manihot esculenta* Crantz) is a root, shrubby and perennial plant with erect stems of about four metres tall, with dark green leaves with reddish or whitish veins. The stem has whitish hollow pith while flowers are creamy in colour. Roots are fleshy, firm and tapered and surrounded by a rough yellow, white or pink coloured mass, which is the most consumed component of the plant. Leaves are usually deep green, palmate and divided into 3-7 lobes without creation of a leaf blade. Seeds are contained in hard covered fruit and are mostly used for breeding purposes (http://plants.usda.gov, visited Dec 2017).

2.2 Uses of cassava

Cassava has become a staple and food security crop in many African countries such as in the DRC, Nigeria, Tanzania, Malawi and forty other countries. Production of cassava is globally increasing due to its various economic uses including its use as staple food in the tropics and around the globe (Balagopalan, 1988; Legg *et al.,* 2014). From the early days, cassava has been used as a food crop for poor societies in the humid and semi- humid tropics, eaten raw or cooked (Howeler, 2007). The storage roots are peeled, chopped and boiled to make dessert, salad dressing and soup thickener and or fried to make ready crisps, but chopped edible roots can be dried and ground to produce flour which is used to make stiff porridge or Ugali in Kiswahili. Cassava leaves are a rich source of proteins, vitamin C and other minerals where fresh leaves are ground and cooked as sauce or dried and packed for storage (Mabasa, 2007).

The root, due to its high starch content that has sticky properties, is also used to make binding agent/gum (Mabasa, 2007). This is achieved by gelatinizing the starch either by

supplementing additives. The first approach is by cooking liquid starch and adding water and preservatives. After the starch, has cooled and gelatinized, copper sulphate is added to prevent microbial growth and damage to the product. The product can be used in bag making, bill pasting and in tobacco products (Plucknett, 1998). The second approach involves addition of inorganic salts: calcium, magnesium, chloride, borax, urea, glycerol and carboxymethyl cellulose which improves viscosity, water content and flowability of the product. The products obtained are used in paper lamination, wall pepper printing and water resistant formulations (Balagopalan, 1998).

Cassava has become a partial substitute of wheat flour and products made from it, for example in bread and biscuits, where its flour substitutes up to 100% of wheat flour and increases the viscosity/quality of products, thus, reducing the use of eggs and milk (Uchechukwu-Agua, 2015; FAO, 2013). This has reduced the production costs and product prices compared to when using wheat flour alone (IFAD and FAO, 2000). Cassava chips can be dried and ground into small pellets that are fed to animals when mixed with other feeds. Cassava leaves and stems are also used in the tropics to feed cattle. The cassava root is particularly suitable as starch carrier because it contains lower levels of amylose and higher levels of energy, which makes it attractive as animal feed. Other technologies exploit cassava for making ethanol by yeast fermentation. Cassava starch has pharmaceutical uses too, by its bond strength at wide temperature and pH range which can be used for making medicinal tablets (Gunorubon, 2012).

2.3 Challenges to cassava production

Cassava development in Tanzania and other African countries is facing challenges particularly at production, marketing and in processing. Cassava producers lack machinery like tractors for timely tilling the land which saves the time in its production. There is still an

opportunity of using machinery to an increase cassava production in the region (Gaffney *et al.,* 2012). Harvesting of cassava need mechanical and breeding effort that will enable a specific root size and shapes that will be harvested by specific machines without any damage of the produces (Tarawali *et al.,* 2013). Cassava producers are facing difficulties of accessing the virus free cassava cuttings. This is because there are few cassava planting material multipliers that can meet the demand of the cassava producers (Ezedinma *et al.,* 2007). In Tanzania, the only cassava multipliers are public institutions which include Agricultural Seed Agency (ASA) and some Agricultural Research Institutions; whereas the private sector may engage in it multiplication to fulfill the demand (Mkamilo and Jeremiah, 2005).

Cassava is mostly used for human food by most cassava producers and in developing countries. Cassava producers have a very limited access to local and international markets (Tounessi, 2008). The report shows that 4,547,940 metric tonnes produced in 2010 was completely not sold to international market but less was locally sold and the rest wasted (Nicholas, 2010).

2.4 Cassava brown streak disease symptoms

As the name suggests, the disease refers to the brown lesions that appear on the bark of the cassava stem although symptoms may be exhibited in all parts of the plant (foliar/leaves, stems, fruits and roots) (Rwegasira *et al.,* 2011; **Plate 2.1**). Leaves develop yellow patches, chlorotic spots, chlorotic blotches and veinal and interveinal chlorosis along the secondary and tertiary veins and pronounced mottling occur mainly on the lower older leaves. Stem symptoms are manifested as scratch-like wounds, dark brown spots, and streaks. Severe systemic necrosis expressed in stems and leaves may be associated with dieback. Roots get constricted, and in some cases various discolourations (brown, black or yellow, or chalky) may be observed in the storage roots (Munganyika *et al.,* 2017).

Plate 2. 1: Cassava brown streak disease symptoms on cassava plant; **A-**Yellow chlorotic blotches on leaves, **B-**die back on cassava growing tip, **C-**brown necrotic spots and lesions on stem, **D-**constriction on root and **E-**brown cerotic rot on root cut longitudinally.

2.5 Cassava brown streak disease epidemiology

Involvement of cassava brown streak viruses (CBSVs) as causal agents for CBSD was shown through sap inoculation from cassava to herbaceous plant hosts such as *Nicotiana benthamiana*, *Nicotiana tabacum*, and *Nicotiana occidentalis* and to healthy cassava plants (Lister, 1959). The disease occurs both on cultivated cassava and wild relatives and between hybrid *Manihot esculenta* and *M. glaziovii* (Mbanzibwa *et al.*, 2011).

The disease is caused by the two distinct virus species namely; *Cassava brown streak virus* (CBSV) and *Uganda cassava brown streak virus* (UCBSV) (Alicai *et al.*, 2007). The main disease transmission vehicle is the use of CBSD infected cassava cuttings as planting materials, which introduces and spreads the viruses to new areas (Rwegasira *et al.*, 2015). The viruses are also transmitted by the whitefly (*Bemisia tabaci*) in a semi-persistent manner (Maruthi *et al.*, 2005; Mware *et al.*, 2009). The viruses can also be transmitted mechanically

through farm equipments in plant sap (Maruthi *et al.,* 2005). Moreover, the viruses can be transmitted to many herbaceous plant species by sap and/or grafting (Calvert *et al.,* 1996; Mohammed *et al.,* 2012).

2.6 Distribution of the cassava brown streak disease

From the foot hills of Usambara Mountain in Tanzania where the disease was first reported (Storey 1936), the disease has now spread in several zones of Tanzania; Northwest, coast, part of central and south zone (Mbanzibwa *et al.,* 2009a). The disease has been reported in other countries including, Uganda, Kenya, Mozambique, Burundi and Malawi (Mbanzibwa *et al.,* 2009b; Monger *et al.,* 2010, Bigirimana *et al.,* 2011; Legg *et al.,* 2011). The disease was initially confined to low altitudes below 1000 m (Hillocks *et al.,* 1999; Nichols, 1950; Storey, 1936). Later the disease was reported to occur in mid altitudes ranging from1200- 1500 m in DR-Congo (Mahungu *et al.,* 2003), Uganda and Tanzania between 1200- 1500 m (Alicia *et al.,* 2007; Mahungu *et al.,* 2003) and in Kenya (Mware *et al.,* 2009). In Tanzania, the disease was confined in North-East region for some-time and later at the coast and Southern zone (Hillocks *et al*., 2001). The disease incidence was reported to be 25.6 and 36% in Masasi and Mtwara, respectively (Jeremiah and Legg, 2008). In Lake Victoria zone and West zone, the incidence ranged from 5.9- 31.6% in year 2006 and 2008 in mid and higher altitudes (Legg *et al.,* 2011; Jeremiah and Legg, 2008).

2.7 Diversity of Cassava brown streak viruses

CBSD is caused by at least two distinct virus species namely *Cassava brown streak virus* and *Ugandan cassava brown streak virus* both members of the same genus Ipomovirus, family *Potyviridae* (Alicai *et al.,* 2007; Mbanzibwa *et al.* 2009b; Monger *et al*., 201; Winter *et al.,* 2010). The difference in the two species was found to be in the coat protein nucleotides and amino acids sequence identities from samples found in different locations of East and

southern parts of Africa (Mbanzibwa *et al*., 2009a). The two viruses together often are referred to as cassava brown streak viruses (CBSVs).

2.8 Genome organization of cassava brown streak viruses

The CBSVs contain a single molecule of +ssRNA of about 8.9 to 10.8 kilobase (**Plate 2.2**). They contain two endings both consisting of a viral genomic- linked protein-VPg (Mbanzibwa *et al.,* 2009b; Winter *et al.,* 2010). The genetic structure of Ipomoviruses shows that member viruses have single polyprotein with autoproteolytic cleavage of 10 mature proteins (**Table 2.1**).

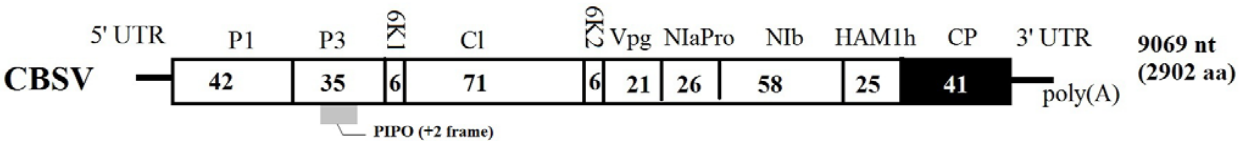


Plate 2. 2*:* Genome structure of cassava brown streak viruses (Adopted from Rajabu, 2012)

T**able 2. 1**: Functions of viral protein of genus Ipomovirus, family *Potyriviridae*

Protein	**Size (aa)**	**Functions**	**References**
P1	362	Virus replication and protease	Valli *et al.* (2008)
P1a		Protease	Valli *et al.* (2006)
P1b		Virus replication, Protease and RNA silencing suppression	Valli *et al.* (2006); Janssen *et al.* (2005)
P3	294	For movement and genome replication	Hjulsager *et al.* (2006)
6K1	52	Virus replication	Riechmann *et al.* (1992)
CI	628	For cell to cell movement and RNA helicase	Lain *et al.* (1990)
6K2	52	Symptom, RNA helicase	Spetz and Valkonen (2004); Restrepo-Hartwig and Carrington (1994)
NIa-VPg	185	Virus replication, RNA silencing suppression and Cell to cell and systemic movement	Schaad *et al.* (1997); Rajamaki and Valkonen (2009)
NIa-Pro	234	Virus replication and Protease	Daros and Carrington (1997); Dougherty *et al*. (1989)
Nib	502	RNA dependent and RNA polymerase	Hong and Hunt (1996)
CP	367	Virus replication, Aphid transmission and Cell to cell and systemic movement	Haldeman-Cahil *et al.* (1998); Atreya *et al.* (1995); Hofius *et al.* (2007)

Source: Mbanzibwa *et al.* (2009a)

2.9 Management of cassava brown streak disease

Major strategies to manage CBSD are mainly through the use of integrated disease management that combines detection of the CBSD-causing viruses in planting materials and phytosanitation approaches. Phytosanitation approaches include (i) selection of planting materials that are free from the disease based on the absence of CBSD symptoms. This strategy can be used to minimize disease spread to healthy plants, (ii) roguing of symptomatic plants has been reported to be effective. (iii) isolation of planting materials by locating cassava multiplication sites away from other cassava producing fields. This reduces chances of virus spread from infected fields (Rwegasira *et al.,* 2015), (iv) the use of resistant cassava varieties has not yet shown success and it is still being researched including natural host

resistance and the transgenic approach to engineer resistance into cassava against CBSVs infection (Winter *et al.,* 2010; Hillocks *et al*., 2001; Patil *et al.,* 2010). Effective use of CBSVs-free cassava planting materials in disease management depends on availability of reliable diagnostic tools.

2.10 Detection of cassava brown streak viruses

2.10.1 Serological techniques

These are diagnostic tools that use specific antibodies/antiserum to detect their corresponding viruses in a sample. They are biochemically based techniques where most of the results are noted by colour changes in test reaction (Clark and Adams, 1977). There are different serological techniques which include; (a) Solid phase serological assays such as micro-titre plate (plate trap) double and triple-antibodies sandwich enzyme-linked immune assay (DAS and TAS-ELISA). This includes the use of nitrocellulose membrane (NCM-ELISA) (Smith *et al.,* 1987; Clark and Adams, 1977). (b) Liquid serological tests which includes; Precipitin, micro precipitin test, gel diffusion assays, immunosorbent electron microscopy (SSEM) and agglutination of cells called agglutination methods (Brundaban and Schmitt, 2006; Marja and Richard, 2006).

The antibodies are developed through two main approaches namely, immunization process and coat protein expression antibodies process (Liu *et al.,* 2010; Köhler *et al.,* 1975). Immunogenicity approach involves production of antibodies through immunizing an animal (rabbit and mouse) which gives polyclonal antibodies. Monoclonal antibodies under immunization process are obtained by culturing the positive reacted cells against specific foreign microorganisms/viruses which gives the cells called myeloma. The myeloma cells diffuse with spleen cells of the immunized animal to form cells called hybridomas. Lastly screening procedure is done to latter cells to get uniform cells called monoclonal antibodies

(Ling *et al.,* 2000). The latter approach gives rise to monoclonal antibodies through expression of specific coat proteins of CBSVs such as those developed by Deutsche sammlung von mikroorganismen und zellkulturen (DSMZ) GmbH.

Serological tools have been popularly used in detection of plant viruses (Van Regenmortel and Dubs, 1993). This is because the techniques are simple to use by simple trained personnel in the field and or in ordinary laboratory. Using polyclonal antibodies (PAbs), Sweetmore *et al*. (1994) detected CBSVs using NCM-ELISA in only a few CBSD infected cassava leaf samples. The monoclonal antibodies raised through coat protein expression developed by DSMZ were evaluated on detection of CBSVs in cassava leaf sample in Kenya (Osogo *et al.,* 2014). Moreover, different immunosorbent assays have been used to detect several plant viruses. Several successful reports on the uses of virus antibodies have also been made in the detection of *Potato virus Y* in potato leaf, stem and tubers (Karasev *et al.,* 2008) and when detecting nine grapevine viruses using DAS-ELISA (Gambino and Gribaudo, 2006), and in the detection of *Tomato spotted wilt virus* in peanut (Dang *et al.,* 2009). The TAS-ELISA tool was studied in CBSVs detection in cassava leaf by Sweetmore (1994) and then by Osogo *et al.* (2014).

2.10.2 Molecular techniques

Molecular techniques used for detection of CBSVs include Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Monger *et al.,* 2010; Mbanzibwa *et al.,* 2011; Mohamed *et al.,* 2012), real time PCR (Moreno *et al.,* 2011; Adams *et al.,* 2013) and Loop mediated isothermal amplification (LAMP) (Tomlison *et al.,* 2012). RT-PCR is the most commonly reliable technique for detection of CBSVs. The technique involves reverse transcription of total RNA into complementary DNA (cDNA) by reverse transcriptase enzyme. The virus is PCR-amplified using virus species specific primer pair. The technique has been used to screen cassava viruses for CBSVs in every part of cassava plant including leaves, stem and

roots (Abarish *et al.*, 2010; Monger *et al*., 2010; Mbanzibwa *et al.*, 2011). Real time PCR is another technique which is more sensitive than RT-PCR, since it can detect and quantify amount of viral copies present in a sample infected with CBSVs. However, Real time PCR is relatively costlier and requires more technical know-how. It also requires a host reference gene with stable expression to normalize the data (Moreno *et al.,* 2011).

The reverse transcription Loop–mediated isothermal amplification technique (RT-LAMP) involves the identification of specific nucleotides of CBSVs using a set of virus specific primers in a short time without the use of conventional PCR machine. The amplifications can be detected through lateral flow devices with the specific antibodies incorporated in labels to react with a special sequence to develop color thus avoiding the need for fluorescence detection or through gel electrophoresis approach (Tomlison *et al.,* 2012).

Molecular techniques are reported to be specific in detecting and discriminating the CBSV species (Mbanzibwa *et al.,* 2009a). The qRT-PCR technique enables quantification of the virus particles number in each sample and determine disease development in growing infected plants (Husted and Bech, 1996; Webster *et al.,* 2004; Mohammed *et al.,* 2007; Ogwok *et al.,* 2014). Despite the benefit of high efficacy of molecular techniques in the detection of CBSVs, the techniques have some limitations (Kox *et al.,* 2007). They are very expensive if used on a daily basis, and the cost of buying operating equipments and the reagents used in running the tests are also high (Fang and Ramasamy, 2015; Gambino and Gribaudo, 2006; Cullen *et al.,* 2005; Costa *et al.,* 2004; Bertolini *et al.,* 2001). They all require very highly skilled personnel to run the tests and results interpretation. This also includes skills of handling of toxic reagents like Ethidium bromide, Chloroform and Phenol used in tests (Lamour and Finley 2006). Moreover, there is high risk of sample contamination when using these techniques if the samples are not properly handled or stored resulting in

false results. This makes the techniques difficult to be used either by ordinary personnel or in a farm environment (Dreo *et al.,* 2007).

2.11 Cost-effectiveness Analysis

This is a financial technique used to determine the worthiness of different alternatives that aid in making decisions and appraising a least cost option/s with reasonable desirable impacts. There are three major financial analytical methods; cost-benefit analysis, financial evaluation and cost-effectiveness analysis (Commonwealth, 2006). Cost analysis only provides a total accounting of the expenses of a given alternative; it considers costs only. Fiscal impact analysis (FIA) is an exhaustive study of all revenue expenditures and savings which is predictive. It shows if the available budget can sustain the available detection technique option within stipulated time. Cost effectiveness analysis (CEA) evaluates which alternative option is efficient in its use of resources with the most desired results at the lowest cost (WHO, 2003; Black, 1990). Cost benefit analysis was used in the current study to evaluate the cost of standard technique (RT-PCR) used as a standard in comparison with TAS-ELISA to determine if it is cost effective to be used in place of RT-PCR technique. The CEA quantifies the intangible benefits, time, quality, satisfaction level and works with a limited number of factors (Commonwealth of Australia, 2006; New York State of Opportunity, 2015). Use of CEA approach enables an estimate of cost and quality of a desired output when using TAS-ELISA. The most cost-effectiveness ratio means that the technique has less cost to attain unit effectiveness of true positive detection than one with least cost-effectiveness ratio (Josiah, 2014).

Different disease diagnostic techniques and steps involve different reagents/materials which give different effectiveness on their performance. Knowing the effectiveness and the cost used in the process of disease diagnosis allow the use of most cost effective method in disease management. Materials used are of different costs, as Abarish *et al.* (2010)

diagnosing CBSD in cassava leaf samples using one and two-steps RT-PCR approaches found the latter approach was more effective but costly and time consuming. The comparison of cost effectiveness of diagnostic techniques gives highlight on where the samples should be taken with reliable diagnostic technique. This was shown to be important when Karasev *et al.* (2008) validated the polyclonal antibodies using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RP-PCR) in detection of *Potato virus Y* (PVY) in leave, tubers and stems.

CHAPTER THREE

3.0 SENSITIVITY AND SPECIFICITY OF ANTIBODIES FOR DETECTION OF CASSAVA BROWN STREAK VIRUSES IN CASSAVA

3.1 Abstract

A study was carried out to determine the sensitivity and specificity of monoclonal antibodies for the detection of viruses causing cassava brown streak disease (CBSD). Symptomatic fresh cassava leaf samples were used to determine efficacy of triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) using monoclonal (MAbs) and mixed antibodies for detection of *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV), the causal agents of CBSD. Reverse transcription polymerase chain reaction (RT-PCR) was used as a gold standard. In addition, the best leaf position for the detection of CBSVs using TAS-ELISA was also determined. Results showed that RT-PCR was more sensitive (100%) than CBSVs monoclonal antibodies in TAS-ELISA while specific MAbs for CBSV and UCBSV were more sensitive with 57.14%, and 85.57%, respectively. The specificity of MAbs were 54.54% and 72.72% for CBSV and UCBSV, respectively and had 62% positive detection when antibodies were mixed (CBSV and UCBSV). Both MAbs and Mixed Ab had more detection levels in co-infected than in single infected plants. Viruses in leaf samples from lower position were readily detected by MAbs at 46.6% and 64.7% for CBSV and UCBSV-MAb, respectively. RT-PCR is more sensitive and specific than MAbs in detection and discrimination of CBSVs. Monoclonal antibodies were more reliable in detecting CBSVs in lower plant leaf positions than in upper and middle leaves. RT-PCR should be used in routine screening of planting materials. However, monoclonal antibodies and the mixed antibodies using TAS-ELISA can be used in academic and research institutions for teaching purposes. However, CBSVs-MAbs can also be used elsewhere where there is no access to RT-PCR for screening of cassava materials.

3.2 Introduction

Cassava brown streak disease (CBSD) is the second most important disease of cassava and is caused by two distinct virus species: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak viruses* (UCBSV) which belong to the genus Ipomovirus, family *Potyviridae* (Alicai *et al.,* 2007, Mbanzibwa *et al.,* 2009b, Winter *et al.,* 2010). The disease is a threat to food security as it affects roots of cassava and renders them inedible. CBSD is mainly spread through planting CBSVs-infected planting materials (Maruthi *et al.,* 2005). The viruses causing the disease are also transmitted by whiteflies in a semi-persistent manner (Legg *et al.,* 2014). The disease has spread in East, Central and southern Africa since being reported for the first time at Amani, Tanga (Storey, 1936; Hillocks, 1997; Hillocks *et al.,* 2001 and 2002).

There have been several efforts to manage the spread of CBSD to disease-free areas through isolation of multiplication sites for planting materials (Frison, 1994), minimizing chances for virus spread by whitefly vectors as well as the use of asymptomatic cassava cuttings. Development and use of resistant cassava varieties against CBSVs either through conventional breeding or genetic engineering is still at research level (Hillocks *et al.,* 2001; Patil *et al.,* 2010; Winter *et al.,* 2010).

Osogo *et al*. (2014) compared TAS-ELISA monoclonal antibodies and RT-PCR in the detection of CBSVs in symptomatic leaf samples and reported that TAS-ELISA could only detect the viruses in 26.7% of the samples. This was the only recent work done on CBSVs test using serology before this study. Currently, detection of CBSVs in cassava planting materials are mainly done using molecular based techniques (Mbanzibwa *et al.,* 2011; Monger *et al.,* 2010; Abarshi *et al.,* 2010). Molecular techniques (RT-PCR, qRT-PCR and LAMP) have also been used to study the accumulation of CBSVs in different cassava plant organs, leaves, stems and roots (Kaweesi *et al.,* 2014; Ogwok *et al.,* 2014; Rwegasira *et al.,*

2011). Therefore, this study was done to determine the efficacy (sensitivity) of CBSVs monoclonal antibodies and specificity of both monoclonal and mixed monoclonal antibodies in detection of CBSVs in cassava. The study also determined the best leaf sampling position/zone for CBSVs detection which was not yet been determined using TAS-ELISA.

3.3 Materials and Methods

3.3.1 Source of antibodies

Five sets of antibodies including: two monoclonal antibodies (MAbs) each for detection of CBSV and UCBSV, and one combined antibody which simultaneously detect both CBSV and CBSV (**Table 3.1**) were procured with TAS-ELISA kits from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ), Germany.

3.3.2 Source of virus isolates

Cassava cuttings from Kiroba variety was obtained from a cassava multiplication field in Chambezi Research Substation, which is located E 38°54'S 06°32', 38 m.a.s.l in Bagamoyo, Coastal region. Cuttings were established in a screenhouse and screened by RT-PCR using virus species specific primers for CBSV and UCBSV (Mbanzibwa *et al.,* 2011) at four months after sprouting. Plants with single infection of CBSV, UCBSV, co-infection and healthy (CBSV-free) were selected and maintained in separate rooms inside the screenhouse. In addition, 32 and 100 cassava leaves with CBSD-like symptoms from four month old plants were collected from Chambezi multiplication field and used to validate the specificity and determine the cost-effectiveness ratio of antibodies using TAS-ELISA kit.

Table 3. 1: Antibodies and conjugates used in the evaluation of efficacy and cost-effectiveness of CBSVs monoclonal antibodies using TAS-ELISA kit for detection and discrimination of CBSVs in cassava planting materials

S/N	Virus species	Trapping antibody	Detecting antibody	Labelled antibody
1	CBSV	IgG-AS-0925(BN:5374A)	MAb-AS-0942/2A (BN:3854),	RAM-AP, BN:5080A
2	CBSV	IgG-AS-0925 (BN:5374B)	MAb-AS-0949/2B (BN:3854)	RAM-AP, BN: 5080B
3	UCBSV	IgG-AS-0912 (BN:5377A)	MAb-AS-0912/2A (BN:5116)	RAM-AP, BN:5377A
4	UCBSV	IgG-AS-0912 (BN:5116B)	MAb-AS-0912/2B (BN:5116)	RAM-AP, BN: 5377B
5	CBSV+UCBSV*	IgG-AS-1153(BN:5540)	MAb-AS-1153(BN:5540)	RAM-AP, BN:5080

*Mixed antibodies. **Source of antibodies:** Deutsche sammlung von mikroorganismen und zellkulturen (DSMZ) GmbH, Germany

3.3.3.1 Sample preparation and RNA extraction

Total RNA was extracted from fresh leaves collected from both screenhouse plants and field collected samples using CTAB procedure as described by Chang *et al.* (1993) and modified by Rajabu *et al.* (2012). One gram of leaf tissue was ground in 1000µl CTAB buffer (2%) pre-warmed at 65^{o}C using a mortar and pestle. Aliquots of 700µl from each sample were transferred to 1.5 ml Eppendorf tubes, mixed and incubated at 65^{o}C for 30 minutes in a heating block. The extract was mixed with an equal volume (700µl) of phenol: chloroform: Iso-amyl alcohol (25:24:1), mixed thoroughly and centrifuged at 12,000 rpm for 10 minutes in 4^{o}C. The resultant supernatant was then transferred to a new 1.5 ml Eppendorf tube and precipitated by adding 0.7% of the supernatant volumes (350 µl) of isopropanol in ice cold ($^{-}$20^{o}C). Samples were then incubated at $^{-}$20^{o}C for 30 minutes and centrifuged at 12,000 rpm for 10 minutes at 4^{o}C where the upper layer of each sample was discarded to remain with the pellet. Finally, the pellet was washed in 500 µl of 70% ethanol, centrifuged at 12,000 rpm for 5 minutes in 4^{o}C. Ethanol was discarded and pellets dried at room temperature. The pellet

was then dissolved in DEPC water and stored at $^{-}80^{o}$C before further analysis. The quality of extracted RNA was assessed in 1% of agarose gel. The gel was made by warming the 1X Tris- Acetic Acid and EDTA (TAE) - buffer in a microwave (Supra Service) for 4 minutes and cooled at room temperature and mixed with 0.02% Ethidium bromide, mixed thoroughly before being poured in the gel casting chamber to solidify with combs to shape the sample loading wells. The integrity of total RNA was checked by electrophoresis in a 2% agarose gel, which contained 2µl of Ethidium bromide (10 mg/ml) in 1XTris–borate–EDTA (TBE) buffer at 100 volts for 45 minutes. RNA bands were viewed under the gel documentation unit (BioDoc-It® 210 Imaging System- US, M-20V Trans illuminator) for the presence of 28s and 18s RNA bands.

3.3.3.2 cDNA synthesis

Complementary deoxyribonucleic acid (cDNA) was synthesized using 4µl of total RNA of 200µg/µl was then mixed with the following to make 17.5 µl per sample of mix I (SddH_2O with 10.5 µl, 10 mM and 10 µM OligodT18 mM of 1 µl (Thermo Scientific, MA, US) and 10 mM dNTPs of 2 µl (Thermo Scientific)). The mixture was then incubated at 65^{o}C for five and immediately chilled on ice for one minute. Then mixture -I was then spun down and 17.5 µl were mixed with 4.5 µl of Mix- II making a total of 22 µl per sample which contained; SddH_2O with 02.0 µl, 0.45X of 10X RT buffer M-MuLV-RT (Lucigen), 1 U/ µl of RNAse incubator (40 U) (Thermo Scientific) and 10 U/ µl of Reverse Transciptase (200U/µl) (Thermo Scientific). Then the mixture- II was incubated at 42^{o}C for 50 minutes and finally reactions were terminated by heating at 85^{o}C for 5 minutes the resulting cDNA were stored at -20^{o}C until PCR analysis.

3.3.3.3 Polymerase Chain Reaction

The cDNA was PCR-amplified using virus species specific primer pair (CBSDDR/CBSDDF_2) that simultaneously amplifies both CBSV (~344 bp) and UCBSV

(~440 bp) (Mbanzibwa *et al*., 2011). PCR master mix contained 25µl per sample reaction including; sterile double distilled water, 10X Dream Taq buffer, 2.5mM of dNTPs and 1.0µM each of the primers (CBSDDR/CBSDDF$_2$) (10 µM), and Dream Taq DNA polymerase (Thermo scientific, USA). PCR reaction was run into a Gene Amp PCR system 9700 (Biosystem, USA) using thermal cycling parameters: 94^{o}C for 2 minutes for initial denaturation, 94^{o}C for 30 seconds, and annealed for 35 cycles, each at 51^{o}C for 30 seconds, 72^{o}C for 30 seconds for extension and final extension time at 72^{o}C for 10 minutes. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel, which contained 2µl of Ethidium bromide (10 mg/ml) in 1XTris–borate–EDTA (TBE) buffer at 100 volts for 45 minutes. The DNA bands were observed under the gel documentation unit (BioDoc-It® 210 Imaging System-USA, M-20V Transilluminator).

3.3.3.4 Triple Antibody Sandwich-Enzyme Linked Immunosorbent Assay

Triple Antibody Sandwich-Enzyme Linked Immunosorbent Assay (TAS-ELISA) was done using kits and CBSVs antibodies following manufacturer's instructions (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ GmbH, German) (DSMZ 1998, Germany) with minor modification following optimization on time of antibodies incubation. Antibody incubation was done for 3 hours instead of 2 hours as recommended by the manufacturer. ELISA plates were loaded with 100µl of coating polyclonal (IgG) antibodies for the respective CBSV species at 1:1000 with coating buffer and incubated at 37^{o}C for 3 hours. Washing of microtiter plates was done manually using PBS-Tween 20 in a wash bottle and soaked for 3 minutes followed by blotting the plate upside down on tissue paper and repeated 3 times in 3 minutes.

Blocking of unbound places was done using 200 µl/well of blocking solution containing 5% skimmed milk in PBS-T and incubated at 37^{o}C for 30 minutes and then solution was discarded and the plate briefly dried by blotting onto a tissue paper. One hundred microliters

of crude sap of fresh cassava leaf samples previously ground in extraction buffer containing 0.05M Tris-HCl, 0.06M Na_2CO_3 2% of PVP at 1:20 (w/v) at 8.5pH clarified at RT (25-30^oC) for 30 minutes were loaded in two wells per sample, covered in plastic bags and incubated overnight (maximum of 9 hours) at 4^oC. After incubation, leaf extracts were discarded and plates washed using PBS-T as above. Unlabeled monoclonal antibodies (MAbs) of the respective CBSVs species (Table 3.1) were diluted in conjugate buffer as described by the manufacturer and loaded onto the plates, each well with 100 µl and incubated at 37^oC for 3 hours and followed by washing as in coating step. Then a labeled Ab, (Rabbit anti mouse-alkaline phosphatase conjugated- RAM-AP) was diluted in conjugate buffer as per protocol and 100µl of its solution were loaded in each well and incubated at 37^oC for 3 hours, followed by final washing as in previous steps. In the final 10 minutes of incubation, substrate buffer was prepared by dissolving 5mg of Nitro phenyl phosphate (Npp) in 5mls of substrate alkaline phosphate buffer and briefly stored in 4^oC.

After washing the plate, 100µls of substrate was added in each well covered by aluminium foil and incubated at room temperature (RT). Readings were recorded after every 30 minutes until 120 minutes using microtiter plate reader (Global Diagnostic and Medical Solutions). Successful reactions were considered only when the well with no sample (blank wells) remained colourless at each reading. Positive readings were taken as any value twice the reading of negative control (CBSVs-free cassava leaf samples confirmed by RT-PCR and qRT-PCR techniques). Mean absorbance values were calculated from the duplicate values of each sample. Each test was repeated three times each with fresh leaf samples collected from the same plant and using the same antibodies.

3.3.3.4.1 Determination of sensitivity of TAS-ELISA for the detection and discrimination of CBSVs in infected cassava plants

The sensitivity of CBSVs monoclonal antibodies (MAbs) using TAS-ELISA technique was determined with two sets of antibodies (**Table 3.1**, S/N 2 and 3) using a total of 9 screenhouse cassava samples including, 3 CBSV, 3 UCBSV, 1 co-infected, 1 healthy and 1 positive control samples with pre-known CBSVs status and confirmed by RT-PCR (Mbanzibwa *et al.,* 2011) as a gold standard. All 9 samples were duplicated into plate wells and diluted five times in ten folds. Both RT-PCR and TAS-ELISA were performed as described in section 3.3.3.3 and 3.3.3.4, respectively. Crude cassava sap were serially diluted starting with recommended dilution by kit manufacturer of 1:20, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$ and $1:10^{-5}$ using its extraction buffer, while total RNA extracted from the same leaf samples were also serially diluted in the same dilution factors using sterile double distilled water. Sensitivity test was determined as percentage calculated as [total number of true positive samples/ {total true positive + total false negative}] X100 (Kox *et al.,* 2007).

3.3.3.4.2 Determination of specificity of TAS-ELISA for the discrimination of CBSV and UCBSV in infected cassava plants

In testing for specificity of CBSVs antibodies, a total of 16 CBSV pre-inoculated screenhouse cassava plants that included CBSV (7), UCBSV (4), co-infected (5) were used in mixed antibodies (**Table 3.1**, SN 5). Specificity was further validated using a field survey samples, 34 cassava leaf samples from four months old plants which included CBSV (10), UCBSV (10), co-infected (10) and a set of monoclonal antibodies (**Table 3.1**, serial numbers 1 and 3). In all the tests, two CBSVs positive samples, one from the TAS-ELISA kit and the other four pre-confirmed by RT-PCR plus one negative control were included. The TAS-ELISA procedure described in section 3.3.3.4 was followed.

The specificity (Spe) was determined as correctness of each antibody to specifically discriminate the negative samples from virus infected cassava leaf samples, and was calculated as a percentage number of true negative (TN) over the total false positive (FP) plus true negative multiplied by one hundred. Percentage specificity= [Total true negative detected/ {total true negative detected + total false positive}] x100 (Kox *et al.,* 2007). The specificity results determined by TAS-ELISA antibodies were compared with those obtained with the RT-PCR (Standard) results.

3.3.3.4.3: Determination of best leaf position for reliable detection of CBSVs in cassava plants using TAS-ELISA

To test for best leaf position for detecting CBSVs infection, 65 leaf samples were collected from five CBSVs pre-inoculated screenhouse plants of six months old. The status of the plants with respect to CBSD viruses were: CBSV (2), UCBSV (2) and CBSV (co-infection (1). Leaf samples were collected from each node from the lowest leaf closest to the base of the top fully open youngest leaves of each tested plant irrespective of having CBSD symptoms. The lower, middle and upper zones had a total 21 samples each. TAS-ELISA and RT-PCR were performed as described in 3.2.3.4 and 3.2.3.3 sections, respectively.

3.4 Data analysis

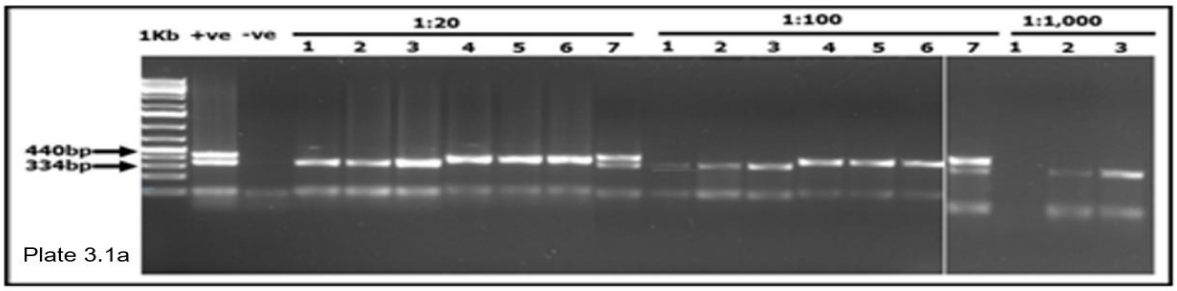
Screenhouse experiment was arranged in a complete randomized design (CRD) with four treatments CBSV, UCBSV, co-infected samples and healthy plantlets which had three entries each and replicated three times, giving a total of 36 plantlets. Antibody specificity, sensitivity and leaf position were subjected to analysis of variance using Genstat statistical software (2006 version 15) and absorbance means among treatments were separated using Fisher's Unprotected LSD.

3.5 Results

3.5.1 Sensitivity of TAS-ELISA for the detection and discrimination of CBSVs in infected cassava plants

Majority of CBSVs-infections were detected by RT-PCR in low dilutions with 100% detection were from the 1:20 up to $1:10^{-4}$dilutions. However, sensitivity decreased with an increase in dilution, where as 28.57% CBSVs were detected when dilution reached 10^{-5} and no detection (0%) was observed at 10^{-6} (**Plates 3.1a, 3.1b** and **3.1c**). Using the same dilution series, CBSV and UCBSV-MAbs showed higher sensitivity in highly diluted samples than with RT-PCR using screenhouse samples. Moreover, the UCBSV-MAb detected more viruses in more diluted samples than CBSV-MAb (**Table 3.2**).

Statistical results showed that there was significant difference ($P \leq 0.05$) between dilutions in both CBSV and UCBSV-MAbs using screenhouse samples (Table 3.3). The CBSV and UCBSV-MAb had the highest absorbance means (nM) of 0.619 nM and 2.524 nM at $1:10^{-5}$ and 1:20 (w/v) dilutions respectively. Generally, the CBSV-MAb had low absorbance means compared to UCBSV-MAb.



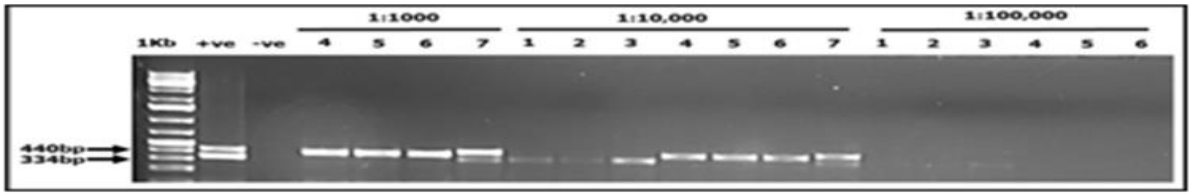


Plate 3.1b

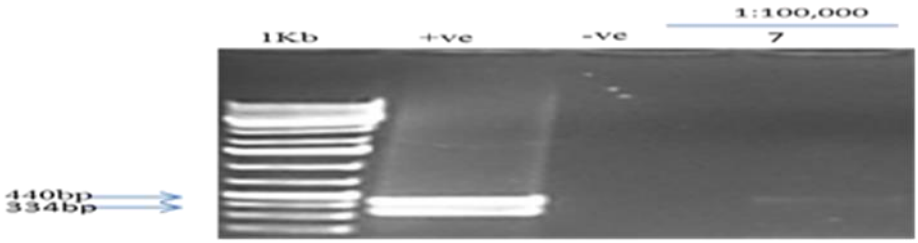


Plate 3.1c

Plate 3. 1a, 3.1b and 3.1c: Agarose gel electrophoresis of cassava leaf samples infected with CBSV isolates collected from the screenhouse and screened by RT-PCR prior to antibody sensitivity testing by TAS-ELISA.

Table 3. 2: Positive samples at different serial dilutions of crude saps from cassava leaf samples used to test for sensitivity of TAS-ELISA kit using Monoclonal antibodies (MAbs) for CBSV and UCBSV confirmed by RT-PCR

Assay/Ab	**Virus Species**	**Dilutions (Leaf sap:extraction buffer- w/v)**				
TAS-ELISA		**1:20***	**1:100**	**1:1000**	**1:10000**	**1:100,000**
CBSV-MAb	CBSV	0/3	0/3	0/3	1/3	3/3
	UCBSV	0/3	0/3	0/3	0/3	1/3
	Co-infection	0/1	0/1	0/1	0/1	0/1
	Healthy	0/1	0/1	0/1	0/1	0/1
Total detection		**0/7=0.0%**	**0/7=0.0%**	**0/7=0.0%**	**1/7=14.2%**	**57.14%**
UCBSV-MAb	CBSV	3/3	3/3	3/3	2/3	2/3
	UCBSV	3/3	3/3	3/3	2/3	3/3
	Co-infection	1/1	1/1	1/1	1/1	1/1
	Healthy	0/1	0/1	0/1	0/1	0/1
Total detection		**7/7=100%**	**7/7=100%**	**7/7=100%**	**5/7=71.4%**	**6/7=85.57%**
RT-PCR	CBSV	3/3	3/3	3/3	3/3	1/3
	UCBSV	3/3	3/3	3/3	3/3	0/3
	Co-infection	1/1	1/1	1/1	1/1	1/1
	Healthy	0/1	0/1	0/1	0/1	0/1
Total detection		**7/7= 100%**	**7/7= 100%**	**7/7= 100%**	**7/7= 100%**	**2/7= 28.75%**

Table 3. 3: Mean Absorbance of serial dilutions of cassava leaf samples tested by CBSV and UCBSV-Mabs, in TAS-ELISA using screenhouse cassava leaf samples

	Treatments	
Dilution (w/v)	**CBSV-MAb**	**UCBSV-MAb**
1:20	0.6848	2.820
1:100	0.4459	2.464
1:1,000	0.3576	2.584
1:10,000	0.8039	2.500
1:100,000	0.8039	2.253
Mean	**0.619**	**2.524**
LSD(0.05)	**0.3072**	**0.4375**
CV%	**70.4**	**24.6**

Dilutions with absorbance mean difference less than 0.3072 and 0.4375 LSD at ($P \leq 0.05$) for CBSV and UCBSV-MAb respectively along the columns are not significantly different

3.5.2 Specificity of TAS-ELISA kit for the discrimination of CBSV and UCBSV in infected cassava plants

The specificity of CBSV and UCBSV-MAbs was tested using a total of 32 field-collected cassava leaf samples. Results showed that both CBSV and UCBSV-MAbs had a specificity (negative selectivity) of 52.5% and 72.7%, respectively and positive selectivity was 62% for CBSV and UCBSV (**Table 3.4 and Appendix 1**), while for the RT-PCR specificity of discrimination was 100%. In comparison to RT-PCR, CBSV and UCBSV-MAbs were 31.1% and 23.8% less specific. Moreover, UCBSV-MAb was more specific than CBSV-MAb by difference of 18.18%. In the mixed antibodies, the specificity of mixed antibodies (**Table 3.5**) was determined using 16 screenhouse-raised cassava leaf samples. Results showed that mixed

antibodies detected 100% total true negative and 62.5% for true positive detection (**Plate 3.5**), while RT-PCR had specificity of 100% (**Table 3.5 and Appendix 2**).

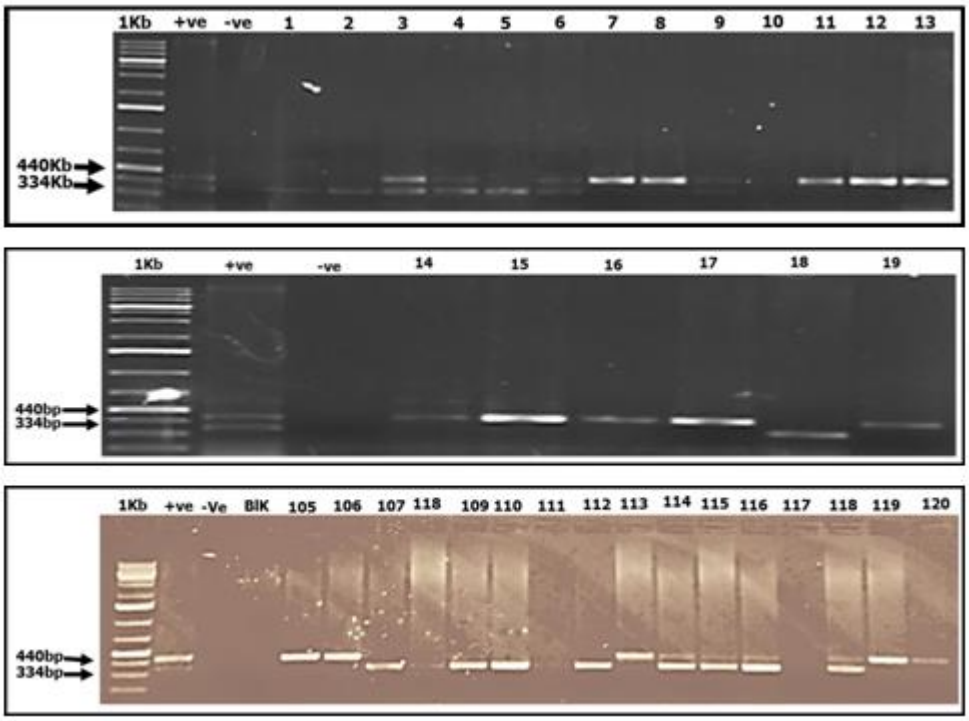


Plate 3. 2: Agarose (1.5%) gel electrophoresis of RT-PCR products from cassava leaf samples collected from breeders' field plots used to validate the specificity of TAS-ELISA kit

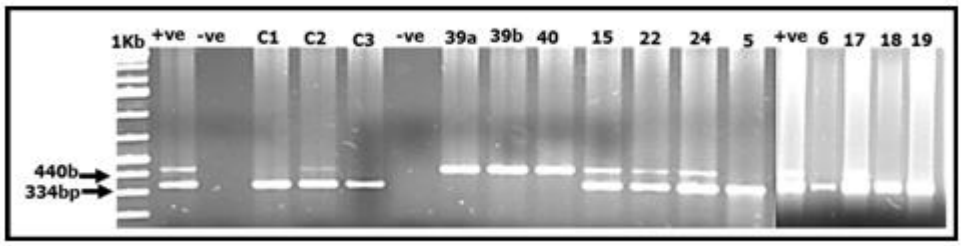


Plate 3. 3: Agarose (1.5%) gel electrophoresis of CBSVs- infected cassava leaf samples collected from screenhouse and screened by RT-PCR and used to validate mixed antibodies for the detection of CBSVs using TAS-ELISA.

Table 3. 4: Percent specificity of CBSV and UCBSV-MAb on CBSVs-infected cassava leaf samples from field pre-confirmed by RT-PCR

Detection categories	**TAS-ELISA**		**RT-PCR**		
	CBSV	**UCBSV**	**CBSV**	**UCBSV**	**U+CBSV**
Total detection	18/32	18/32	10/10	10/10	11/11
Total true +Ve	13/21	13/21	10/10	10/10	11/11
Total true –Ve	6/11	8/11	21/21	21/21	21/21
Total false +Ve	5/32	3/32	0/0	0/0	0/0
Total false –Ve	9/32	6/32	0/0	0/0	0/0
% Specificity	**54.54**	**72.72**	**100**	**100**	**100**

Table 3. 5: Percent specificity (% Spe) combined antibodies using TAS-ELISA for the detection and discrimination of CBSVs in screenhouse raised cassava plants infected with CBSVs in single and double infection confirmed by RT-PCR

Detection categories	**TAS-ELISA**	**RT-PCR**		
	Mixed antibodies	**CBSV**	**UCBSV**	**CBSV+UCBSV**
Total detection	15/16	7/7	4/4	5/5
Total true +ve	15/16	7/7	4/4	5/5
Total true-ve	1/1	1/1	1/1	1/1
Total false +ve	0/15	0/16	0/0	0/0
Total false-ve	4/15	0/1	0/1	0/1
% Specificity	**100**	**100**	**100**	**100**

Statistical analysis revealed that the highest absorbance mean increased in co-infected samples when using CBSV-MAb and UCBSV-MAb than in singly infected samples (**Table 3.6**). The highest absorbance means were observed in co-infected samples (1.634 nM) and the lowest in CBSV- infected samples (1.1173 nM). There were significant differences at LSD ($P \leq 0.05$) in absorbance means of the three infection types.

Using mixed antibodies in the screenhouse higher absorbance mean of 2.056 nM was recorded in the co-infected samples than in single infections of UCBSV and CBSV with 1.42

and 1.524 nM respectively, (**Table 3.7**). There was no significant difference in different infections at 1.1023 LSD (P≥0.05). The lowest mean absorbances (1.421 nM) was observed in UCBSV infected samples.

Table 3. 6: Mean absorbance means for specificity of CBSV and UCBSV-monoclonal antibodies (MAb) in TAS-ELISA assay for detection and discrimination of CBSVs using field collected samples naturally

Sample infection status	**CBSV-MAb**	**UCBSV-MAb**
Blank	0.008	0.0060
Health	0.648	0.4100
CBSV	1.326	0.6691
UCBSV	1.617	0.8870
Co-infection	1.634	1.1173
Mean	**1.0454**	**0.61788**
LSD (P≤0.05)	**0.5513**	**0.4791**
CV (%)	**25.6**	**37.9**

*CBSVs infection types with absorbance mean difference less than 0.5513 and 0.4791 LSD at (P≤0.05) for CBSV and UCBSV-MAbs respectively along the columns are not significantly different.

Table 3. 7: Mean absorbance results for specificity of combined antibodies in TAS-ELISA assay for CBSVs using screenhouse collected samples

Sample Infection Status	Mean absorbance (combined CBSV+UCBSVMAbs) (nM)
Blank	0.016
Health	0.7791
UCBSV	1.42
CBSV	1.524
Co-infection	2.056
Mean	**1.1592**
LSD (P≤0.05)	**1.1023**
CV%	**33.6**

*CBSVs infection types with absorbance mean difference less than 1.1023 LSD at (P≥ 0.05) along the columns are not significantly different

3.5.3 Determination of best plant leaf position for reliable detection of CBSVs in cassava plants using TAS-ELISA Kit

Of the 65 leaf samples collected from 5 CBSVs-infected plants and used to determine best leaf position for detecting CBSVs, 60 samples were positive to at least one of the CBSVs antibodies used (**Table 3.8** and **Appendix 3, 4**). CBSV-MAb detected 27% and UCBSV-MAb 40.9% true positive samples (**Table 3.8** and **Appendix 3, 4**). In both MAbs, samples from lower leaf position had high detection percentage of 46.6% and 64.7% for CBSV and UCBSV-MAbs respectively.

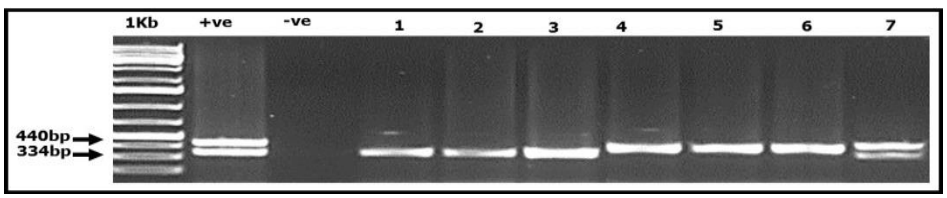


Plate 3. 4: Agarose (1.5%) gel electrophoresis of PCR amplified products for CBSV, UCBSV and dual infection of cassava leaf samples pre-inoculated with CBSVs isolates collected from screenhouse with CBSD like symptoms, used to determine best plant leaf position for detection of CBSVs by TAS-ELISA assay.

Table 3. 8: Mean absorbance of CBSV and UCBSV-monoclonal antibodies (MAbs) using TAS-ELISA detecting CBSVs on three plant leaf positions with and without CBSD-like symptoms

Leaf position/zone	CBSV-MAb	UCBSV-MAb
Low	7/15=46.6%	11/17=64.7%
Middle	5/13=38.5%	11/17=64.7%
Upper	6/13=46%	5/15=33.3%

The lowest mean absorbance were observed in upper leaf position for CBSV-MAb and UCBSV-MAb at 1.076 nM and 2.210 nM, respectively. The low leaf positions had the highest absorbance mean of 2.21 nM in UCBSV- MAb significantly different (P≤0.05) from the upper samples but not with middle leaf zone (**Table 3.9** and **Appendix 3, 4** and **Table 3.8**). However, the lower zone had the higher number of samples detected positive but with less differences in other plant positions using both monoclonal antibodies.

Table 3. 9: Mean absorbance values of three plant leaf positions used during sampling and detection and discrimination of CBSVs in the cassava plants using TAS-ELISA kit with monoclonal antibodies for detection of CBSV and/or UCBSV

Leaf position	CBSV-MAb	UCBSV-MAb
Upper leave	1.030	1.730
Lower leaves	1.076	2.210
Middle leaves	1.092	2.055
Mean	1.067	2.102
LSD (P≤0.05)	0.1392	0.3557
CV (%)	30.8	41.7

Leaf positions with absorbance mean difference less than 0.1392 and 0.3557 LSD at (P≥0.05 and P≤ 0.05) for CBSV and UCBSV-MAbs respectively along the columns are not significantly different

3.6 Discussion

This study was to determine the efficacy of CBSVs monoclonal antibodies developed and recently refined by the DSMZ for their sensitivity and specificity in detection and discrimination of CBSVs in cassava infected with CBSD. The results showed that the monoclonal antibodies were as sensitive as RT-PCR up to a dilution of $1:10^{-5}$ (w/v), but less sensitive in low dilutions with no difference of detection between the antibodies. Similarly, both MAbs and mixed monoclonal antibodies produced around 50% specificity for the targeted CBSVs species, with MAb-UCBSV being more specific than MAb-CBSV and not for the combined antibodies. The results also showed the sensitivity of the MAbs to increase with sample dilution above the recommended 1:20 w/v to $1:10^{-5}$ w/v, which increased CBSVs detection in both antibodies. The present results are consistent with those obtained by Sano *et al*. (1992) and Karasev *et al*. (2008), who reported that TAS-ELISA was more sensitive in more diluted samples with less crude materials such as glycoproteins and proteins that impair immunological reactions between the antigens and antibodies.

The specificity of TAS-ELISA using MAbs for CBSVs was around 50% of the gold standard (RT-PCR). Mixed antibodies increased the chances of recognizing the true positive. The cross-reaction between these closely related virus species is caused by the relatedness of the virus species epitopes (Brunt, 1992; Babu *et al.,* 2012), hence the monoclonal antibodies did not specifically discriminate the virus specific species. In this study, no cross reaction was observed between CBSVs both MAbs and /or mixed antibody. The sensitivity of MAbs and mixed Ab was higher in co-infected samples with high absorbance values than in the single infected samples. This agrees with the CBSVs quantification studies by Kaweesi *et al*. (2014) and Ogwok *et al*. (2014) where co-infected samples had more virus titer values than other infection types. Monoclonal antibodies were not reliable in detecting the true positive from true negative samples.

The results showed that there were significant differences ($P \leq 0.05$) in the absorbance values recorded for different sampling positions. Absorbance values were highest in lower mature leaf positions followed by middle and least for the upper leaf positions. Both CBSV-MAb and UCBSV-MAb detected large numbers of positive samples in lower leaf positions with non-senescence leaves followed by middle and upper plant leaf zones. Previous studies (Husted and Bech, 1996; Mahmoud *et al*., 2007; Moreno *et al*., 2011) using qRT-PCR, a more sensitive assay for CBSV detection, also reported similar results. The DSMZ protocol manual version 2.0 (DSMZ, 1998) and Moreno *et al.* (2011) recommended that mature infected cassava leaf samples with symptoms yield more true positive samples. Results obtained with MAbs using TAS ELISA in the current study detected higher absorbance values on samples collected from lower symptomatic non senesced leaves. Moreover, Kaweesi *et al*. (2014) and Ogwok *et al*. (2014) suggested that to increase the chances of detecting CBSVs on CBSD-infected cassava plants, sampling should be done not less than four months after planting.

From the study, it is clear that RT-PCR should be used in routine screening of planting materials since the method is more effective in detection of CBSVs. However, monoclonal antibodies and the mixed antibodies using TAS-ELISA can be used in academic and research institutions for teaching purposes. However, CBSVs-MAbs can also be used elsewhere where there is no access to RT-PCR for screening of cassava materials.

CHAPTER FOUR

4.0 COST-EFFECTIVENESS OF TAS-ELISA AND RT-PCR TECHNIQUES IN DETECTION OF *CASSAVA BROWN STREAK VIRUSES* INFECTING CASSAVA

4.1 Abstract

This study was carried out to determine the cost effectiveness between the cassava brown streak diasese (CBSD) diagnostic techniques namely reverse transcription polymerase chain reaction (RT-PCR) and triple antibodies sandwich-enzyme linked immunosorbent assay (TAS-ELISA) in routine screening of CBSV and UCBSV infection in cassava planting materials. Ratio analysis was used in this study to allow choosing the most cost effective tool between the RT-PCR and TAS-ELISA when detecting the CBSVs in cassava leaves. Cost estimations for each detection method was done using cost analysis technique on 100 cassava leaf samples. Results showed the cost for testing 100 samples using RT-PCR to be US$ 558.95, while for TAS-ELISA was US$452.39, with an average cost of US$5.58 and US$4.52 per sample, respectively. The true detection capability of RT-PCR for CBSV, UCBSV and co-infection was 100%, while the CBSV and UCBSV-MAbs had 60.87% and 59.09%, respectively. Analysis by RT-PCR saves more time than TAS-ELISA by a difference of 10 hours and 30 minutes for the same number of samples. The TAS-ELISA had a higher cost to attain its single unit of effectiveness (true positive detection capacity) by 7.53 US$% than RT-PCR which had 5.58 US$%. This means that TAS- ELISA technique is not only less effective than the standard RT-PCR technique but also not cost effective. Therefore, RT-PCR is expensive but cost effective and should be used in detecting CBSVs in cassava to ensure good management of the CBSD.

4.2 Introduction

Cassava brown streak disease (CBSD) is caused by two distinct virus species, *Cassava brown streak virus* (CBSV) and/or *Uganda cassava brown streaks virus* (UCBSV), genus Ipomovirus; family *Potyviridae* (Mbanzibwa *et al.,* 2009a). The disease is a threat to food security in sub-Saharan Africa (SSA) and is reported to cause loss of up to100 million USD per year (Hillocks *et al.,* 2002; Ndunguru *et al.,* 2015). However, losses are not only limited to food insecurity, its management is also costly due to lack of durable resistant varieties. Different methods have been developed and are being used for the detection of the CBSVs including reverse transcription polymerase chain reaction (RT-PCR) (Mbanzibwa *et al.,* 2009a; Abarshi *et al.,* 2010; Monger *et al.,* 2010), triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) (Winter *et al.,* 2010) and loop-mediated isothermal amplification assay (LAMP assay) (Tomlison *et al.,* 2012). However, no single practical cost-effective technique is available for comprehensive routine screening of cassava planting materials for CBSVs infection in seed multiplication schemes.

Cost Effective Analysis (CEA) is a financial technique used in analysing the benefit of alternative techniques available for decision making (Fenwick *et al.,* 2005; Karasev *et al.,* 2008). The financial analysis technique can be integrated in plant disease diagnosis to analyse costs and effectiveness of available diagnostic techniques. Financial analysis on the cost and effectiveness of techniques used in the disease diagnosis is crucial to ensure the use of less costly and reasonable effective technique among the techniques available. Under this study, financial analysis involved TAS-ELISA and RT-PCR in CBSD diagnosis. The study involved analysis of incremental costs and effects and their ratios between the studied techniques as well as cost effectiveness ratios. The cost-effectiveness ratio is used to determine the costs used to attain a single unit of detecting true positive samples (effectiveness) and hence used by decision makers to either adopt or reject new technique

(Prime Minister's Strategy Unit, 2004; Sanjeev *et al.,* 2013; Josiah, 2014). Finally, the technique with the least cost, is effective and easily accessed by the end user will be adopted by cassava seed multipliers for their routine CBSD diagnosis.

4.3 Materials and Methods

4.3.1 Source of samples and sample collection

A total of 100 cassava leaf samples with CBSD-like symptoms were collected from three released varieties including: Kiroba (86), Kibaha (05), Kizimbani (06), and one breeding line KBH0363 (03) by field survey from Chambezi substation in Coast region (E 38°54'S 06°32', 38 m.a.s.l). Negative and positive control samples were obtained from screenhouse-grown cassava plants at Mikocheni Agricultural Research Institute (MARI), Dar es Salaam. Leaf samples were collected in plastic bags and stored at 4°C in a cool box and shipped to MARI for analysis on the same day of sampling.

4.3.2 RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis was performed as described in section 3.2.3.1 and 3.2.3.2, respectively using buffers prepared at MARI using recommended chemicals as described in the CTAB protocol (Chang *et al.,* 1993) and RT-PCR kit.

4.3.3 RT-PCR and TAS-ELISA

All field collected samples were first screened for their CBSVs status using RT-PCR with virus species specific primers as described in section 3.2.3.3, PCR kit (Thermo scientific, USA) was procured from Inqaba Biosciences, South Africa. Similarly, serological analysis was carried out using TAS-ELISA kit containing monoclonal antibodies for CBSV and UCBSV, Rabbit anti Mouse (RAMs) (Batch Number: (i) CBSV:5374, 3854 and 5080 (ii) UCBSV: 5116, 5116 and 5377 for IgG, MAb and RAM, respectively) and lyophilized positive control was procured from DSMZ, Germany. TAS-ELISA buffers and procedures

were prepared at MARI using chemicals and procedure described by the manufacturers (Section 3.3.3.4). Both RT-PCR and TAS-ELISA tests were repeated three times using the same reagents and buffers prepared from same source of chemicals, respectively.

4.3.3.1 Determination of effectiveness of CBSVs monoclonal antibodies using TAS-ELISA for the detection and discrimination of CBSVs in cassava

To determine the effectiveness of CBSVs monoclonal antibodies S/N 5 **(Table 3.1)** using TAS-ELISA in relation to cost was evaluated by testing 100 field collected cassava leaves with CBSD-like symptoms and asymptomatic samples. Samples were pretested in three independent RT-PCR tests using CBSVs specific primers (Mbanzibwa *et al.,* 2011) and confirmed to contain 27 (CBSV), 30 (UCBSV), 18 dual infections (CBSV and UCBSV) and 25 (healthy samples) and TAS-ELISA assay was done 3 times each using same MAbs for CBSV and UCBSV. TAS- ELISA procedures were done as described in section 3.3.3.4. Sensitivity and specificity were also measured as the number of correct true positive and true negative samples detected respectively, compared to the RT-PCR results.

4.3.3.2 Determination of the cost of TAS-ELISA and RT-PCR techniques

The overall measure of the difference between the cost effectiveness /or output yielded by each assay; TAS-ELISA and RT-PCR in detecting CBSVs in cassava leaf samples was done by comparing the costs involved in analyzing 100 samples by each assay. Costs were categorized based on the step in which materials were used in the assays. Reagent costs and other materials for TAS-ELISA were obtained from the kit manufacturer, DSMZ Plant Virus Department (Invoice Number of 05th June, 2014 and of 27th May, 2015), whereas the currency was converted from Euros into US-Dollar using "Easy currency exchange rate application" version 2.1.8., at a rate of 1EURO = 1.1335USD on September 13th, 2015.

Steps and materials involved in TAS-ELISA included; (1) buffers preparation phase-PBS-Tween 20, conjugate, substrate, coating and extraction buffers, (2) Biochemical-Antibodies

IgG, MAb and AP-RAM, Positive controls and Skimmed milk, (3) Glassware: Tips, trough, microtiter plates, extraction bags and Aluminium foil, and (4) Other charges; Shipping costs from the supplier to MARI including Bank charges.

Cost categories for the two steps RT-PCR were based on the quotation from MARI laboratory reagents main supplier, Inqaba Biotech EA Ltd, Nairobi, Kenya (Quotation Number: 20151233 and 20151901 of August 18th, 2015). Main categories include; (1) Sample extraction buffer, cost for each constituent of 2% CTAB buffer as described by Chang *et al.* (1993), (2) Polymerization phase involving costs for each biochemical contained in reverse transcriptase kit and PCR kit as indicated by the supplier. Other chemicals procured separate from the kits including; dNTPs and Primers; Forward primer (CBSDDF2) and reverse primer (CBSDDR) (Mbanzibwa *et al.,* 2011) and OligodTs $_{(18)}$ primer, (3) Gel electrophoresis phase; agarose powder, gel running buffer-TAE buffer components, loading dye, ladder and ethidium bromide (4) consumables tips, eppendorf tubes, and (5) Other charges including shipping costs and bank charges when ordering kits and reagents. All capital expenses for machines such as thermal cycler and microtiter plate readers and other machines were excluded as each laboratory has its established bench fee costs which cover for machine and utilities.

Time taken by each assay to analyse 100 fresh samples was calculated based on the time recommended for each step in the respective protocols by manufacturers and optimized in MARI laboratory. For TAS-ELISA time taken included sample extraction, serological testing including ELISA plate washing, colour development and absorbance reading for 120 minutes. While, in RT-PCR involved time from extraction of total RNA using CTAB method (Chang *et al.,* 1993), cDNA synthesis, PCR amplification and finally gel electrophoresis and photo band reading.

4.3.3.3 Cost-effectiveness of RT-PCR and TAS-ELISA ratios determination

The cost/effectiveness ratio of both techniques were determined by using the formulae described by Josiah (2014) and WHO (2003) whereas; *Cost- effectiveness ratio = [cost of a tool to analyse 100 samples / total true positive (sensitivity) percent of samples detected in 100 samples]…….Equation (i).*Comparison of positive samples selection capacity (sensitivity) of each technique was done using mean sensitivity of both antibodies.

4.4 Data analysis

Antibody absorbance differences in different virus infections were analysed using Genstat statistical software (2006 version 15) and absorbance means separated using Fisher's unprotected least significant difference (LSD). The cost, effectiveness and cost effectiveness ratios of antibodies and RT-PCR were analyzed as described by Josiah *et al.* (2014).

4.5 Results

4.5.1 The effectiveness of commercial CBSVs monoclonal antibodies using TAS-ELISA and RT-PCR for detection and discrimination of CBSVs in cassava plant infected with CBSD

Both MAbs had high percent sensitivity in reliable detection of CBSVs. Monoclonal antibody for CBSV had 60.9 and UCBSV had 59.1% sensitivity of the previously confirmed CBSVs infected samples by RT-PCR (**Table 4.1, Plate 4.1 and Appendix 5 and 6**).

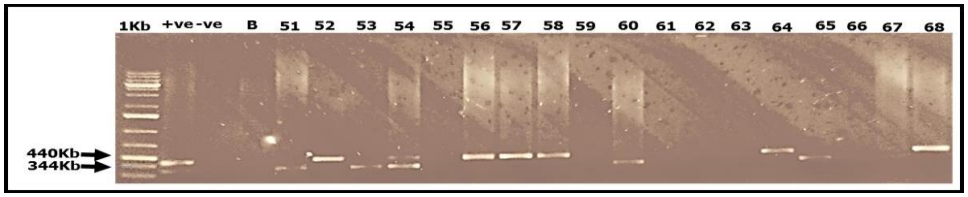


Plate 4. 1: Agarose (1.5%) gel electrophoresis of RT-PCR amplified products of field collected cassava leave with CBSD-like symptoms and asymptomatic samples used to evaluate effectiveness of CBSVs monoclonal antibodies using TAS-ELISA for the detection and discrimination of CBSVs.

Statistical analysis showed that there was no significant difference in detection sensitivity between MAbs for CBSVs and UCBSV at (P≥ 0.05). The overall mean absorbance values for MAbs was 1.477 and 1,207nm (Table 4.2). UCBSV-MAb had the highest mean absorbance of 1.64nm followed by MAb-CBSV with 1.48nM. In general, the co-infected samples had higher mean absorbance than other infections with no relation between absorbance mean differences with number of samples detected to be true positive for each infection type (**Table 4.1 and 4.2**).

Table 4. 1: Effectiveness of CBSVs Monoclonal antibodies TAS-ELISA and RT-PCR on detection of CBSVs on CBSVs-pre-confirmed CBSD infected cassava leaf samples

	TAS-ELISA		**RT-PCR**		
	MAb-CBSV	**MAb-UCBSV**	**CBSV**	**UCBSV**	**U+CBSV**
TD	77/100	39/100	27/100	30/100	18/100
TP	77/45	39/48	27/27	30/30	18/18
TN	23/52	61/52	83/83	70/70	83/83
TTP	28/45	26/48	27/27	30/30	18/18
TTN	18/52	42/52	83/83	70/70	82/82
Positive (%)	**60.8**	**59.09**	**100**	**100**	**100**
Negative (%)	**39.0**	**95.5**	**100**	**100**	**100**

*N=100 leaf samples, *TD-total detection, TP=Total positive samples, TN-total negative, TTP=total true positive, TTN=total true negative, Se-sensitivity and Spe-Specificity

Table 4. 2: Mean absorbance value of TAS-ELISA assay using MAbs for CBSV and UCBSV to detect CBSVs in CBSD-infected pre-confirmed samples using RT-PCR technique

Infection Status	**CBSV-MAb**	**UCBSV-MAb**
Health	0.8945	0.8775
CBSV	1.379	1.011
Co-infection	1.487	1.647
UCBSV	1.703	1.294
Mean	**1.477**	**1.207**
LSD (P≤ 0.05)	**0.687**	**0.9805**
CV%	**29.5**	**19.3**

*Virus infections with different absorbance mean less than 0.687 and 0.9805LSD (P≥0.05) for CBSV and UCBSV-MAb respectively along the columns are not significantly different

4.5.2 Determination of cost between TAS-ELISA and RT-PCR for detection and discrimination of CBSVs

The cost of analyzing 100 fresh cassava leave samples using RT-PCR was estimated at US$ 558.95 and took 19 hours and 30 minutes, while TAS-ELISA cost was estimated at US$ 452.05 and took 29 hours and 36 minutes to complete the analysis on the same number of samples (**Table 4.3** and **4.4**). The RT-PCR cost was US$ 106.90 higher than TAS- ELISA, cost that can be used to test 24 samples using TAS-ELISA. Shipping costs for reagents from the suppliers overseas is the most expensive part in both techniques, where it took 80% and 41.6% of the total cost in TAS-ELISA and RT-PCR assays, respectively. Polymerization phase is the second most expensive part in RT-PCR, and incurred 38% of the assay cost. Moreover, TAS-ELISA takes much longer to finish a test of 100 samples with 29 hours and 36 minutes, which is almost 2 times longer than RT-PCR which takes 10 hours and 6 minutes.

Table 4. 3: Results showing the cost in used for analyzing 100 cassava fresh leaf samples in US$ using CBSV and UCBSV-MAbs in TAS-ELISA

TAS-ELISA assay	Reagents	Amount required	Costs (US$)
(a) Samples extraction phase			
	$NaCO_3$	0.015gms	
	$NaHCO_3$	0.0293gms	
	NaN_3	0.002gms + 0.6gms	
	Na_2SO_3	0.232gms	
	$KHPO_4$	0.6gms	
	Na_2HPO_4	3.45gms	
	KCl	0.6gms	
	NaCl	24gms	
	Tween 20	2mls for 2L	
	Egg Albumin	0.0232gms	
	PVP	0.232gms	
	Diethanolamine	0.97mls	
Subtotal cost			**44.66**
(b) Serological phase	IgG antibody	23.2µl	14.28
	Mab-CBSVs	46.4µl	14.28
	RAM antibody	11.6µl	14.28
	Positive control	2000µls	1.81
	Skimmed milk	0.2gms	
	Substrate (Npp tablets)	2 tablets	
Subtotal cost			**44.65**
(c) Consumables	Aluminum foil	$100cm^2$	0.01
	White tips	3 tips	0.15
	Tips(100uls)	1,435 tips	9.81
Consumables			**Costs USD**
	Tips (1000uls)	3 tips	0.71
	ELISA-plates	2 plates	4.53
	Plastic bags	102bags	17.34
Subtotal cost			**32.57**
(d) Other charges	Shipping cost		328.71
	Bank charges		34.00
Subtotal cost			**362.72**
Grand total			**452.05**
Assaying time			29 hours 36 minutes

*All buffers were ready made as for its price, the actual cost were derived from the ratio of amount of reagent required and amount of buffers.

* Total time for each assay exclude time for buffer and solution preparations

Table 4. 4: Results showing the cost used for analyzing 100 cassava fresh leaf samples in US$ using RT-PCR

RT-PCR Assay	Reagents	Amount required	Costs (USD)
(a) Samples extraction phase	CTAB powder	1.4mgs	4.6
	Tris-HCl	7mls	2.8
	EDTA	5.5mls	1.77
	NaCl	5.7gms	0.78
	PVP	1.4gms	0.32
	Chloroform	70mls	12
	Iso-Amylalcohol	2.8mls	0.32
	Ethanol	24.5mls	0.72
	Iso-propanol	49mls	4.39
	2-Mecarptoethanol	1.4mls	0.36
Subtotal cost			**28.06**
(b) Polymerization Phase	dNTPs (10 & 2.5mM from 100mM)	27µl	23.2
	RiblockRNAse	54 µl	9.02
	10xRT Buffer	108µl	5.1
	Dream Taq buffer		12.78
	RT (Transcriptase)	108µl	114
	Taq-Polymerase	32.4µl	41.4
	Primers (F&R)-CBSDDF2/CBSDDR	200 µl	0.26
	OligodT (18)	100µl	6.49
Subtotal cost			**212.25**
(c) Gel electrophoresis Phase	Agarose powder	7.5gms	13.4
	Tris- base	4.84gms	0.31
	Acetic acid	1.142gms	0.0024
	Loading dye	200µls	3.36
	Ladder	4 gel lanes	2.82
	Ethidium bromide		
	*EDTA (costs included in extraction		
Subtotal cost			**19.8924**
(d) Consumables	Eppendorf tubes	200 tubes	4.99
	Tips 100uls)	324 tips	14.53
	Tips (1000uls)	124 tips	2.47
	PCR-tubes 200pcs	115 tips	2.43
	Blue tips		
Subtotal cost			**35.028**
RT-PCR Assay	**Reagents**		**Costs (USD)**
(e) Other charges	Shipping cost		232.56
	Bank charges		31.15
Subtotal cost			**263.71**
Grand total			**558.95**
Assaying time:	10 hours, 6 minutes		

4.5.3 Determination of cost-effectiveness ratios of RT-PCR and TAS-ELISA

Cost effectiveness ratio was calculated using the following equation. *Cost- effectiveness ratio = [cost of a tool to analyse 100 samples / total true positive (sensitivity) percent of samples detected in 100 samples]……..Equation (i).*

Cost-effectiveness ratios analysis: The cost of testing 100 cassava leaf samples using was 452.06 US$ and 558.98 US$ for TAS-ELISA and RT-PCR respectively (**Table 4.3 and 4.4).** Where TAS-ELISA and RT-PCR cost 4.52 US$ and 5.59 US$ testing a one sample respectively. The true positive effectiveness of CBSV-MAb and UCBSV-MAb mean percent was calculated as (60.87% + 59.96%)/2=59.98%. The TAS-ELISA cost-effectiveness ratio=452.06 US$/59.98%=7.53 US$/%effectiveness. The RT-PCR cost-effectiveness ratio=558.98 US$/100%=5.59 US$/%effectiveness. The RT-PCR has the most cost-effectiveness compared to TAS-ELISA by difference of 1.94US$.

4.6 Discussion

This study determined the cost-effectiveness of Triple Antibody Sandwich- Enzyme Linked Immunosorbent Assay (TAS-ELISA) and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The findings showed that TAS-ELISA technique had a lower cost compared to RT-PCR. However, RT-PCR had the most cost-effectiveness in testing a sample than the TAS-ELISA technique. CBSV-infected samples had lower absorbance means in CBSV and UCBSV-antibodies, and highest values were found in co-infected samples in UCBSV-antibodies with significant differences to other infection status. In terms of time taken to test the same number of samples, the RT-PCR technique took shorter time than TAS-ELISA by a difference of 19 hours and 30 minutes. True positive selection (sensitivity) of both CBSV-MAb and UCBSV- MAb were half and above the RT-PCR selection capacity.

The low effectiveness of the antibodies is probably due to; (i) the presence of plant proteins and other organic compounds such as nucleoproteins and deactivation of nuclease, lipid and

carbohydrates that impair the reation of the antibodies with the viruses' epitopes (Karasev *et al.,* 2008; Tan and Yiap, 2009). The TAS-ELISA technique has no centrifugation procedures to separate and remove those compounds from viruses in the samples As compared to RNA purification processes in RT-PCR technique, where organic compounds are removed. (ii) Incompatibility of antibodies' isotopes with virus is another reason for the immuno-reaction not to take place. Such incompatibilities occur due to differences on nucleotide sequences of epitopes during RNA replication of the virus among virus isolates, making limited isotopes recognition of some nucleotide sequences of virus epitopes (Brunt, 1992; Mbanzibwa *et al.,* 2009; Monger *et al.,* 2010; Ndunguru *et al.,* 2015). There was no correlation between number of positive samples detected and mean absorbance values by antibodies (MAbs). This is because there are different virus species and virus load/accumulation which are not directly proportional to each species in the samples. The co-infected samples seem to have high virus load, implying a high rate of virus multiplication than in single CBSV or UCBSV infection in cassava plants (Kaweesi *et al.,* 2014; Ogwok *et al.,* 2014). Earlier studies using the antibodies found less detection capacity (Osogo *et al.,* 2014) compared to RT-PCR technique.

Previous studies reported that TAS-ELISA is less costly but less effective compared to RT-PCR when testing PVY in potato tubers, stems and leaves (Karasev *et al.,* 2008), and that TAS-ELISA technique doubles the cost compared to DAS-ELISA (Vettrano *et al.,* 2009). The triple monoclonal virus detection fashion in the study doubled the cost since each antibody was tested in its own ELISA plate than polyclonal fashion. Moreover, the shipping cost contributed the highest cost in serological tests by taking 80%, otherwise the cost would drop to half the cost calculated. However, RT-PCR is the most expensive assay, mostly due to high shipping cost as well as polymerization phase covering 38% of the cost. Tomlison *et al.* (2012) reported that the antibody development process is more expensive than primer development, but not for the process of sample testing in laboratory.

Depending on the kind of ELISA used, time consumed by TA-S ELISA is longer than RT-PCR (Sharman *et al.,* 2000; Huang *et al.,* 2001) when detecting viruses in banana. This study has shown that TAS-ELISA takes longer than RT-PCR to analyse the same number of samples in fresh state. This is due to the type of ELISA used, where TAS-ELISA has three phases of incubating antibodies for 3 hours each time, and the incubation of samples overnight for approximately 6 hours.

RT-PCR assay uses less cost to detect a single CBSVs positive sample (effectiveness) as compared to CBSVs monoclonal antibodies (MAbs) using TAS-ELISA (Josiah, 2014). Therefore, CBSVs- MAbs are 38% less effective to RT-PCR technique using the same number of samples detecting same CBSVs. The cost-effectiveness of RT-PCR assay is favoured by, (i) high positive selection capacity over TAS-ELISA, (ii) The multiplex nature of the assay, where more than one virus species is detected in one reaction, hence reducing the cost using primers that detects both species. This is different from TAS-ELISA assay where the cost is double because of using two reactions (of antibodies) to detect different virus species; this increases the cost. Similar findings were also observed by Gambino and Gribaudo (2006) when analysing nine grapevine viruses using multiplex RT-PCR and ELISA. Since this study had no standard data to establish the slope for the willing to pay point (k) as per Black (1990), RT-PCR was used as the standard technique. This study indicates that RT-PCR technique is most cost effective and should be used to screen cassava planting materials for CBSVs, while CBSVs mixed monoclonal antibodies can be used where there is no access for RT-PCR in screening the CBSVs in cassava planting materials.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

This study was to determine the efficacy and cost effectiveness of monoclonal antibodies (MAbs) in the detection of CBSVs infecting cassava. The validation of the ELISA kit involved determination of the sensitivity, specificity, best plant leaf sampling position/zone and the cost-effectiveness in comparison with RT-PCR technique.

The study found out that sensitivity of monoclonal antibodies for the detection and discrimination of CBSV was high at above 50%. The antibodies were able to detect the viruses in cassava leaf samples in more diluted concentration below manufacturer's recommended ratio of 1:20 w/v. The antibodies' sensitivity increased as the sample dilution increased before starting to decrease, as it was also reported by Karasev *et al.* (2008). This study is the first to report on the sensitivity of CBSVs-MAbs on CBSVs beyond the recommended ratio. This is because the highly concentrated sample contains high levels of unwanted plant proteins, glycoproteins and carbohydrates that inhibit the immune-reactions between the viruses and antibodies than in more diluted samples (Sano *et al.*, 1992). To get more true positive samples in consignment of samples using antibodies, more dilution of up to $1:10^{-4}$ (w/v) can be used when using this kit for both antibodies.

In this study the specificity of MAbs was further evaluated to determine their ability to detect true positive and true negative samples from different combinations of CBSVs infections. The specificity was shown both in inoculated plants in screenhouse and in naturally infected samples from the field. The percent of false positive and negative samples was due to either background reaction or low virus titre below detection limits by TAS-ELISA.

In order to increase the chances of reliable detection for CBSVs from cassava leaf samples, appropriate samples at proper sampling stage is a prerequisite. The present study evaluated the best leaf position for reliable detection of CBSVs using TAS-ELISA. This study also found out that the best leaf position was lower mature symptomatic leaf. The efficacy of detection was high in mature symptomatic leaves collected from lower position than those from the middle and the least were from the top position. The findings agree with previous findings (Kaweesi *et al.,* 2014; Winter *et al.,* 2010) using RT-PCR assay for detection.

Molecular based assay for detection of CBSVs such as RT-PCR and qRT-PCR are highly sensitive and specific in detection and discrimination of CBSVs than serology based assays. However, the practicability of molecular based assays on routine screening of CBSVs is limited by the high cost due to volume of samples analyzed daily. Therefore, RT-PCR is a more reliable and effective technique for detection of CBSVs in cassava planting materials and can be used for certification and disease indexing on routine basis.

5.2 Conclusions

This study confirmed that CBSVs-monoclonal antibodies using TAS-ELISA can be used in the detection of the viruses, even while using concentrations below the recommendation ratios of antibodies. However, both CBSV and UCBSV monoclonal antibodies were only able to detect the positive samples up to 50% as compared to RT-PCR technique. Both CBSVs monoclonal antibodies have cross-reactions between virus species that leads to false negatives and positive yielding to low specificity as compared to RT-PCR. RT-PCR was more specific than CBSVs-MAbs in discriminating the two CBSVs virus species in cassava sample leaves. The study is the first to report the reliable plant leaf position for sampling for CBSVs detection using CBSVs-MAbs using TAS-ELISA. There were significantly higher numbers of cassava leaf samples detected and virus load determined in lower mature leaf samples than in the middle and upper plant leaf positions when using CBSV-MAbs in TAS-

ELISA technique. Moreover, the CBSVs-MAbs using TAS-ELISA were less costly in testing CBSVs in 100 cassava leaf samples than RT-PCR but less effective in discriminating the virus species, leading to the false positive and negative results.

5.3 Recommendations

The following are the recommendations which are made from this study

- CBSVs-MAbs in TAS-ELISA can be used to detect CBSVs in infected cassava leaf samples below the recommended sample dilution (1:20 w/v) by antibodies' manufacturer.
- Further research needs to be done to improve the specificity of monoclonal antibodies in discriminating the individual cassava brown streak virus species to acceptable levels to be readily available to regulatory authorities and producers of cassava planting materials.
- Cassava leaf samples for CBSVs testing using MAbs in TAS-ELISA technique should be taken from lower plant parts, which have not yet started to senescence to increase chances/reliability of antibodies to detect the viruses if plants are infected with the viruses.
- RT-PCR should be used in detection of CBSVs in cassava due to its reliability and effectiveness in screening the cassava planting materials. However, TAS-ELISA using the MAbs can be used in academic and research institutions for teaching purposes and to places where there is no access to RT-PCR.

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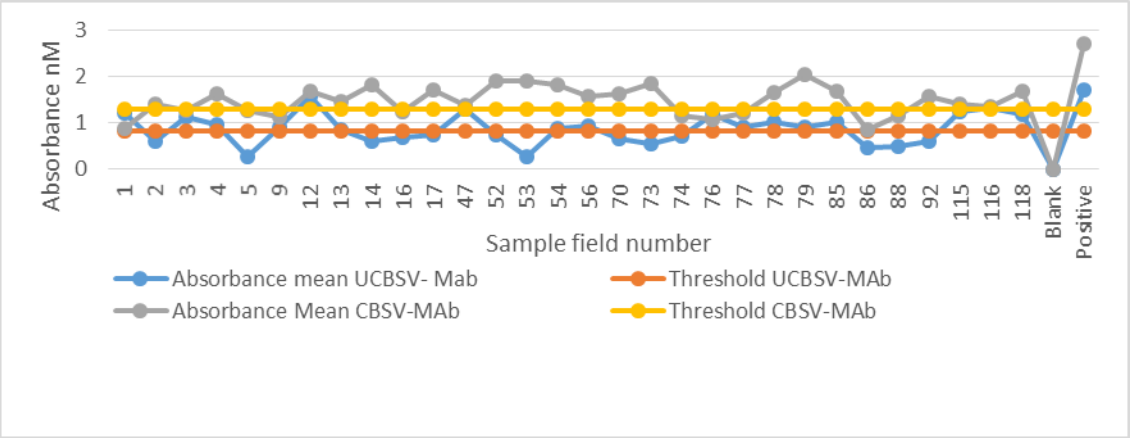
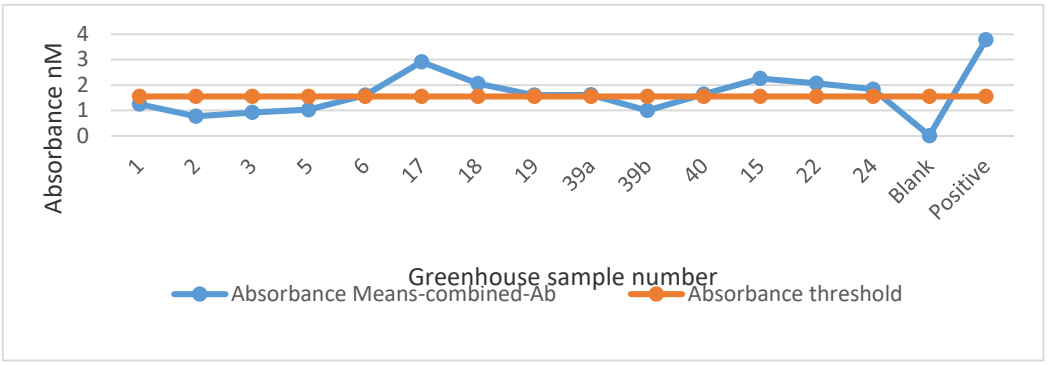
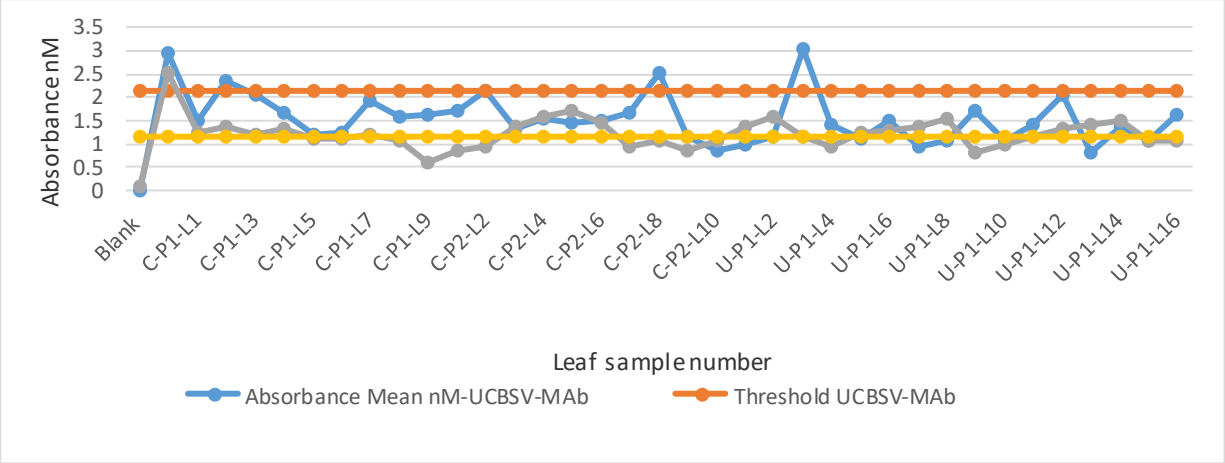
APPENDICES



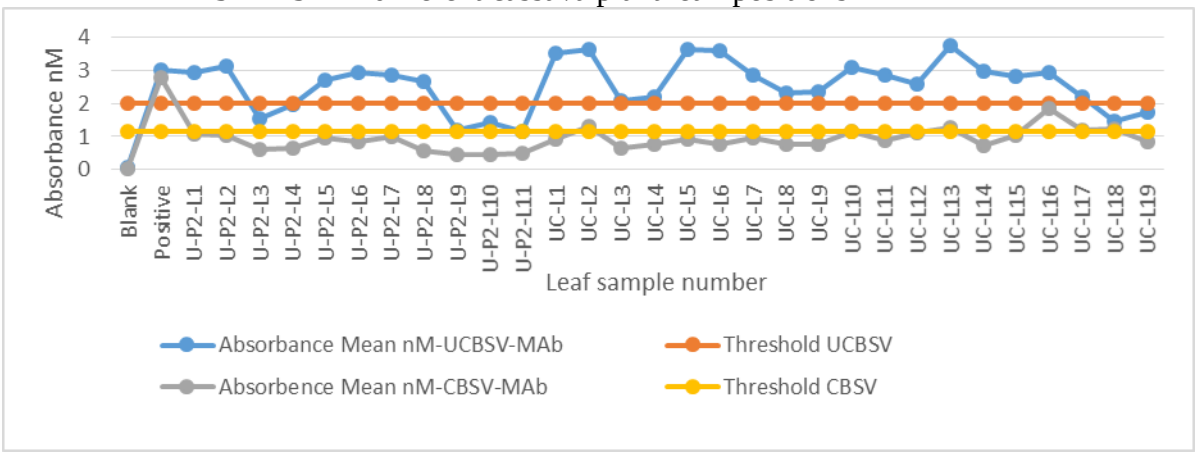
Appendix 1: CBSV and UCBSV-MAb specificity on CBSVs-infected cassava leaf samples from field pre-confirmed by RT-PCR



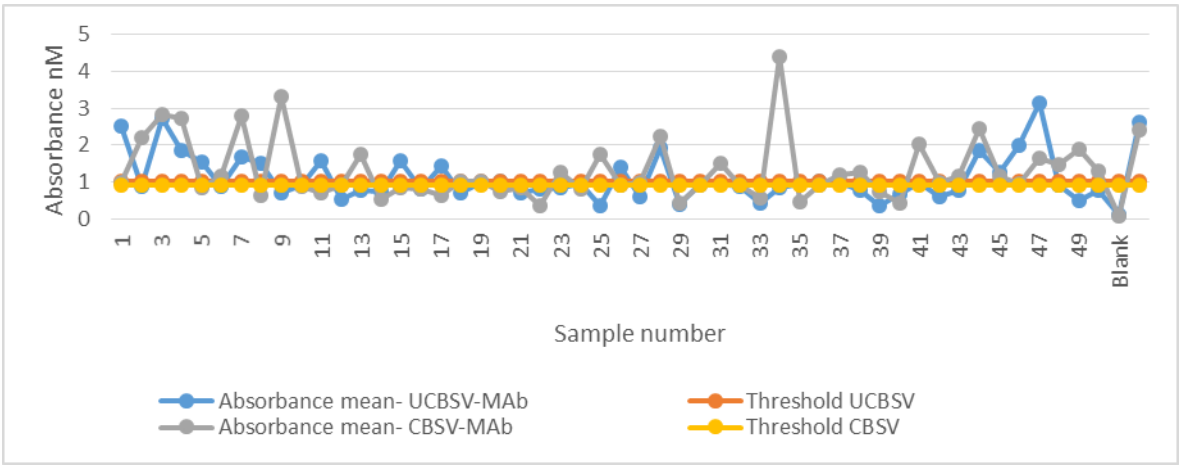
Appendix 2: Specificity (% Spe) of TAS-ELISA combined antibodies for the detection and discrimination of CBSVs in screen house raised cassava plants infected with CBSVs in single and double infection confirmed by RT-PCR



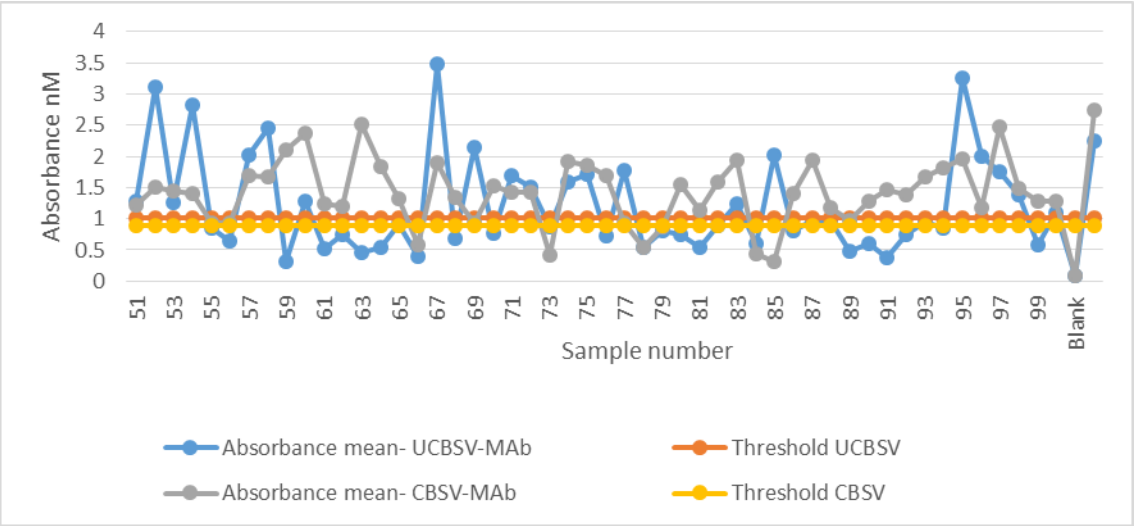
Appendix 3: Mean absorbances of CBSVs using CBSV and UCBSV-MAbs in TAS-ELISA in different cassava plant leaf positions



Appendix 4: Mean absorbances of CBSVs using CBSV and UCBSV-MAbs in TAS-ELISA in different cassava plant leaf positions



Appendix 5 : Mean absorbances of CBSVs using CBSV and UCBSV-MAbs in TAS-ELISA determining the effectiveness of the antibodies in detection and discrimination of virus species



Appendix 6: Mean absorbances of CBSVs using CBSV and UCBSV-MAbs in TAS-ELISA determining the effectiveness of the antibodies in detection and discrimination of virus species