LAMP AND REAL-TIME PCR FOR DETECTION OF PASSION FRUIT WOODINESS DISEASE (PWD) CAUSAL VIRUSES

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Lamp and Real-Time PCR for Detection of Passion Fruit Woodiness Disease (PWD) Causal Viruses

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature…… Date……

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DEDICATION

I dedicate this work to my beloved family; my husband Chris and sons Allan and Ian. "Your moral support, patience and encouragement made me come this far."

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ABBREVIATIONS, SYMBOLS AND ACRONYMS

BecA	Biosciences east and central Africa
bp	Base pair(s)
CABMV	*Cowpea -aphid -borne -mosaic virus*
cDNA	Complementary Deoxyribonucleic acid
DAS-ELISA	Double-antibody Sandwhich Elisa
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EAPV	*East Asian passiflora virus*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbant assay
FTA	Fast Technology in Analysis of nucleic acids
g	Gram(s)
ILRI	International Livestock Researc1h Institute
kb	Kilo base(s)
KEPHIS	Kenya Plant Health Inspectorate Service
LAMP	Loop-mediated isothermal amplification
M	Molar
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
mg	Milligram(s)
min	Minute(s)

ml	Millilitre(s)
mM	Millimolar
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PWD	Passion fruit woodiness disease
PWV	*Passion fruit woodiness virus*
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse Transcription -PCR
s	Second(s)
SAPV	*South African passiflora virus*
SMV	*Sugarcane Mosaic Virus*
TE	Tris-Cl/EDTA
UPV	*Ugandan passiflora virus*
V	Volt (s)

ABSTRACT

Passion fruit *(Passiflora edulis* [Sims]) is an important economic crop grown for both domestic and export market world-wide. In Kenya, passion fruit productivity is low due to both biotic and abiotic constraints. Passion fruit woodiness disease (PWD) is the most important viral disease and the most limiting factor for passion fruit production worldwide. *Cowpea Aphid Borne Mosaic Virus* (CABMV) reported to be present in Brazil, South Africa, Uganda and Rwanda is the major causal agent of woodiness disease in Kenya. This and other pests contribute largely to non-availability of clean planting material. This study aimed at improving the diagnostics of passion fruit woodiness disease through development of molecular-based diagnostic assays. SYBR Green-based real-time PCR and loop-mediated isothermal amplification (LAMP) were optimized and evaluated for detection of the causal agents of woodiness disease in passion fruit. The effect of different sample storage conditions for passion fruit leaf samples on RNA quality and suitability for passion fruit virus diagnostics were also evaluated. This was to determine the most reliable method for handling sample materials from field to the laboratory with the aim of achieving rapid detection of the viruses. To evaluate the different sample storage methods, passion fruit leaf samples were collected from the field and stored for 1 and 2 weeks using FTA® cards, RNAlater, cold ice followed by transfer to -80℃ freezer, drying on silica gel and drying in between the sheets of newsprints (as herbarium) before RNA extraction and subsequent downstream assays. Good RNA yield and quality were obtained from samples stored in silica gel for 1 and 2 weeks after collection. Further analysis confirmed that RNA extracted from samples stored in silica gel was suitable for RT-PCR amplification. The assay conditions of LAMP and SYBR Green real-time PCR assay were optimized followed by specificity and sensitivity determination. The lowest limit of detection for the LAMP assay and real-time PCR was determined using tenfold dilution series were found to be 10^{-7} and 10^{-6}, respectively. No cross-reaction was observed when other viruses were included in the assay indicating that the assays were 100% specific. There is need for adoption of these molecular diagnostic methods in Passion fruit nursery certification programs for early stage screening of planting materials.

CHAPTER ONE

INTRODUCTION

1.1. Background information

Passion fruit *(Passiflora edulis* [Sims]) is an important fruit crop grown worldwide for both export and domestic markets (FAO, 2011).In Africa, it provides smallholder farmers with a diversification enterprise to secure income due to its high market value and short maturity period of the crop. Passion fruit is widely consumed as fresh fruit or processed into juice. When sweetened and diluted it is very palatable and blends well with other fruit juices. Typical processed products include ice cream, sherbet, nectar, juices, concentrate, squash, jams and jellies. Passion fruits are very rich in beta-carotene making it an important health food as source of vitamin A and C (Sema & Maiti, 2006).

Passion fruit has great commercial potential in Kenya since demand for both fresh fruit and processed juice is on the increase. In 2013, passion fruit contributed Kshs 2.25 billion under an area of 4,377.2 Ha and production of 62,207 Metric Tons (HCDA, 2013). However, although there is great potential in passion fruit production, pests, diseases and inadequate disease-free planting materials continue to impact negatively on the production (Barros, 2011; Trevisan *et al*, 2006). Among other pathogens limiting production, Passionfruit woodiness disease (PWD) has been reported as the most devastating diseases of passion fruit production in the world (Novaes and Rezende,

2003). A combination of *Fusarium wilt*, woodiness and brown spot diseases has been reported to cause up to 100% loss in fruit yields in Kenya (Amata *et al.*, 2009).

In Kenya, *Cowpea Aphid Borne Mosaic Virus (*CABMV*)* has been reported as one of the major causative agents of the woodiness disease (Kilalo *et al.*, 2013). CABMV is also reported to be present in Brazil, South Africa, Uganda and Rwanda (McKern *et al.*, 1994; Ochwo-Ssemakula *et al.*, 2012). The disease spread is forcing farmers to abandon passion fruit production in areas where it was first introduced or move into new areas where the disease is not reported. Since the use of vegetative cuttings usually leads to accumulation of pathogens, particularly the viruses and the same are vectored by aphids species present in the environment; the disease quickly builds up in the newly planted orchards (Kilalo *et al.*, 2013).

Nursery certification has been identified as one of the effective management strategy of woodiness disease. However, in Kenya, for a long time this has been carried out based on visual inspection due to lack of a diagnostic protocol especially for viruses causing woodiness disease. This has led to increased cases of the disease at farmers' level (Wangungu *et al.*, 2010). Use of symptoms alone has been reported to be unreliable due to inconsistent expression of the disease symptoms in leaf, stem and fruits (Nascimento *et al.*, 2006). The other available virus detection method relies on serological assay utilizing ELISA which requires development of antibodies and in Kenya, the available plant virus ELISA assays rely on imported antisera which is a time consuming process.

In order to implement effective nursery certification, accurate and precise diagnostic tool for detection of the disease needs to be incorporated in routine nursery inspections.

1.2 Problem statement

Woodiness viral disease is the most important biotic constraints to passion fruit production worldwide (Moreira, 2008). In Kenya, up to 90% yield losses been recorded due to the disease. A combination of Fusarium wilt, woodiness disease and dieback disease have been reported to cause up to 100% loss in fruit yields in Kenya (Amata *et al.,* 2009). Identification of *Cowpea Aphid-Borne Mosaic Virus* (CABMV) and other potyviruses causing woodiness disease based on symptoms is reported as unreliable (Njuguna *et al.,* 2005). The current diagnostic methods rely on the use of biological assays on indicator plants and Enzyme-Linked Immunosorbent Assay (ELISA). The use of biological assays on indicator plants is time consuming, and may not confirm the specific identity of the viral causative agents (Omatsu *et al.,* 2004). The use of serological-based assays give false negatives because of low concentrations of the pathogen in tissue samples or false positives where cross reaction of the viruses occurs especially where potyviruses are involved (Ward *et al.,* 2004; CIP 2007). This has largely contributed to lack of clean health planting materials to farmers in Kenya.Therefore the aim of this study is to develop molecular diagnostic assays based on Loop Mediated Isothermal Amplification (LAMP) and real-time reverse transcription (RT)-PCR to accurately identify the presence of PWD causal viruses, as well as provide

a valuable diagnostic tool for supporting certification programs and early stage screening of passion fruit planting materials in nurseries.

1.3 Justification

Application of molecular (nucleic acid-based) diagnostics methods in plant pathogen detection has greatly accelerated identification of disease causing pathogens. These methods provide reliable, rapid testing alternative to traditional biological and serology-based techniques for instance where antisera of sufficient quality or specificity is unavailable (Mumford *et al.,* 2000). Polymerase Chain Reaction (PCR) is the most widely used molecular diagnostic technique due to its fast and easy to use protocol. Variants of PCR like real-time Polymerase Chain Reaction has been widely applied for detection of main plant pathogens including fungi (Bohm *et al.,* 1999), bacteria (Schaad *et al.,* 1999), viruses (Mumford *et al.,* 2000), viroids and phytoplasma (Bianco *et al.,* 2004). The real-time technology has also been developed for the specific identification of virus vectors (Walsh *et al.,* 2005). Real-time PCR based methods provides greater sensitivity, specificity and also reduces risk of cross contamination since no post-amplification processing required. Loop-mediated Isothermal amplification (LAMP) is a molecular detection method capable of amplifying DNA under isothermal conditions with high specificity, efficiency and speed. It is simple, utilizing minimal equipment and has faster reaction time which makes it particularly suitable and much easier to adapt in the diagnostic laboratories with little resources (Okuda *et al.,* 2005). The method has been used and adapted in the diagnosis of a variety of plant pathogens and pests

including viruses (Fukuta *et al.,* 2003a; Almasi *et al.,* 2013; Fukuta *et al.,*2004; Tomlinson & Boonham 2008). For these reasons, development of molecular diagnostic assays based on Loop Mediated Isothermal Amplification (LAMP) and real-time reverse transcription (RT)-PCR would be useful to accurately identify the presence of PWD causal viruses, as well as provide a valuable diagnostic tool for supporting certification programs and early stage screening of passion fruit planting materials in nurseries.

1.4 Null Hypothesis

1. Sample preservation methods have no effect on RNA quality and suitability for passion fruit virus detection
2. SYBR green based real-time PCR assay is not effective for detection of PWD causal viruses
3. Loop-mediated Isothermal Amplification (LAMP) is not effective for detection of PWD causal viruses

1.5 Objectives

1.5.1 Overall objective

To optimize LAMP and real-time PCR Methodologies in the detection of passion fruit woodiness disease causal viruses

1.5.2 Specific objectives

1. To compare the effect of selected sample preservation methods on RNA quality and suitability for passion fruit virus detection using LAMP and real-time PCR

2. To evaluate the effectiveness of a SYBR green based real-time PCR assay for detection of the PWD causal viruses in Kenya

3. To evaluate the effectiveness of Loop-mediated Isothermal Amplification (LAMP) for detection of the PWD causal viruses in Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of passion fruit

Passion fruit (*Passiflora edulis*) is a shallow-rooted, woody, perennial vigorous vine, climbing by means of tendrils. It belongs to the family passiflora L. (*passifloraceae*) with a wide genetic base of about 400 species (Vander-Plank, 2006). It flowers and produces fruits within a year of establishment. Passion fruit is a native of southern Brazil where it grows on the edges of rain forests and is widely cultivated in Australia, New Zealand, Hawaii, Indonesia, South Africa, Zimbabwe, Uganda and Kenya (Ochwo-Ssemakula *et al.,* 2012). There are about 60 species of passion fruit producing edible fruits (Manicom *et al.,* 2003) and the most important include *Passiflora edulis var.flavicarpa* the yellow passion fruit and *Passiflora edulis var. purplar (*purple passion fruit). The purple passion fruit is native to southern Brazil, Paraguay and Northern Argentina (Souza *et al.,* 2008), whereas the yellow form has been stated to be of unknown origin (Manicom *et al.,* 2003).

2.2 Global production of passion fruit

The total world passion fruit production is estimated to be nearly 805,000 tons with Brazil producing 56% of this (Manicom *et al.,* 2003). Ecuador and Colombia follow with production of 250,000 tons and 30,000 tons, respectively Fig 2.1 (ITIS Tropocols

2007)**.** Other major producers include Colombia, Peru and Ecuador (Manicom *et al.*, 2003).

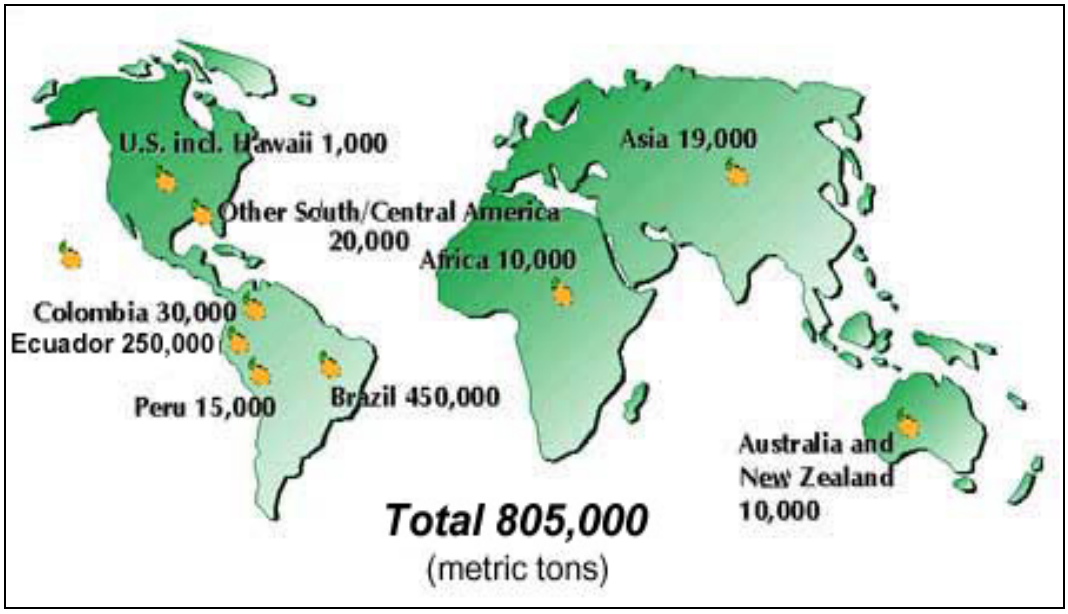

Figure 2.1: World production of passion fruit in 2007 Source: (ITIS Tropocals, 2007)

2.3 Production of Passion fruit in Kenya

Commercial farming of passion fruit in Kenya began in 1933 and was expanded in 1960, when the crop was also introduced into Uganda for commercial production (Ochwo-Ssemakula *et al.*, 2012). Although a large proportion of the passion fruit crop is produced on small farms by small scale out-growers with average plot sizes of 0.5 to 2 acres, the farmers market the fruits collectively. Reports from Horticulture Development Authority (HCDA, 2013) indicate that passion fruit contributed Kshs 2.25 billion under an area of 4,377.2 Ha with production of 62,207 MT. The area and production of passion

fruit increased in 2011-2012 and 2012-2013 by 16.9% and 52.2%, respectively with leading counties in 2013 being Kwale, Embu and Migori at 22.7%, 20.9% and 16.8%, respectively (Table 2.1)

Table 2.1: Production of passion fruit (in Metric tons) in Kenya 2011-2013(HCDA 2013)

Regions	**2011**			**2012**			**2013**		
County	**Area (Ha)**	**Quantity (MT)**	**Value (Kshs-million)**	**Area (Ha)**	**Quantity (MT)**	**Value (Kshs-million)**	**Area (Ha)**	**Quantity(MT)**	**Value (Kshs-million)**
Embu	102	1,661	109.58	127	1,539	95.9	89	12,981	757.64
Kwale	115	1,309	39.28	306	3,441	103.2	886	14,108	282.34
Migori	1,073	10,037	238.75	1,103	10,309	253.27	1,121	10,462	222.18
Meru	207	6,658	216.38	208	4,155	152.73	205	5,569	191.48
Elgeyo marakwet	220	2,899	113.99	270	3,248	129.82	346	4,565	180.26
Uasin gishu	230	2,000	120.7	351	5,779	209.14	222	3,190	119.6
Others	1,219	10,368	415.65	1,378	11,096	442.13	1,508	11,332	501.2
National Total	**3,166**	**34,932**	**1,254.33**	**3,743**	**39,567**	**1,386.19**	**4,377**	**62,207**	**2,254.69**

The most common varieties grown in Kenya are the purple passion fruit *(Passiflora edulis var. purplar)* and the yellow passion (*Passiflora edulis var.flavicarpa)* (Annon, 2006).The purple variety has deep purple skin colour with an aromatic flavor at maturity and is about 4.5cm in diameter. It is predominant of the two and grows well in mid-attitude regions (1200m-1800m). It has quick market returns for both local and export market, though susceptible to *Fusarium wilt*, brown spot and woodiness disease (Njuguna *et al.,* 2005). The yellow passion (*Passiflora edulis var.flavicarpa)* is most suited for the coastal lowlands. It has a larger fruit of 5-7cm and turns from green to yellow at maturity. It is more acidic and used for juice extraction. Yellow passion fruit is resistant to *Fusarium wilt*, tolerant to phytopthora blight, nematodes and brown spot (Amata *et al.,* 2009) and is used as root stock to purple passion fruit. Other varieties grown in Kenya include sweet passion *(Passiflora ligularis),* Banana passion *(Passiflora mollissima)* and Giant passion *(Passiflora quadrangularis).*

2.4 Importance and utilization of passion fruit in Kenya

Passion fruit production is gaining popularity in Kenya due to the changing consumer preferences as Kenyans move from carbonated soft drinks to fresh juices. According to reports from FAO, (2011), passion fruit was ranked the third most popular fruit in Kenya after mangoes and bananas. The fruit is mostly consumed fresh although the pulp is also extracted and processed. Like many fruits, passion fruit has been cited as an excellent source of fibre, vitamins and minerals (Sema & Maiti, 2006).

Passion fruit has quick financial returns because it takes only one year for the crop to mature and has a lifespan exceeding 3 years depending on how the crop is managed. Data from the Ministry of Agriculture indicates that it is possible to earn over 2 million Kenyan shillings from one hectare, an earning exceeding that of maize, beans and many other farming enterprises (KHDP 2006). For export market, Kenya supplies passion fruits to the European markets including UK, Holland, Germany and Belgium. According to HCDA reports in 2011, passion fruit contributed about USD27 million accounting for 1.1% of the domestic value for Kenya's Horticultural produce and 3.1% value of the fruit sub-sector.

2.5 Constraints to passion fruit production in Kenya

Despite the high potential, passion fruit in Kenya faces many challenges affecting productivity. The most important constraints are pests and diseases. Important diseases of passion fruit include dieback (*Fusarium spp* and *Phytopthora spp*), brown spot (*Alternaria passiflorae*), collar and stem rot (*Fusarium solani*), anthracnose (*Colletotrichum passiflorae*), bacteriosis caused by *Xanthomonas campestris pv. Passiflorae* and Woodiness viral disease (Gesimba *et al.,* 2008). Yield losses of 80-100% have been attributed mainly to multiple occurrences of those diseases (Amata *et al.,* 2009: Wangungu *et al.,* 2010) which contribute to lack of certified disease free planting materials (HCDA, 2013). Other constraints include high establishment costs, inadequate planting materials, drought, declining soil fertility, poor yielding varieties, poor agronomic practices and poor post-harvest handling (Gaturuku *et al.,* 2012).

2.5.1 Passion Fruit Woodiness Disease

According to Moreira (2008), Woodiness Viral Disease is the most limiting factor for passion fruit production worldwide. The viral pathogens designated as putative etiological agents of woodiness disease include *Passion Fruit woodiness virus* (PWV) in Australia and Brazil, (Iwai *et al.,* 2006), *East Asian Passiflora Virus* (EAPV) in Japan (Iwai *et al.,* 2006) *South African Passiflora Virus* (SAPV) in South Africa (Brand *et al.,* 1993), which was later found to be synonymous with *Cowpea Aphid Borne Mosaic Virus* (CABMV) (McKern *et al.,* 1994; Nascimento *et al.,* 2006) and *Ugandan passiflora virus* (UPV) in Uganda (Ochwo-Ssemakula *et al.,* 2012).

In Kenya, *Cowpea Aphid Borne Mosaic Virus* (CABMV) has been reported as one of the causal agents of woodiness disease (Kilalo *et al.,* 2013). CABMV is also reported to be present in Brazil (Nascimento *et al.,* 2006; Cerqueira-Silva *et al.,* 2008; Moreira 2008), Uganda and Rwanda (Ochwo-Ssemakula *et al.,* 2012). *Cowpea aphid-borne mosaic virus* (CABMV) belong to the genus *potyvirus*, family *potyviridae*; one of the largest groups of plant viruses (Berger *et al.,* 2005). Its genome is constituted by a single, positive RNA strand, with approximately 10,000 nucleotides, an ORF coding a polyprotein that originates 10 functional proteins when cleaved and a second ORF called PIPO, overlapping the genomic region corresponding to protein P3. Most proteins in potyviruses have multiple functions. The Coat protein (CP) encapsidates the genome, and it is also associated with intracellular transport of the virus, transmission by aphid vectors, and replication of the viral genome, apart from being an important taxonomic

tool used to classify potyviruses at species level (Adams, 2012).The core fragment of the Coat protein (CP) gene contains conserved and unique regions hence highly useful for establishing identification and classification of many viruses including CABMV and other potyviruses.

2.5.2 Symptoms, mode of spread and management of CABMV

CABMV, causing woodiness disease in passion fruit, is known to be transmitted naturally by several aphid species including, *Aphis gossypii, A.fabae, Myzus persicae* in a non-persistent, non-circulative manner (Kilalo *et al.,* 2013; Costa *et al.,* 1995). However, the major means of the disease spread is vegetative propagation of infected plants, often contributing to multiple virus infections. If any of the mother plants are infected during vegetative propagation, it consequently increases the number of virus infected plants. Symptoms induced by CABMV on passion fruit leaves include severe mosaic, yellow spots, flecks or mottling, rugosity and distortion (Plate 2.1; a and b). Infected plants produce small, misshapen fruits with a thick hard and woody rind, cracked fruits (Plate 2.2; a and b) and small pulp cavity (Nascimento *et al.,* 2006). The stems also become shortened.

Currently, several cultural practices have been recommended to help prolong the life of orchards and minimize disease problem and include most importantly use of virus-free seedlings from certified nurseries, avoiding intercropping with leguminous plants which harbor the viruses and complete elimination of old and infected plants before new planting as they serve as new sources of infection (Gioria *et al.,* 2003*).* Pruning and

thinning to prevent mechanical transmission of the viruses, systematic elimination of symptomatic plants within the first seven months after transplantation to the field is also a common practice (Cerqueira-Silva *et al.*, 2014).

(a) leaf yellow spots, flecks or mottling

(b) mosaic, rugosity and distortion

Plate 2.1: (a) and (b) symptoms of passion fruit leaves infected with woodiness disease (Fischer *et al.,* 2007)

(a) small fruit pulp cavity

(b) thick hard and woody rind, cracked fruits

Plate 2.2: (a) and (b) symptoms of passion fruits infected with woodiness disease (source: Kilalo et al., 2013**)**

2.6 Techniques used for preservation of passion fruit leaf samples for virus diagnostics

Recent advances in molecular biology have led to increased demand for methods of preserving nucleic acids from point of sample collection to the laboratory. Ribonucleic Acid (RNA) based assays like passion fruit virus diagnostics require extraction of RNA with high quality and integrity. This is challenging especially for samples collected in remote areas (Ndunguru *et al.,* 2005) since RNA can be degraded by the presence of environmental and endogenous ribonucleases during transit if the storage conditions are not ideal (Perez-Novo *et al.,* 2005). Therefore, identification of a suitable technique for collection of samples in the field and subsequent preservation in the laboratory is crucial for diagnostic applications. Different plant sample storage methods have been described for samples destined for molecular based studies both pre-and post processing to maintain the integrity of the nucleic acids in tissues. Some of these methods are as discussed below;

2.6.1 Fast Technology for Analysis of nucleic acids (FTA® cards)

FTA® card is a chemically treated filter paper designed for the collection and room temperature storage of biological samples for subsequent nucleic acid analysis. Whatman patented FTA® cards provide safe, secure and reliable method for the room temperature collection, transport and storage of nucleic acids. When a virus infected or a healthy tissue is applied to FTA cards, the fibers of the card are conditioned with chaotrophic and other reagents that lyses cells, release and immobilize the nucleic acids while inhibiting their degradation (Whatman, 2004). The successful use of FTA® cards

has been reported in samples like blood, bacteria, viruses, plant, materials, microorganisms (Zhong *et al*., 2001).The method has proven useful for plant DNA viruses and for plant gene expression studies (Ndunguru *et al.,* 2005).

2.6.2 Herbarium

This storage method involves placing the samples in between sheets of newsprint and drying the material (Taylor & Swann, 1994). Sampled leaflets are arranged on a piece of plain newsprint and further covered with two more sheets of newsprint. The completed sheets are usually placed on herbarium press covered with a sheet of corrugated cardboard. The objective of the press is to flatten and evenly dry the leaves. It is recommended that the materials be dried as quickly as possible and be pressed tightly enough while drying to preserve the nucleic acids from degradation (Staats *et al.*, 2011). The samples are then stored at room temperature in the laboratory.

2.6.3 Cold storage

Maintaining samples in cold storage like -20 °C, -80 °C or snap freezing in liquid nitrogen is commonly used in many equipped laboratories. Freezing prevents the activity of nucleases that degrade RNA. The method can be quite successful when the plant are nearby and in easily accessible localities and has been reported by Mutter *et al,* (2004) as convenient to process samples at a later date. However, the integrity of RNA is compromised using this method particularly when collecting samples from locations far away from the laboratories due to possibilities of shipment delays and sub-optimal packaging (Straube & Juen, 2013) Additionally, frozen samples need to be homogenized

quickly to avoid rapid RNA degradation during thawing of the previously frozen samples (Michaud & Foran, 2011).

2.6.4 Silica gel preservation

Preservation of plant tissues by silica gel desiccation has been reported to be effective for many plant species with rapid rates of desiccation following the use of proper leaf material to silica gel ratio (Adams *et al.*, 1992) Samples can be stored in silica gel at room temperature with occasional changing of the silica gel for proper drying of the materials. Although some species do not respond well to this type of sample preservation, the technique is generally simple and efficient since at dehydrated state, RNA is less susceptible to chemical or enzymatic degradation (Chase & Hillis, 1991).

2.6.5 RNAlater preservation method

There are new technologies that help preserve RNA at room temperature, such as RNAlater® (Ambion, Carlsbad, CA, USA) and RNA stable® (Biometrica, San Diego, CA,USA), which also keeps isolated RNA in anhydrobiosis (Mutter *et al.,* 2004). RNAlater® stabilizes and protects cellular RNA in intact unfrozen samples by penetrating fresh tissue and deactivating nucleases (Gorokhova, 2003). The reagent can also be applicable for DNA preservation (Johnson *et al.,* 2003). According to manufacturer's instructions, samples can be stored in RNAlater for up to 1 day at 37 °C, for up to a week at room temperature, for a month or more at 4 °C, or stored indefinitely at -20 °C without nucleic acid degradation (Ambion, 1999). However, these recommendations vary depending on sample material. Some organisms are not suitable

for preservation in RNAlater (Ambion, 1999) and therefore there is need to explore the effect of RNAlater in field collection and room temperature storage of passion fruit leaf samples intended for virus diagnostic assays. This would be applicable in laboratories where no freezing facilities are available. The major draw-back with this method is that the reagents are costly and need to be available in the laboratory at the moment of use.

Lack of preservation methods remains an obstacle for nucleic acid work in the field studies including collection of samples for virus diagnostics. It is therefore necessary to determine the most suitable method that would be convenient and reliable for handling of passion fruit samples collected from farmers fields to the laboratory while aiming to achieve successful diagnostic applications. In the present study, the effects of different sample preservation methods on the quality of the RNA from virus infected passion fruit leaf samples for further cDNA synthesis and RT-PCR amplification were evaluated.

2.7 Methods used for detection of plant viruses

Diagnosis of plant viruses for disease prevention and control is vital in any agricultural production systems and regulatory frameworks. Therefore, ensuring freedom from viral diseases in planting materials is mandatory in certification schemes for production of plant propagation materials like passion fruit. The detection and identification of plant viral pathogens relies on a very diverse range of techniques and skills from symptomatology, biological assay on indicator plants, serological or immunological methods to modern molecular based techniques (Torrance, 1997)

2.7.1 Symptomatology

Use of symptoms on plants is one of the earliest methods in detection of plant viruses and is still practiced today. This involves differentiation of plant viruses using a range of symptoms. The method is commonly used in cases where roguing of diseased plants is applied in attempt to control diseases (Mathews, 1980). This visual inspection is easy when symptoms are clearly characteristic of a specific disease. However, many symptoms such as mosaics, stunting, twisting, chlorosis, leaf strapping may be caused by a number of other factors which include herbicide/growth regulators effect on plants, nutrient deficiencies or excesses, mutations, phytoplasmas and certain other non-viral plant pathogens, and insects or mites (CIP, 2007).

Although use of symptoms can provide the first clue in diagnosing diseases caused by viruses, adequate field experience is required in making a decision based on symptoms alone (Bock ,1982). Therefore, a combination of symptomatology with other confirmatory tests like serology and molecular based methods are required for accurate detection of virus infections (Akinjogunla *et al.*, 2008).

2.7.2 Use of Indicator plants

Use of indicator plants has been one of the earlier methods of virus detection in plants (Jones, 1993). Viruses have been reported to cause a wide variety of symptoms on inoculated indicator test plants including mosaic/mottling, stunting, leaf deformation, chlorotic and necrotic lesions and ringspots. Several plant families including *Solanaceae*

species such as *Nicotiana benthaniana*, *Passiflora* species, *Chenopodiaceae* and some *Curcubitaceae* species have been commonly used as diagnostic plants for many viruses (Omatsu *et al.,* 2004; Nascimento *et al.,* 2006). However, in many cases, symptom expression on the indicator plants are dependent on the conditions of the host, environment and species/strain of the virus and may be obscured when the plants are healthy and vigorously growing (Trevisan *et al.,* 2006). These indicator plant based tests do not give definitive answers on virus identification and have therefore been replaced with nucleic based techniques, or used in conjunction with other diagnostic procedures.

2.7.3 Serological-based methods

Serological techniques are well established and widely used for routine detection of various plant viruses. These include Enzyme Linked Immuno-sorbent (ELISA) (Clark & Adams, 1977). Tissue Blot Immunosorbent assays (TBIA) and Lateral flow devices. The techniques are based on an antigen-antibody binding reaction between epitopes on the surface of the virus particles and the binding sites of specific anti-virus antibodies (Van Regenmortel, 1982). The basic principle of ELISA technique involves immobilizing the antigen onto a solid surface or captured by specific antibodies bound to the surface, and probing with specific immunoglobulin's carrying an enzyme label (IITA, 2009).

ELISA detects pathogens from their protein material. The enzyme retrained in the case of positive reaction is detected by adding a suitable substrate. The enzyme converts to a product which can then be recognized by color (IITA, 2009). They are simple to perform, sensitive if there is availability of virus-specific monoclonal antibodies, and

easily used in large-scale analysis. However, development of monoclonal antibodies can be time consuming and in some cases the antibodies that are developed may display insufficient sensitivity and specificity, or may not reliably detect all species (Ward *et al.*, 2004). Furthermore, difficulty may be encountered in the detection of low-titer viruses, for example in asymptomatic or latent infections.

Serological methods for passion fruit virus identification using ELISA is most commonly done using double antibody Sandwhich ELISA (DAS-ELISA) using polyclonal antibodies for broad-spectrum potyviruses (AS-0573/1) (Clark & Adams, 1977). These ELISA assays are not specific for PWD causal agents, sometimes giving false negatives in cases where there is low concentrations of the pathogen in tissue sample used or false positives where cross reaction of the viruses occurs. The cross-reaction is due to high level of homology between different potyviruses. The presence of many shared epitopes makes it difficult to distinguish between two closely related strains/species (CIP, 2007). There is, therefore, need to develop more sensitive diagnostic assays based on molecular detection to complement ELISA assays in such cases.

2.7.4 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR), a sensitive technique used for detection and amplification of specific sequences of nucleic acids in a sample was introduced by Karry Mullis in 1983 (Mullis & Faloona, 1987). The availability of PCR assay has greatly assisted in the detection of the disease pathogens which are difficult to diagnose with the

traditional biological assays and is sensitive to amplify very low concentrations of the disease pathogens in the plant tissue sample used. Polymerase chain reaction (PCR) detects pathogens from their genetic material. The process utilizes a pair of synthetic oligonucleotide primers each hybridizing to one strand of the double stranded deoxyribonucleic acid (dsDNA) target with the pair spanning the target region and multiplies exponentially (Mackay *et al.,* 2002). The PCR process occurs in 3 different stages namely; the dsDNA denaturation, the primer annealing and the extension phase. Repeated cycles result in an exponential increase in target DNA to a point where there are enough copies of the sequence for it to be visualized (Rebecca & Lynn, 2010).

In endpoint (Conventional) PCR, the detection and quantification of the amplified target sequences are performed at the end of the reaction, and involves additional steps such as gel electrophoresis and image analysis. The assay has proven to be a sensitive, specific and reliable method for the detection and identification of plant disease causing agents including viruses. However, the method is hampered by carry-over contaminations during the post amplification manipulations (Mumford *et al.,* 2006).

2.7.5 Real-time PCR

Several studies targeting plant viruses have concluded that real-time PCR offers greater sensitivity than conventional PCR, molecular hybridization or serological methods (Korimbocus *et al.,* 2002). Real-time PCR has also been applied to detect RNA and DNA viruses for its higher sensitivity (Rocha *et al,* 2015). This technique is based on fluorescence detection, with the use of either fluorescent probe or an intercalating dye

such as SYBR Green. In contrast to the conventional end-point detection PCR, quantification in real-time PCR occurs during the exponential phase of amplification. The ability to monitor the reaction during exponential phase enables users to determine the initial target with great precision (Wong & Medrano, 2005).

Various real-time PCR chemistries have been developed but Taq Man® probes and SYBR Green are the most commonly used for detection of pathogens (Mumford *et al.,* 2006). Taq Man® probes primarily consist of oligonucleotide sequence complementary to some regions of the target DNA. The probe is complexed with a quencher and a reporter fluorophore dye at its 3' and 5' ends, respectively (Livak & Schmittgen, 1995). During amplification, the probe is associated to its complementary target DNA and is then cleaved by Taq DNA Polymerase 5'- 3' exonuclease activity (Fig 2.2; B). This cleavage releases the reporter dye and generates a fluorescent signal that increases with each cycle (Bustin *et al.*, 2009). An increase in florescence intensity propositional to the amount of amplicon produced (Rebecca & Lynn, 2010). Taq Man® provides higher specificity than DNA intercalating dye. However, the main draw-back of this system is the requirement to synthesize the specific probes to each target sequence, increasing the cost of the assay (La Cruz *et al.,* 2013).

SYBR Green 1 is an intercalating dye that gives a fluorescent signal when bound to double-stranded DNA, while being otherwise virtually non-fluorescent (Fig 2.2; A). Once the reaction proceeds and the PCR product is accumulated, the fluorescence levels increase proportionally to the amount of DNA present in the original sample (Livak &

Schmittgen, 1995; Pabla & Pabla, 2008). This dye is used to monitor amplification of any DNA sequences and dispenses the use of a probe, thus reducing the cost of amplification and providing a great advantage in its application. It is widely used because of the ease of designing the assays and its relatively low running costs. Unlike Taq man fluorescent probes, SYBR Green Dye binds not only to the target DNA, but also to all dsDNA formed during the PCR, therefore, while simple to use, the assays lacks specificity.

The non-specific amplification by SYBR Green assays can generate false positive signals if non-specific products or primer-dimers are present in the assay. Such challenges can be addressed by carefully designing the primers and validating the PCR products with melting curve analysis immediately after PCR (Dheda *et al.,* 2004). Targets for real-time PCR are typically short (60 to 120 bases), allowing faster thermal cycling to be used for real-time PCR than conventional PCR.

In this study, SYBR Green based real-time PCR assay was tested for detection of CABMV causing woodiness disease in Kenya. The figure below illustrates the principle of SYBR Green 1 based real-time chemistry (A) whereby the intercalating dye bind between the two strands of DNA emitting a fluorescence signal. Taqman detection chemistry is demonstrated in (B) below as decribed by Livak and Schmittgen, (1995).

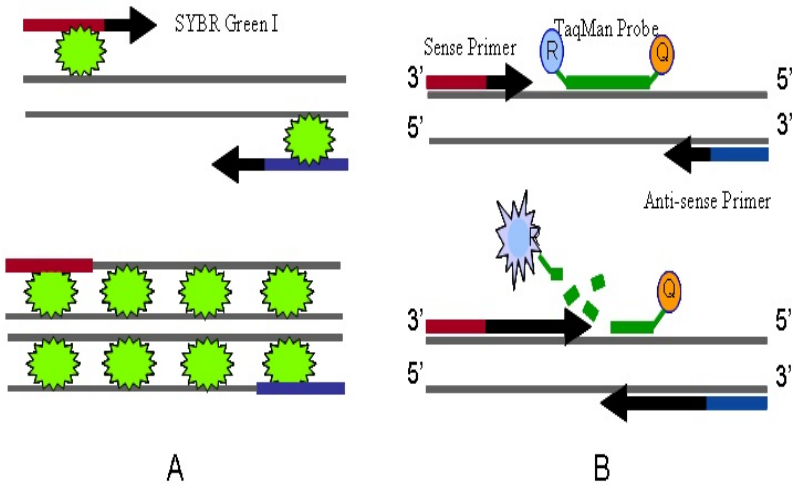

Figure 2.2: showing the principle of SYBR Green and TaqMan assay (Livak & Schmittgen, 1995).

2.7.6 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a novel technique, capable of amplifying DNA under isothermal conditions with high specificity, efficiency and speed (Notomi *et al.*, 2000). The most significant advantage of LAMP is the ability to amplify specific sequences of DNA under isothermal conditions between 60-65 °C. LAMP can be carried out with a simple system and can be monitored in real time. This makes LAMP much easier to adapt in diagnostic laboratories with little resources. The method has been used and adopted in the diagnosis of many plant pathogens and pests (Tomlinson & Boonham, 2008), including viruses (Tomlinson *et al.*, 2012; Almasi *et al.*, 2013). LAMP primers can bind without denaturation (Nagamine *et al.*, 2001) and the method has the potential to overcome some of the cost barriers limiting uptake of PCR-

based testing while exceeding sensitivity and/or specificity of ELISA based methods (Fukuta *et al.*, 2003b; Zhong *et al.*, 2011).

Loop-mediated isothermal amplification (LAMP) uses four to six specially designed primers to generate amplification products that contain single-stranded loops, allowing primers to bind without the need for repeated cycles of thermal denaturation (Notomi *et al.*, 2000; Nagamine *et al.*, 2001). Forward and reverse internal primers, each consisting of a 3' region that binds to the target and a 5' region that matches the target, interact with upstream external primers, resulting in the displacement of strands containing self-complementary regions that form stem-loop structures. Figure 2.3 below demonstrates the principle of LAMP as described by (Tomita *et al.*, 2008).

As shown in diagram 2.3 **(a)**, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c and B3 from 5' end. C represents a complementary sequence. F1c is complementary to F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. Figure 2.3 **(b)** demonstrates DNA synthesis initiated from FIP which proceeds as follows; The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 5' end (structure 4). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to

generate structure 5, which possesses the loop structure at both ends (dumbbell like structure) (Tomita *et al*., 2008).

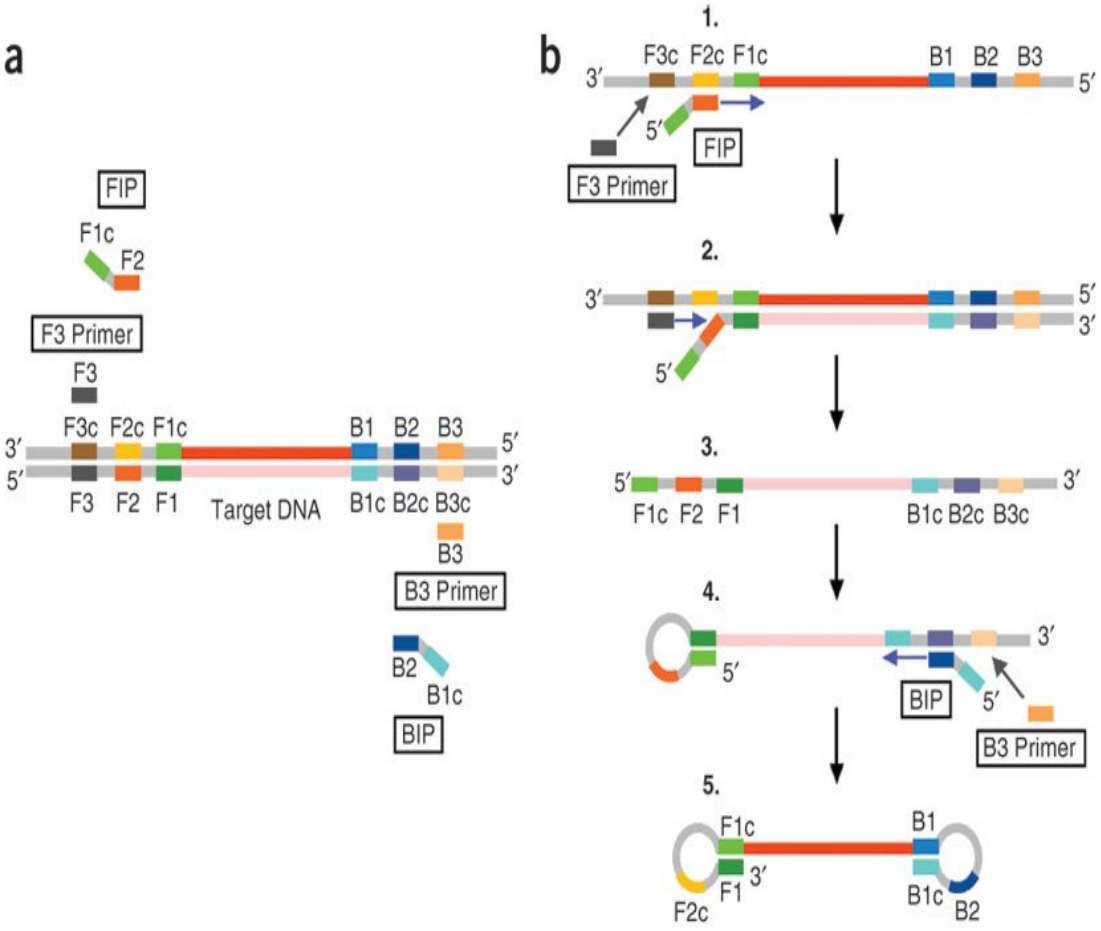

Figure 2.3: Principle of Loop-mediated isothermal amplification (LAMP) (Tomita *et al*., 2008).

Several alternative methods are available for detection of amplified LAMP product including addition of intercalating dyes such as SYBR Green and PicoGreen at concentrations sufficient to produce visible colour change (Iwamoto *et al.*, 2003), incorporation of hydroxylnapthol blue into the reagent mix (Goto *et al.*, 2009), use of

agarose gel electrophoresis (Notomi *et al.,* 2000) and real-time detection systems (Tomlinson *et al.,* 2010). The study utilized real-time LAMP detection system using a portable Genie II machine (Optigene,UK) and detection using hydroxylnapthol blue based on colour change as well as use of agarose gel electrophoresis to confirm the typical ladder-like appearance of the LAMP amplified products.

There is no reported diagnostic assay for CABMV based on LAMP and hence this research aims to establish LAMP protocol for use in passion fruit certification process and virus indexing to avail clean planting materials to farmers.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The research was conducted at Biosciences east and central Africa-International Livestock Research Institute (BecA-ILRI -Hub) laboratories in collaboration with Kenya Plant Health inspectorate service (KEPHIS). BecA-Hub laboratory is a shared Agricultural research and biosciences platform located and managed by ILRI in Nairobi, Kenya. It offers access to world class current molecular and sequencing platforms facilitating research for African and International scientists. KEPHIS is the National Plant Protection Organization (NPPO) of Kenya mandated to carry out regulation of Agriculture including certification services.

3.2 Experimental Design

The study was cross-sectional which involved sample collection in different preservation methods to determine the suitable method for passion fruit sample collection. Completely randomized design was used whereby the sampling field was examined diagonally at random and plants suspected to be diseased counted along the 2 diagonals. Screening of the collected samples was done using conventional RT-PCR for detection of CABMV was carried out followed by sequencing of selected positive samples to determine the specific amplicon. The reaction conditions for RT-LAMP and SYBR

Green-based real-time PCR was done and the sensitivity and specificity of the developed assays determined.

3.3 Determination of a suitable preservation method for CABMV diagnostics

Passion fruit virus diagnostics is an RNA-based assay which requires extraction of RNA with high quality and integrity. Since samples are collected from farmers' fields followed by transportation to the laboratory for analysis, RNA can be easily degraded by the ubiquitous presence of endogenous Ribonucleases during transit if the storage conditions are not ideal. In this study, the effect of different storage conditions for passion fruit leaves on RNA quality and suitability for RT-PCR was evaluated and the suitable method was used to collect samples for subsequent development of diagnostic methods for passion fruit woodiness disease.

3.3.1 Leaf sample collection and preservation

Passion fruit leaf samples for evaluation of different storage methods were collected from a greenhouse collection at the University of Nairobi; Department of crop protection and Plant Science. Purposive sampling was done targeting leaf samples with typical virus-like symptoms showing different levels of wrinkling, mosaic, chlorosis, vein clearing and ring spots. Sampling was done uniformly by collecting three young and topmost leaflets and stored using five different preservation methods including herbarium, -80 °C freezer, RNAlater, FTA cards and use of silica gel. A total of 25 samples were collected for evaluation of the storage methods. This was determined by

having 5 samples per the five different storage methods. The samples were then divided into two sets per method for one week and two week's storage before subsequent assays.

For herbarium, sampled leaflets were arranged on a piece of plain newsprint and covered with two sheets of newsprint. The completed sheets were placed on herbarium press covered with two more sheets of newsprint and a sheet of corrugated cardboard. The samples were stored at room temperature in the laboratory. Samples to be kept in -80 °C freezer were collected in Zip Lock polythene bags (size 12 x 8cm), put in a cool box with ice packs before snap freezing in -80 °C freezer.

Collection in silica gel involved harvesting the symptomatic passion fruit leaf samples into Zip Lock bags and silica gel (Sigma S-5631) was added in the ratio of one gram of leaf material to approximately 10 grams of silica. The Zip Lock bags were shaken to distribute the silica gel between the layers of leaves for sufficient drying. The samples were stored at room temperature with regular checking for dryness after every 12 hours. After samples were sufficiently dried, they were removed from the silica gel and stored at room temperature in the laboratory.

Sampling onto FTA cards was done by gently pressing the leaf material on FTA® cards and allowing them to air-dry for one hour. The FTA cards were transferred to the laboratory and kept at room temperature until used for RNA extraction. For RNAlater, sampled leaflets were sliced with a sterile blade before immersing in RNAlater solution. Approximately one gram of leaf material was used per 5 ml of the RNAlater solution

according to the manufacturer's instructions (Ambion, Inc. Austin, TX). The materials in RNAlater were transferred to the laboratory and kept at 4 °C awaiting RNA extraction.

3.3.2 RNA extraction from samples preserved using different methods

All samples from the different preservation methods were subjected to total RNA extractions after one and two week's storage following the TRIzol method according to (Chomczynski and Mackay, 1995). For samples stored in -80 °C, silica gel and in herbarium, approximately 100 mg of leaf sample was ground using mortar and pestle. Samples in FTA cards were processed by making a 3 mm diameter punch from the chlorophyll-stained regions and placed in a sterile microtube for subsequent RNA extraction.

Samples stored in RNAlater were processed by first removing the leaves with a sterile forceps into a clean Eppendorf tube, centrifuging at 12,000xg for one minute to remove the excess RNAlater solution and ground a fine paste. Eight hundred microlitres of TRIzol-reagent (Invitrogen, Calsband, CA, USA) was added into each of the sample in a sterile microtube with shaking moderately for 30 seconds. The homogenate was incubated at room temperature for 5 minutes and centrifuged at 12,000 xg for 10 minutes at 4 °C. The supernatant was transferred to a fresh Eppendorf tube and 200 µl chloroform added to remove the proteins, lipids and DNA. The upper aqueous layer containing RNA was transferred to a fresh Eppendorf tube and RNA precipitated using 250 µl of isopropanol followed by 250 µl of 1.2 M NaCl/0.8 M sodium citrate and centrifuged at 12,000 xg for 10 minutes. The precipitated RNA pellet was washed with 1

ml of 75% ethanol and the RNA was briefly air-dried. RNA pellet was dissolved in 30 µl of nuclease-free water and kept at -80 °C freezer till use.

3.3.3 Determination of quantity and quality of RNA from different preservation methods

The quantity of the extracted RNA from the samples was determined using Nanodrop ND-2000C spectrophotometer (Thermo scientific, Wilmington, DE, USA). The machine was blanked using 1µl nuclease-free water before 1µl of RNA sample was loaded. The nanodrop measurements were based on the optical density (OD) readings at 260 and 280 nm. The quantity was determined using the readings from the Nanodrop machine. The data obtained after quantification was exported on excel sheet and stored on a computer for further analysis (Annex 6). The integrity of extracted RNA was determined using denaturing (formaldehyde) gel electrophoresis. This was carried out by preparing the samples by mixing 4µl of RNA for each sample with 6µl deionized formamide, 1µl of 10x loading dye and 1µl gel red (1:100) (Biotium, Hayward, CA, USA) as described by (Masek *et al.*,2005). The samples were then denatured by heating for 5 minutes at 65 °C on a heater block and immediately chilled on ice for 5 minutes before loading onto the already cooled 1.2% gel. The gel was left to run at 100 volts for 40 minutes and visualized under UV transilluminator (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA).

3.3.4 Semi-quantitative RT-PCR

First strand Complementary Deoxyribonucleic acid (cDNA) was synthesized using Maxima First Stand cDNA synthesis kit for RT-PCR (Thermo Scientific) according to the manufacturer's instructions. This was performed in a 20 µl reaction consisting of 4 µl of reaction buffer mix, 2 µl of Maxima enzyme mix, 2 µg of total RNA and nuclease-free water. The mixture was incubated for 10 minutes at 25 °C followed by 15 minutes at 50 °C, and the reaction was terminated by heating the mixture at 85 °C for 15 minutes. The cDNA was stored at -20 °C until use.

To evaluate the effect of the different sample storage methods on RT-PCR assays, semi-quantitative polymerase chain reaction (PCR) was conducted using primers specific for housekeeping gene nadhB; using AtropaNad2.1a/2b primers amplifying NADH dehydrogenase region **(Table 3.1)**

Table 3.1: Conventional RT-PCR primers for internal control gene (Schmits-Linne weber *et al.,* 2002).

primer	**Primer Sequence (5' to 3'):**	**Target gene**	**Annealing temp**	**Expected product (base pairs)**
Forward	5'-GGACTCCTGACGTATACGAAGGATC-3'	nadhB;	55 °C	188 bp
Reverse	5'-AGCAATGAGATTCCCAATATCAT-3'	nadhB;		

Amplification was done using Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Cycling conditions consisted of initial denaturation

of 95 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 seconds, annealing at 55 °C for 40 seconds, extension at 72 ℃ for 45 seconds and final extension at 72 °C for 10 minutes. The PCR products were visualized by gel electrophoresis in a 2% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) and visualized under UV transilluminator (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA).

The effect of the different methods on virus detection was determined by conducting semi-quantitative polymerase chain reaction (PCR) to amplify the coat protein gene for *Cowpea Aphid Borne Mosaic Virus* (CABMV). The expected amplicon was 500 bp product (Table 3.1). Accupower PCR premix (Bioneer Corporation, Daejeon, Korea) was used to perform the PCR in a 20 µl reaction. Two microlitres of cDNA in a 20 µl reaction was used with a final concentration of 1X buffer, 1.5 nM $MgCl_{2,}$ 250 µM dNTPs, 0.6 µM Forward and Reverse primer for 1 µM for (CABMV) and I U Taq polymerase. Amplification was carried out in an Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with these conditions; 95 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 1 minute; and extension at 72 °C for 10 minutes. The PCR products were visualized by 2% agarose gel electrophororesis stained with GelRed (Biotium, Hayward, CA, USA).

3.4 RT-LAMP and real-time RT-PCR assay development, optimization and validation

3.4.1 Sample collection, RNA extraction, determination of quality and quantity

Silica gel method identified as suitable for field collection of passion fruit leaf samples for virus diagnostics in section 3.3 was used for collection of passion fruit leaf samples from various parts of passion fruit growing areas in Kenya. These areas included Nakuru (S00.27303, E035.86749), Trans Nzoia (N00.97257, E034.86390) Uasin Gishu (N00.59130, E035.43787) and Central region in Kenya (Annex 7). A total of 38 representative leaf samples with characteristic virus-like symptoms were collected into sealable Zip Lock bags with silica gel as described in section 3.3.1. The sample size was determined as described by (Sin-Ho, 2014). The samples were transported to the laboratory and RNA extraction, quality and quantity determination carried out as described earlier in sections **3.3.2** and **3.3.3** respectively.

3.4.2 Screening of the samples for CABMV using Semi quantitative RT-PCR

Initial screening of the samples was carried out using semi-quantitative RT-PCR to amplify the coat protein gene for *Cowpea Aphid Borne Mosaic Virus* (CABMV) at 500 bp product as described in section 3.3.4. This was aimed at selecting samples that amplified for CABMV for use in the subsequent assays development and optimization of RT-LAMP and real-time RT-PCR and as well for use as positive controls in the assays. The conventional RT-PCR primers used for CABMV detection are as described in **table 3.2.**

Table 3.2: Conventional RT-PCR primers for CABMV (Kilalo *et al.,* 2013)

primer	Primer Sequence (5' to 3')	Target gene	Annealing temp	Expected product (bp)
CABMV F2	CACCAGAGCATCAAAGACACAGCTCA	CP	55 °C	500
CABMV F3	CAGCTCAGTAAATGGTTTGAGGCCA	CP		

3.4.3 Purification of PCR products for sequencing

Purification of the PCR products from the selected samples that amplified for CABMV was conducted using GenJET PCR purification kit (Thermoscientific) according to the manufacturer's protocol. Approximately 17 µl of binding buffer was added to 17 µl of PCR products and transferred to GeneJET purification column. Centrifugation was done at 12,000 rpm for 1 minute and the flow through discarded. About 700 µl of wash buffer was added to the GeneJET purification column and centrifuged for 1 minute. The empty purification columns were centrifuged for 1 minute to completely remove any residual wash buffer. Subsequently, 5 µl of elution buffer was added to the centre of purification column membrane and centrifuged at 12000 rpm for 1 minute.

The purified products were analysed for quantity and quality using Nanodrop ND-2000C spectrophotometer (Thermo scientific, Wilmington, DE, USA) and 2% agarose gel respectively. The gel was prepared by weighing 2g of agarose in 100mls of 0.5x TBE buffer, boiled in a microwave, added 2.5 µl gel red, poured the gel in a gel casting tray and then allowed to cool before loading the samples.

3.4.4 Sequencing of PCR products of selected samples

The sequencing of the purified PCR products was done using Sanger method. The PCR specific primers for CABMV on an ABI 3370 Genetic analyzer (Applied Biosystems, Foster city, CA) at the BecA-ILRI Hub sequencing unit. Sequencing reactions consisted of 1µl purified products, 4 µl of BigDye terminator (PE Biosystems, CA), 4 µl of 2.5x dilution buffer (containing 200nM Tris-HCl, 5Mm $MgCl_2$) and 6 pmol of reverse or forward primers specific for CABMV to a final reaction volume of 20 µl. The cycling parameters followed closely those of the PCR reactions. The sequencing was performed for both strands of the fragments for the selected samples.

3.5 Establishment of real-time RT-PCR assay for detection of CABMV

The study focused on optimization evaluation of real-time Reverse Transcription Polymerase Chain Reaction (PCR) based on SYBR Green and Loop mediated isothermal amplification (LAMP) for detection of CABMV which causes passion fruit woodiness disease.

3.5.1 Optimization of reaction conditions for SYBR real-time RT-PCR assay

The assay was carried out on a Lightcycler®Nano real-time PCR system (Roche, Germany).The optimization of the real-time PCR reaction was performed according to the manufacturer's instructions (PE Applied Biosystems, user Bulletin 2 applied to SYBR-Green 1 core reagent protocol) but scaled down to 25 µl per reaction. The reaction contained 12.5 µl 2x SYBR Green (Roche)1 master mix, 0.5 µl each of 10 µM

of forward and reverse primers were used initially during optimization of primer concentrations. Nine microlitre of nuclease free water and 2.5 µl of the cDNA were added. The cycling conditions were 95 °C for 10 minutes followed by 35 cycles of 95 °C for 10 seconds 55 °C for 30 seconds and 72 °C for 45 seconds. Melting curve analysis was set at 60 °C to 95 °C±. The primers used were specific for CABMV coat protein gene.

Following amplification, a melting curve analysis programme was performed to verify the authenticity of the amplified products by their specific melting temperatures (Tm) according to the instructions of the manufacturer (Roche, Germany).In addition to optimizations of the primer concentrations and annealing temperature, the PCR products were electrophoresed on a 2% agarose gel to check for the presence of primer dimmers. Reactions containing water instead of template were included as negative controls in every assay.

3.5.2 Sensitivity and specificity determination of SYBR Green I based-real-time RT-PCR

Specificity of the assay was done through inclusion of positive samples for other viruses namely; SCMV (sugarcane mosaic virus) and Maize chlorotic mottle virus (MCMV) that were available at BecA-ILRI Laboratories. Melt curve analysis was done to check for contamination and also if the assay had produced a single, specific amplicon. For sensitivity testing, RNA from a positive sample was diluted in nuclease-free water to

produce a tenfold dilution series prior to testing by two-step real-time RT-PCR on a Lightcycler®Nano using the reaction described in 3.5.1.

3.6 Establishment of RT- LAMP assay for detection of CABMV

3.6. 1 LAMP primer design

LAMP primers were designed based on the alignments of published CABMV sequences retrieved from NCBI (http://www.ncbi.nhi.gov). Alignments were constructed using the clustalW method of MegAlign program MEGA 6 (Tamura *et al.,* 2013).GenBank accession numbers of the sequences used in the alignments were as follows; CABMV: (AF348210, AF241233, D100053, Y18634 and DQ 397532). Primer design was carried out using Primer Explorer V4 (Fujitsu Ltd., Tokyo Japan) http://primerexplorer.j.p/elamp 3.0.0/index.html).Four sets of primers (External Primers F3 and B3, internal primers FIP and BIP) recognizing a total of six sequences of the CP gene were designed. Degenerate bases were incorporated into primers to mitigate against intraspecific variation where necessary. All the primers were synthesized by Bioneer Ltd (Korea).The LAMP primers are shown in Table 3.2.

Table 3.3: LAMP primers for CABMV

Primer ID	Sequence (5’ to 3’)
CABMV-F3	GCAATAGCRCARATGAARGC
CABMV-B3	TAC TAA AAC CAA CCA TTA GCT A
CABMV-FIP	TCA CTA GTT GTT GCC ACR TTY CTBTCGCCAACGTHAACACCA
CABMV-BIP	GATGTNAATCARAAYATGCACCA AGC CTT TAC TGC CCA TGC

3.6.2 RT-LAMP assay using Genie II instrument (Optigene)

Real-time LAMP experiment was carried out on the Genie II instrument (Optigene) using 2.5 µl of primer mix containing primer concentrations of 20 µM each external primer (F3 and B3) and 50 µM each internal primers (FIP and BIP) with 7.5 µl isothermal mastermix (OptiGene), 1.5 µl Nuclease-free water and 1 µl of the sample (template) in 12.5 µl reactions. This was carried out at 65 °C for 1 hour with real-time monitoring. Negative controls containing water instead of template were included in every run. Initially, reactions were carried out in duplicates and each experiment was repeated once. For visualization of typical LAMP products, the amplification products were further analysed by electrophoresis using 1.2% agarose gels containing GelRed (Biotium, Hayward, CA, USA) and visualized in UV transilluminator (Thermo Scientific, Thermo Scientific, UV-VIS Spectrophotometer, California, USA).

3.6.3 RT-LAMP Using Hydroxynaphtholblue (HNB).

RT-LAMP assay was also optimized for monitoring by colour change method using the metal-ion binding indicator dye; Hydroxynaphtholblue on a heater block / thermo block. This was carried out in 25 µl reaction of 2.5 µl of 10X isothermal buffer, 0.25 µl of 20 µM each of F3 and B3, 2.5 µl of 20 µM each of FIP and BIP, 1.5 µl of 100mM $MgSO_4$, 5 µl of 5 M Betaine and 3.5 µl of 10 mM dNTPs. 0.15 µl of 25X Hydroxynapthol blue (HNB), 3.35 µl of Nuclease free water and 2.5 µl of the template (cDNA). Negative and positive controls were included in the assay. The mixture was briefly centrifuged and incubated for 95 °C for 5 minutes. The tubes were transferred to ice to chill for 5 minutes. One µl of *Bst* DNA Polymerase (8 Units) was added to each tube and incubated at 60 °C for 60 minutes followed by 85 °C for 5 minutes to terminate the reaction. The results were visualized with naked eye for colour change.

3.6.4 Sensitivity and specificity determination of RT-LAMP assay.

Sensitivity was carried out by determination of the limit of detection. Ten fold serial dilutions was carried out for RNA from a positive sample and used as template for RT-LAMP assay on Genie II instrument (Optigene) as described in section **3.6.2.** Specificity of the assay was done by inclusion RNA from infected samples with SCMV (Sugarcane mosaic Virus) and samples positive for Maize chlorotic mottle virus (MCMV). Sample containing water instead of template was also added as a negative control.

3.7 Comparison of RT-LAMP and SYBR® Green based real time RT-PCR

To compare the optimized RT-LAMP and real-time PCR assay, 38 field collected passion fruit leaf samples were tested by the two methods separately and the results compared. Positive and negative controls were also included in the assays.

3.8 Data analysis

Data analysis for RNA concentration in ng/µl and absorbance values at $_{A260/280}$ from different sample preservation methods was performed using SAS (Statistical Analysis systems) version 9.3. Two- way ANOVA was carried out and the Fisher's Least Significant Difference (LSD). Comparions of Means Test was used to test for any significant differences (at $p = 0.05$) in the ratio of $A_{260/280}$ and RNA quantity among the different storage methods for week 1 and 2.

Data obtained from sequenced samples was analysed using CLC main Workbench software (CLC Bio, Aarhus, Denmark). Quality control of the sequences was carried out to remove the poor quality reads at the beginning (about 20-30 bases) and towards the end (500-800 bases). For each sample, the overlapping portions of high quality reads from forward and reverse sequences were used to build a consensus sequence. The resulting consensus was compared with other sequences available in GenBank using the Basic Local Alignment Search Tool (BLASTn) (Altschul *et al.,* 1997).

CHAPTER FOUR

RESULTS

4.1 Evaluating different sample preservation methods for virus diagnostic assays

4.1.1 Quantity and quality of RNA from different preservation methods

Relatively large quantity of RNA in ng/µl was isolated from samples stored in silica gel and -80 °C freezer. Samples stored in silica gel gave RNA with a mean yield of 1447.6± 236.44 ng/µl for week 1 and 2336.1 ±100 ng/µl for week 2. Samples stored in -80 °C freezer gave a mean yield of 1161.9±145.42 ng/µl and 4171.8±365.24 ng/µl for week 1 and 2 respectively. Herbarium storage gave mean yield of 1156.9 ± ng/µl in week 1 and 434.6±423.39 ng/µl in week 2. RNAlater storage method gave a mean yield of 271 ±164.99 ng/µl in week 1 and 927.5 ±341.19 ng/µl in week 2 whereas FTA card method gave a mean yield of 830.3±166.02 and 954.5 ±441.46 ng/µl for week 1 and 2 respectively (Table 4.1)

All the extracted RNA gave $A_{260/280}$ nm absorbance ratio of between 1.7 ± 0.23 and 2 ± 0.06 (Table 4.1). The mean $A_{260/280}$ values for samples stored in -80 °C freezer was 2±0.06 for week 1 and 2±0.02 for week 2. For silica gel stored samples, mean $A_{260/280}$ values were 2±0.01 for week 1 and 2±0.03 for week 2. For samples stored in RNAlater, there was a significant difference in mean absorbance values for week 1; 1.7± 0.23 and week 2; 1.9±0.05. Herbarium storage method gave mean absorbance values of 2±0.03 in week 1 and 1.7±0.14 while RNA from samples stored in FTA cards gave a mean

absorbance value of 1.8±0.12 for week 1 and 1.7±0.14 for week 2. In general, except for silica gel and -80 °C freezer storage methods, all the other sample storage had a significant difference in mean $A_{260/280}$ values for week 1 and week 2 (Table 4.1).

Table 4.1: Analysis of RNA quantity and quality from different storage methods

Storage method	**RNA concentration (ng/µl)**		**RNA quality (Absorbance values at $A_{260/A280}$)**	
	Week 1	Week 2	Week 1	Week 2
FTA cards	830.3 ± 166.02	954.5 ± 441.46	1.8 ± 0.12	1.7±0.14
Herbarium	1156.9 ± 156.99	434.6 ± 423.39	2 ± 0.03	1.7±0.14
RNA later	271 ± 164.99	927.5 ± 341.19	1.7 ± 0.23	1.9±0.05
-80 °C freezer	1161.9 ± 145.42	4171.8 ± 365.24	2 ± 0.06	2±0.02
Silica gel	1447.6 ± 236.44	2336.1 ± 100.00	2 ± 0.01	2±0.03

Each data is the average of three independent extractions of each sample and the ± SD.

4.1.2 Determination of suitable preservation method based on RNA integrity.

Qualitative assessment of the rRNA integrity was done by visual inspection of the agarose gel images to confirm the presence of bright, clear bands corresponding to the 28S and 18S ribosomal subunit. Intact RNA was obtained in samples stored using silica gel, -80 °C freezer and RNAlater as evident by the two sharp bands corresponding to 18S and 28S rRNA on denaturing 1.2% agarose gel electrophoresis (Plate 4.1).The clear

distinct bands that were comparable to the ones obtained from RNA isolated from freshly homogenized sample as a control were observed (Plate 4.1 C). This was found to be related to the $A_{260/280}$ ratio values of RNA obtained for samples preserved in silica gel and -80 °C freezer (Table 4.1), as there was no significant difference (*P*=.05) among these values (Table 4.1). According to the $A_{260/280}$ ratio values RNA obtained from the leaf samples preserved in silica gel and -80 °C freezer was of good quality and integrity. The distinct bands obtained in silica gel were stronger than the bands obtained from RNA of samples stored in -80 °C freezer and comparable to the bands on freshly extracted RNA (Plate 4.1 C). RNA isolated from samples stored in the herbarium and FTA cards was relatively of lower quality compared to that of samples stored in silica gel and -80 °C freezer. This was evidenced by the smearing and absence of distinct bands (Plate 4.1).

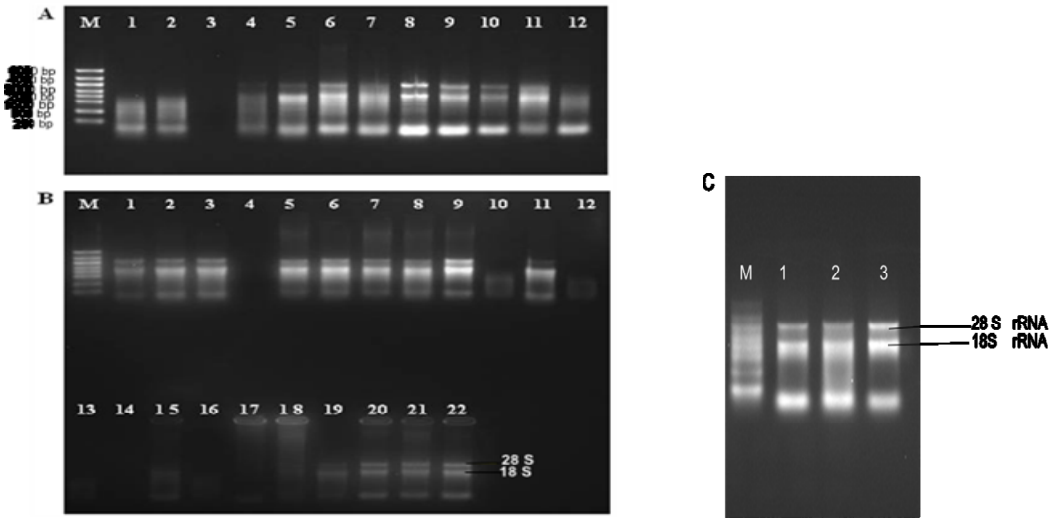

Plate 4.1: Total RNA isolated from passion fruit leaf samples from five different preservation methods after one (A) and two (B) weeks of storage and C (Fresh samples prior to storage) as assessed by denaturing agarose gel electrophoresis. 200 ng RNA per sample was used per lane. For 1 week of storage (A), lanes 1 and 2 (FTA cards), lanes 3 and 4 (herbarium), lanes 5, 6 and 7 (-80 °C freezer), lanes 8, 9 and 10 (silica gel) and lanes 11 and 12 (RNAlater). For 2 weeks storage (B) lanes 1-3 (-80 °C freezer), lane 4 (blank), lanes 5-9 (RNAlater), lanes 10-13 (FTA cards), lanes 14-18 (herbarium) and lanes 19-22 (silica gel). Lane M represents RiboRuler RNA ladder (Thermoscientific) for both A, B and C.

4.1.3 Suitability of RNA isolated from different sample preservation methods for RT-PCR

In this study, the quality of RNA was confirmed by RT-PCR amplification of housekeeping gene AtropaNad2.1a/2b using specific primers targeting ndhB plant internal gene. Amplification product of 188 bp (Plate 4.2) targeting NADH dehydrogenase region was evident from all the samples stored in silica gel, followed by samples stored in -80 °C freezer, and then RNAlater. This was indicated by the observation of clear band signals (Plate 4.2).Faint signals of amplification products were visible in the samples stored in FTA cards and herbarium which had earlier indicated a moderately to complete RNA degradation in the denaturing gel electrophoresis.

Suitability of RNA isolated for RT-PCR was also carried out for amplification of *Cowpea Aphid Borne Mosaic Virus* (CABMV) coat protein gene using specific primers. Amplification was evident for samples stored in silica gel giving specific product of expected band size of 500 bp (Plate 4.3). The RNA from other sample preservation methods gave amplification in either one or two samples which could be related to the low quality of the RNA template or possible inhibition of the RT-PCR by RNA contaminants obtained during RNA extraction.

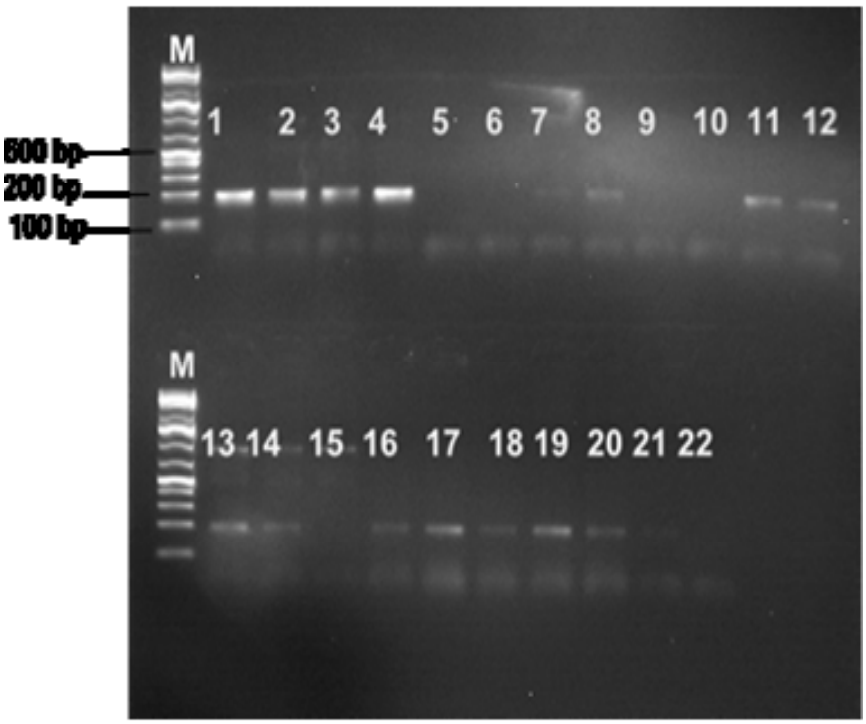

Plate 4.2: PCR products for plant internal control gene using AtropaNad2.1a/2b primers after two week's sample storage.

Lanes 1-4 (silica gel), lanes 5-8 (Herbarium), lanes 9-12 (FTA cards), Lanes 13-17 (RNALater), Lanes 18-21 (-80 °C freezer), lane 22 (blank) and lane M represents 1 kb plus DNA ladder (Thermoscientific).

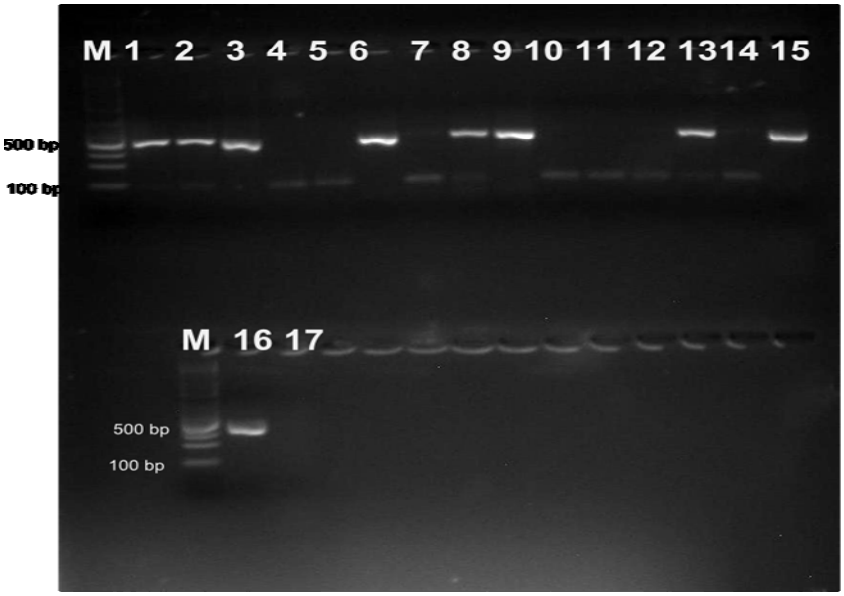

Plate 4.3: PCR amplifications products using virus specific primers for CABMV detection after 2 weeks storage: Lanes 1-3 (silica gel), 4-6 (herbarium), 7-9 (FTA cards), 10-12 (RNAlater), 13-15 (-80 °C freezer), 16 (positive control) and 17 (negative control). Lane M represents 1 kb plus DNA ladder (Thermoscientific).

4.2 RT-LAMP and real-time RT-PCR assay development, optimization and validation

4.2.1 Selection of samples for RT-LAMP and real-time RT-PCR assay development

Five samples were selected from samples that amplified for the coat protein gene for *Cowpea Aphid Borne Mosaic Virus* (CABMV) at 500 bp product using semi quantitative RT-PCR. These samples were sequenced to confirm the identity of the amplicon. Comparison of the resulting consensus sequences in GenBank using the Basic Local Alignment Search Tool (BLASTn) gave CABMV sequences comparable to the

sequences deposited in the Genbank (Table 4.2). Nucleotide sequence analysis of the amplified fragments with Basic Local alignment Search Tool (BLAST) revealed a high identity (91%) to the available CABMV sequences deposited in the Genbank. (Table 4.2).

Table 4.2: CABMV Hits from NCBI-BLAST search

Description	**max score**	**total score**	**query cover**	**e-value**	**identity**	**Accession no.**
Cowpea aphid-borne mosaic virus isolate Lns9 coat protein gene, partial cds	484	484	91%	5.00E-139	91%	KF725714.1
Cowpea aphid-borne mosaic virus isolate Lns3 coat protein gene, partial cds	484	484	91%	5.00E-139	91%	KF725708.1
Cowpea aphid-borne mosaic virus polyprotein gene, complete cds	481	481	91%	6.00E-138	91%	AF348210.1
Cowpea aphid borne mosaic virus RNA for coat protein	481	481	91%	6.00E-138	91%	X82873.1
Cowpea aphid-borne mosaic virus isolate Lns2 coat protein gene, partial cds	479	479	91%	2.00E-137	91%	KF725707.1
Cowpea aphid-borne mosaic virus isolate Lns10 coat protein gene, partial cds	473	473	91%	1.00E-135	91%	KF725715.1
Cowpea aphid-borne mosaic virus isolate LMN4 polyprotein gene, partial cds	473	473	88%	1.00E-135	91%	JF833420.1

From the blastn hits that aligned with our query sequence, it was evident that six best hits had a query coverage ranging from 88% to 91% and the identity was 91%. Athough we could have expected the identity to be 100% with 100% query coverage, this was not so probably due to variability of different CABMV isolates in the Genebank.

4.3 Establishing Real-time RT-PCR for CABMV detection

4.3.1 Development and optimization of real-time RT-PCR assay

Real-time RT-PCR detection of CABMV was carried out using SYBR Green-1 based detection system. During the initial optimization, melting curve analysis performed gave a melting temperature (T_m) value of 80.5℃ for the specific products, while the non specific products gave a characteristically lower temperature of 75℃ and below as shown in (Plate 4.4 A and B).

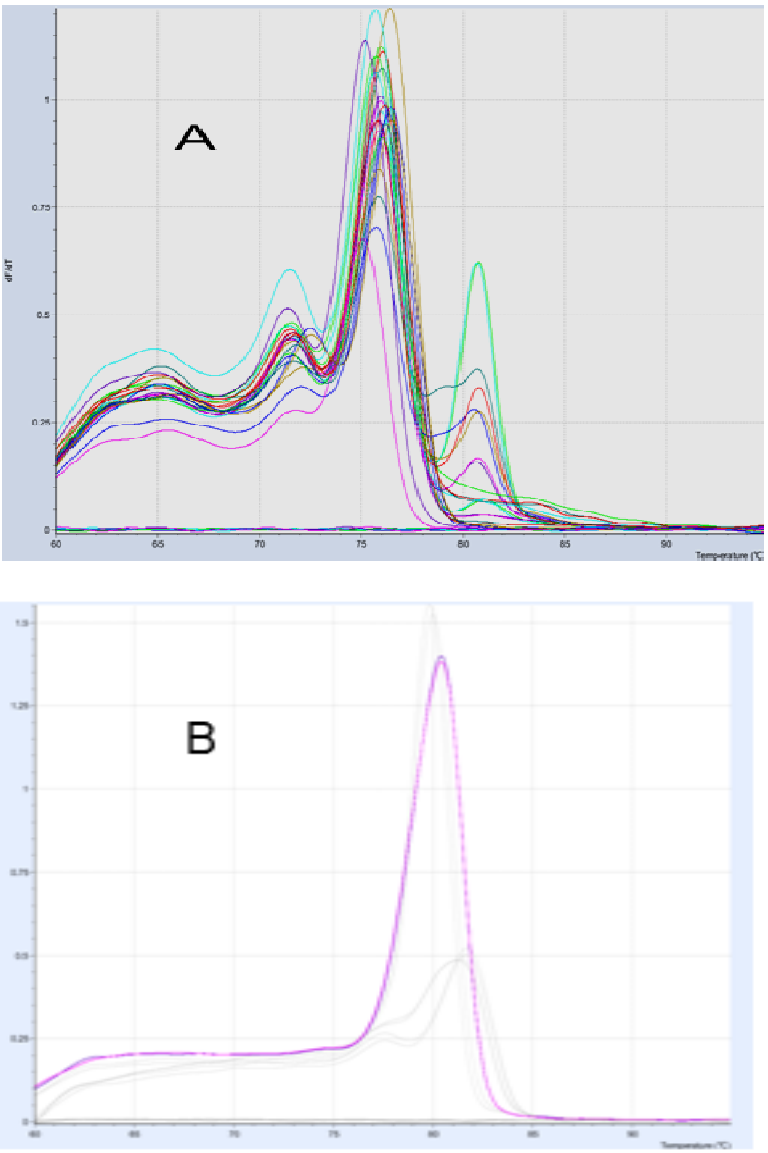

Plate 4.4: Melt-curve analysis of reaction products from SYBR Green® 1 assay; (A) shows the presence of non-specific products with noisy peaks and (B) indicates a specific product.

During optimization of the primer concentration 10µM used for both forward and reverse primers gave noisy peaks similar to the ones observed in plate 4.4 A. When the

amplification products were run on agarose gel electrophoresis, primer-dimers were observed (Plate 4.5 B). Lowering the primer concentration to 5µM led to no primer-dimer formation indicating that the primers worked well at this concentration (Plate 4.5A)

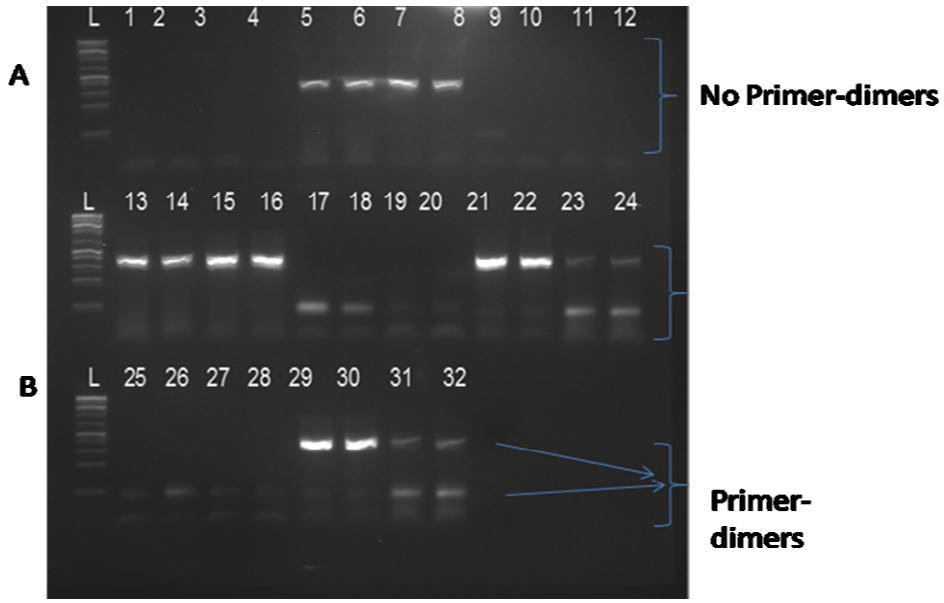

Plate 4.5: Real-time PCR products on 2 % agarose gel electrophoresis of ;(B) shows the presence of primer-dimers when 10µM concentration of primers was used indicated that the primer concentration was not optimal and; A) Lack of primer dimers after lowering the concentration of primers to 5µM which indicated the optimal concentration. Lanes L indicate 1 kb Plus DNA ladder (Thermoscientific). Lanes (5,6,7,8), (13,14,15,16), (21,22,23,24) and (29,30,31,32) are same samples replicated to demonstrate reproducibility.

Further testing of samples using the optimized SYBR Green-1 based real-time RT-PCR gave good sigmoid amplification curves and cycle thresholds ('ct' values) as expected of an optimized assay. Amplification curves of the positive samples with ct values ranging from 12.47 to 24.48 appeared earlier than those of negative samples and non-template controls (Plate 4.6). Any amplification with ct values >30 were treated as negative. The amplification curves indicated the increase in fluorescence caused by the binding SYBR Green dye to the double stranded dsDNA. Once the reaction proceeded and the PCR product accumulated, the fluorescence levels increased proportionally to the amount of DNA present in the original sample.

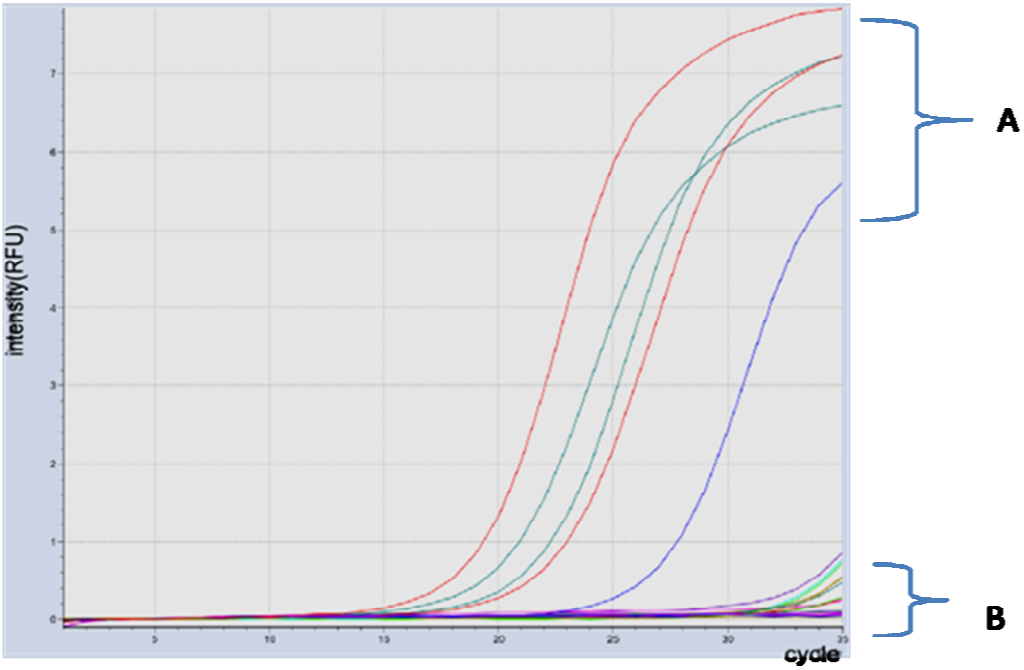

Plate 4.6: Amplification plots following real time RT-PCR assay; A) amplification curves of different positive samples, and B) amplification curves of negative samples and non template water control.

4.3.2 Sensitivity and specificity determination of real-time RT-PCR assay

When samples positive for SCMV (Sugarcane mosaic virus) and Maize chlorotic mottle virus (MCMV) were added in the assay developed for CABMV, results indicated that only CABMV positive samples could be detected with ct (Threshold cycle) values ranging from 20.106 to 25.662 (table 4.3). Positive control gave an average ct value of 18.97. There was no amplification observed in nuclease-free water used as a control as well as no amplification in the samples positive for SCMV and MCMV.

Table 4.3: Specificity determination of real-time PCR for detection of CABMV

CABMV realtime PCR specificity determination			
Position	Sample	SYBR Green I	Cq (ct)
A1	NFW	Target 1	
A2	NFW	Target 1	
A3	K5	Target 1	20.106
A4	K5	Target 1	20.711
A5	K6	Target 1	25.355
A6	K6	Target 1	25.33
A7	SCMV 1	Target 1	
A8	SCMV 1	Target 1	
B1	MCMV1	Target 1	
B2	MCMV 1	Target 1	
B3	K7	Target 1	25.645
B4	K7	Target 1	25.662
B5	POS	Target 1	19.688
B6	POS	Target 1	18.257
B7	NFW	Target 1	
B8	NFW	Target 1	

4.4 Establishing RT-LAMP for detection of CABMV

RNA extracted from passion fruit infected with CABMV and confirmed by conventional RT-PCR was tested in RT-LAMP assay. All tested samples of exhibited ladder-like DNA fragments typical of LAMP assay products after 2% agarose gel electrophoresis (Plate 4.7). Similarly, RNA extracted from a positive control sample showed the RT-LAMP amplified product (Plate 4.7). There was no visible amplification in non-infected sample and in template-less water control, indicating that primer design and reaction conditions were suitable for detection of CABMV.

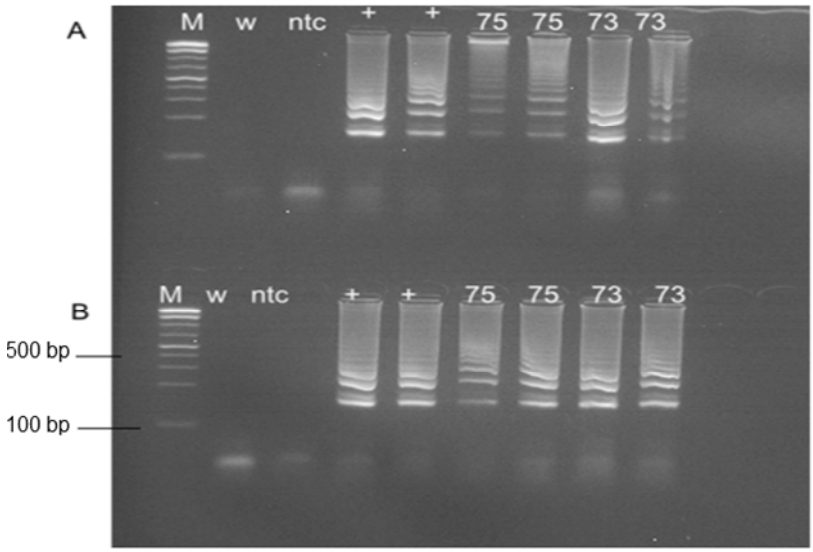

Plate 4.7: RT-LAMP assay amplification products for CABMV as visualized by 2 % agarose gel;M = 1kb plus DNA ladder (Thermoscientific), w = water, ntc = negative control,+ = positive control, 75 and 73 were positive samples.

When RT-LAMP was done on a real-time GENIE 11 detection system, amplification plots as sigmoid curves were observed for a positive reaction and horizontal lines where

no amplification reaction occurred indicated negative reactions i.e. negative samples and negative controls (Plate 4.8 B). For all positive reactions, the annealing temperature of the amplification product was observed to be in the range 85.97-86.77 °C (Plate 4.8 A). There was no product amplification in the non-infected leaf samples (negative control) and the non-template control.

A

Well	sample	Tp	anneal
A1	w		
A2	w		
A3	ntc		
A4	ntc		
A5	75	17:18	86.13
A6	75	17:33	86.13
A7	73	20:18	86.22
A8	73	20:03	86.77
B1	w		
B2	w		
B3	ntc		
B4	ntc		
B5	75	17:18	85.97
B6	75	17:18	86.02
B7	73	20:33	86.27
B8	73	20:18	86.61

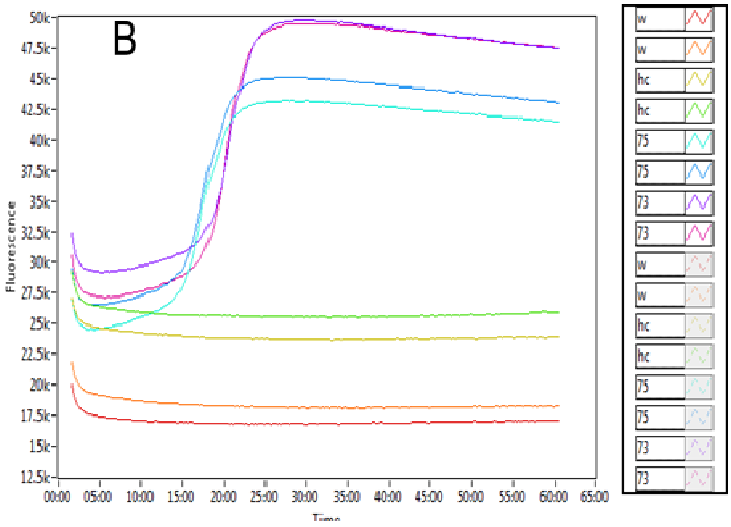

Plate 4.8: Amplification signal for CABMV real-time LAMP results from GENIE 11 detection system.

The sigmoid curves indicate positive reaction whereas the horizontal lines indicate no amplification. The table shows the (Tp values) i.e. time taken to generate a positive result based on the fluorescence of real-time LAMP generated from genie 11 machine; A1-B8 shows the position of the wells, ntc represents a healthy control. 73 and 75 denote samples used in replicates to demonstrate repeatability and reproducibility.

4.4.1 HNB visualization of RT-LAMP products

LAMP product was initially visualized using agarose gel electrophoresis (Plate 4.7); however this method was laborious and contributed to a large amount of contamination. Positive results were then judged colorimetrically **(Plate 4.9)** using a metal ion binding dye; Hydroxynaphthol blue (HNB). To test HNB, positive and negative samples were amplified in LAMP reaction containing HNB (Goto *et al.*, 2009) and tubes were visually monitored for a colour change from purple to blue. Positive results were seen for positive samples, with an easily discernible colour change from purple to sky blue (Plate 4.9 C and D). Neither negative control (non-infected and water) displayed any colour change (remained purple), showing that the colour change is specific to positive samples (Plate 4.9 A and B). These results show that LAMP detection of CABMV is simple and rapid.

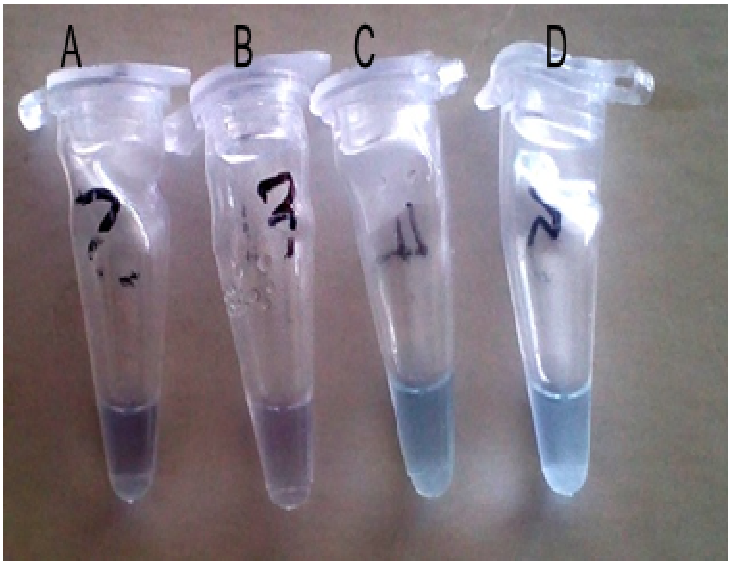

Plate 4.9: Visualization of LAMP products in HNB; A and B (purple colour) indicate negative results; C and D (sky blue) indicate positive results**.**

4.4.2 Specificity of RT-LAMP for CABMV detection

The specificity of the RT-LAMP assay for detection of CABMV was determined by evaluating its cross-reactivity for the closely related Sugarcane Mosaic Virus (SCMV) and unrelated Maize Chlorotic virus (MCMV). Total RNA from leaf tissues infected with SCMV and MCMV was amplified using the RT-LAMP protocol for CABMV. In CABMV infected samples, the RT-LAMP product could be easily visualized after agarose gel electrophoresis, similar to the amplification in positive controls. In contrast, in samples infected with either SCMV or MCMV, there was no detectable amplified product. Similarly, there was no visible DNA amplification in samples from non-infected samples and in template-less control reactions. Thus, the RT-LAMP primer set demonstrated a high degree of specificity for CABMV (Plate 4.10).

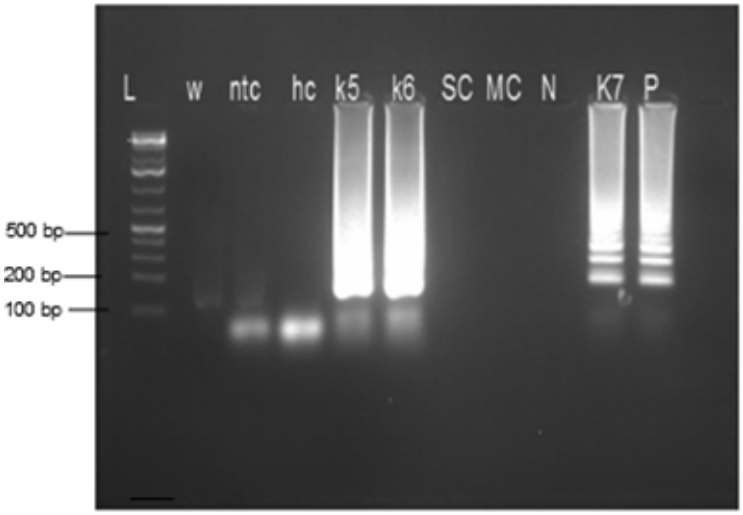

Plate 4.10: RT-LAMP assay amplification products for specificity determination; As visualized by 2 % agarose gel; L = 1kb plus DNA ladder (Thermoscientific), w = water, ntc =template less control, hc =non-infected (healthy) sample, K5, K6 and K7 = positive samples, SC= SCMV infected sample, MC= MCMV infected sample, N represents blank while P = positive control.

Specificity was also demonstrated by the melt curve analysis in the Genie II machine which indicated specific amplicon (Plate 4.11 A and B).

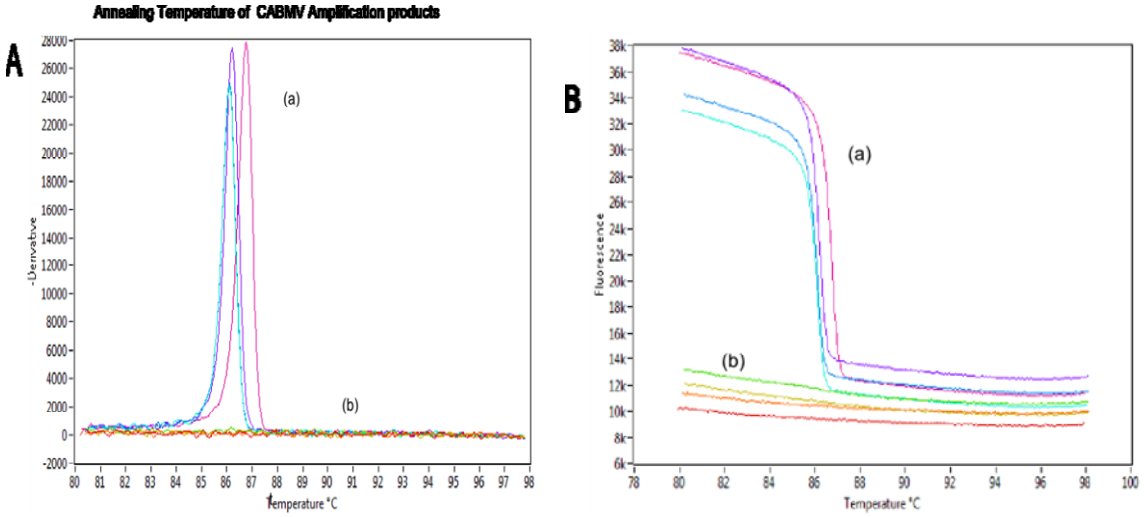

Plate 4.11: Annealing curves showing temperature on x-axis and fluorescence on the Y-axis. The peaks in A indicate Tm values generated by the optimized annealing temperature.

4.4.3 Determination of Sensitivity RT-LAMP assay

Sensitivity was carried out by determination of the limit of detection. Ten fold serial dilutions was carried out for RNA from a positive sample diluted to produce tenfold series ranging from (10^{-1} up to a dilution of 10^{-9}). The RNA at the different dilutions was then used as a template in RT-LAMP assay. The amplification products for the LAMP assay had a ladder-like appearance when visualized by agarose gel electrophoresis. These ladder-like bands could be visualized up to a dilution of 10^{-7}, indicating that this was the limit of detection for the assay (Plate 4.12). At dilution of 10^{-8}, and 10^{-9}, there were no visible amplification as well as in the negative control.

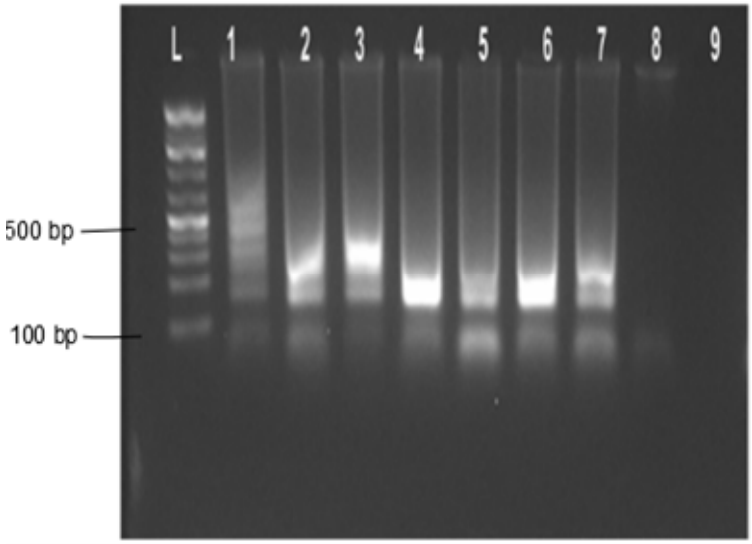

Plate 4.12: RT-LAMP assay amplification products for sensitivity determination visualized by 2 % agarose gel; L = 1kb plus DNA ladder (Thermoscientific),

1 =10^{-1} ,2 = 10^{-2} ,3 = 10^{-3} , 4 = 10^{-4} ,5 = 10^{-5} ,6= 10^{-6} ,7 = 10^{-7}, 8 =10^{-8} and 9 =10^{-9}.

4.4.4 Comparison of RT-LAMP and real-time PCR for detection of CABMV

Thirty eight field collected samples were tested by the developed real-time RT-PCR and by two- step RT-LAMP. The results revealed that ten out of the 38 samples tested positive by RT-LAMP and 8 out of 38 samples tested positive using SYBR Green-based real-time RT-PCR as shown in table 4.4. The results confirm the sensitivity of 10^{-7} of RT-LAMP compared to 10^{-6} of real-time PCR. Amplification was observed in the positive controls as well as no amplification in the negative controls.

Table 4.4: Comparison of detection of CABMV by RT-LAMP and real time RT-PCR

Sample No	RT-LAMP	real-time PCR	No.	RT-LAMP	real-time PCR
1	+	+	21	-	-
2	-	-	22	-	-
3	-	-	23	-	-
4	-	-	24	-	-
5	-	-	25	-	-
6	-	-	26	-	-
7	-	-	27	-	-
8	-	-	28	-	-
9	-	-	29	+	-
10	-	-	30	-	-
11	-	-	31	+	+
12	-	-	32	+	+
13	-	-	33	+	+
14	-	-	34	+	+
15	-	-	35	+	+
16	-	-	36	-	-
17	-	-	37	+	+
18	+	-	38	+	+
19	-	-	ntc	-	-
20	-	-	pos	+	+

+ = positive, - = negative, ntc = no-template control

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Recent advances in molecular biology especially in plant health diagnostics has led to increased demand for methods of preserving nucleic acids from point of sample collection to the laboratory. Since many samples are collected from farmers' fields followed by transportation to the laboratory for analysis, RNA can be easily degraded by the ubiquitous presence of endogenous Ribonucleases during transit if the storage conditions are not ideal. In this study, the effect of different storage conditions for passion fruit leaves on RNA quality and suitability for RT-PCR for two time points; one week and two weeks post-harvest was evaluated. This was to determine the most suitable method for collection of passion fruit leaf samples from the fields to the laboratory with the aim of achieving rapid detection of CABMV. The time point of two weeks was chosen as the ideal maximum duration that plant health inspectors or other researchers would spend in the fields during epidemiological studies or surveillance activities involving sample collection and transit to the laboratory.

The quality of RNA is very important for RT-PCR. A $_{260/280}$ ratio greater than 1.8 is usually considered acceptable indicator of good quality RNA (Sambrook *et al.*, 2000), although this ratio does not guarantee pure RNA. If the ratio is appreciably lower, it is an indication of the presence of protein, phenol, or other contaminants that absorb

strongly at or near 280 nm. Ratios lower than expected could indicate that additional cleaning is necessary (Florell *et al.,* 2001). Results of the mean $A_{260/280}$ ratio from this study indicated that - 80℃ Freezer method and silica gel after one and two weeks' storage were not significantly different (P=.05) and gave mean absorbance values within the acceptable range of 1.8 to 2.0. This indicated that there was low polyphenol, polysaccharides and protein contaminants in these methods. The integrity of 28S and 18S ribosomal RNA is used as the measure of RNA integrity and the lack shows a smear in the agarose gel, indicating that the total RNA is degraded. Visual observation of gel images from the study confirmed intact, bright and clear bands corresponding to 28S and 18S ribosomal subunit in RNA from samples stored in silica gel, - 80℃ freezer and RNAlater, comparable to the bands observed in fresh sample used as a control.

From the findings of this study, good RNA quality and integrity was obtained from samples stored in silica gel, followed by -80°C frozen samples. Further results on RT-PCR analysis demonstrated that the RNA obtained from leaf samples stored in silica gel and -80 °C freezer were well suited for downstream applications, such as gene amplification, which can be further used in studies involving analysis of gene expression. Due to stability and consistency over the two weeks, silica gel preservation proved to be a simple, efficient and low cost passion fruit sample preservation method and can be used for collection of samples from remote locations thus providing alternative to cold chain logistics especially where samples need to be collected and transported from remote areas. These results confirmed similar studies conducted by Staats *et al., (*2011).

Loop-mediated isothermal amplification (LAMP) for detection of *Cowpea aphid-borne mosaic virus* (CABMV) was optimized and evaluated. This was carried out in a simple system for monitoring by the colour change using a metal ion-binding indicator dye; Hydroxynaptholblue (HNB) as well as through real-time monitoring in Genie® II (Optigene, United Kingdom).HNB is known to bind the Mg^{2+} ion and changes colour depending on PH and Mg^{2+} ion concentration present in the mastermix. The colour of HNB is purple at Mg^{2+} ion concentration of 6mM and below or higher. As the DNA synthesis progresses, the concentration of Mg^{2+} ion decreases and when it reaches 6mM and below, the colour changes to sky blue indicating a positive reaction. Comparable to previous report by (Das *et al*, 2012), positive samples from this assay gave a blue colour whereas the negative samples and non-template control retained the purple colour of HNB indicating that the assay worked.

Accurate identification of positive reactions from negative reactions, either from non template (amplification of primers without target DNA) or nonspecific amplification (amplification of non target DNA present in the sample) has been reported as an essential consideration for amplification technique or any diagnostic method (Tanner *et al.*, 2012). The ability to distinguish the positive reaction and a negative reactions in this study was further proven when the LAMP products were run on 2% agarose gel electrophoresis whereby ladder-like patterns from the positive samples were observed but no detectable production of such patterns was observed in negative controls /samples. According to (Notomi *et al.*, 2000),the ladder-like patterns as observed in our study occurs due to the fact that LAMP products consist of products of different lengths

containing alternately inverted repeats of the target sequence, appearing as a ladder-like pattern when visualized on a gel. In agreement with reports from previously published work, anneal curve validation step in the optimized real-time LAMP assay distinguished specific amplification from non-specific amplification, which ruled out of any possible contamination (Abdulmawjood *et al*, 2014). Considering resource limited laboratories, RT-LAMP using HNB could be adopted for easy observation of colour change by naked eye. The LAMP assay was demonstrated to be 100% specific as no cross-reaction was observed when other viruses (related SCMV and unrelated MCMV) were included in the assay.

Several studies have shown the successful use of real-time PCR assays to detect and quantify viral load of infected organisms. However, all these methods depend on the use of sequence-specific oligonucleotide hydrolysis probes, which are more expensive option than using SYBR Green chemistry. SYBR Green-1 based real-time PCR for detection of CABMV in passion fruit leaf samples was evaluated in this study. The assay conditions were optimized including the annealing temperature and primer concentrations. The analysis of amplification plot in conjunction with melting curve resulted in discriminating non-specific amplification. This was further confirmed when the real-time PCR products were run on a 2% agarose gel electrophoresis whereby the presence of primer-dimers were observed when the primer concentration was not optimal. This compared with the published assays and is usually recommended in all SYBR green based real-time PCR assays to avoid false positive results, since SYBR green can also intercalate into non-specific DNA which could co amplify with the

specific products. No- cross reactivity was observed when a panel of 5 samples of known positive and negative samples were included, indicating that the assay was 100% specific.

5.2 Conclusion

1. This study demonstrated that Silica gel is the most suitable method for collecting and preserving passion fruit leaf samples from fields far from the processing laboratories for RNA extraction and subsequent assays. The ability to collect and preserve samples in a simple and convenient manner allows analysis of samples that would otherwise prove difficult. The method will be invaluable for disease diagnostics and epidemiological studies, particularly in developing countries where it is difficult to provide a cold chain from sample collection to processing.
2. Real-time PCR proved to be sensitive and 100% specific since no cross-reaction observed when other potyviruses were included in the assay. The assay was evaluated and found to be sensitive hence suitable for detecting *Cowpea Aphid Borne Mosaic Virus* (CABMV) in passion fruit leaf samples
3. The optimized LAMP assays demonstrated to be simple, rapid and reliable for CABMV detection especially on latent infections of woodiness disease due to its sensitivity.

The study supports the adoption of these methods in certification programs as a valuable diagnostic tool for early stage screening of passion fruit planting materials in nurseries.

This will facilitate increased production and distribution of clean planting materials to farmers.

5.3 Recommendations

1. There is need to evaluate different RNA extraction protocols against the different sample preservation methods
2. There is need for adoption of these molecular diagnostic methods in Passion fruit nursery certification programs for early stage screening of planting materials

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APPENDICES

Appendix 1: RNA extraction protocol

Items Required

- Eppendorf tubes
- Liquid nitrogen
- 250µl Choloroform
- 250µl Isopropanol
- 1.2 M Nacl / 0.8 M sodium citrate
- 75% Ethanol

Procedure

1. Grind 100mg of plant leave samples in liquid nitrogen
2. Transfer the material to Tri-reagent (800µl)
3. Shake moderately for several seconds. Incubate the samples for 5 minutes at room temperature (do not voltex)
4. Centrifuge at 12,000 xg for 10 minutes at 4℃ (supernatant contains RNA &proteins)
5. Transfer upper (clear) supernatant to fresh tube. Add 200µl chloroform, shake for 15 seconds. Allow to stand for 15 min at room temperature (do not voltex)
6. Centrifuge at 12,000 xg for 15 mins at 4℃ (Colourless upper aqueous layer contains RNA)

7. Transfer the upper aqueous layer to fresh tube. Add 250µl isopropanol and 250µl of 1.2 M Nacl/0.8 M sodium citrate.
8. Invert the tubes to mix, let sit for 10 minutes at room temperature
9. Centrifuge at 12,000xg for 10 minutes at 4℃. The RNA will precipitate to form pellet at the bottom of the tube
10. Remove the supernatant & wash the RNA pellet by adding 1ml of 75% Ethanol. Voltex briefly and centrifuge at 8,000 xg for 5 minutes at 4℃
11. Briefly dry the RNA for 5 minutes by air-drying
12. Add 30µl of nuclease-free water
13. Incubate the samples at 65℃ for 10 minutes. Mix by pipetting
14. Read the nanodrop readings and keep the RNA at -20℃ for immediate use and in -80℃ for long term use

Appendix 2: RNA gel electrophoresis procedure and reagents preparation

Preparation of 10 % and 0.5% SDS;

10% SDS;

1. Weigh 10g SDS and pour it in a conical flask
2. Add Ultrapure water - 60ml
3. Allow to mix in a magnetic stirrer
4. After dissolving, top –up with water to 100ml

To prepare 5 % SDS (CIVI=C2V2)

Draw 50 mls pf 10% SDS, mix with 950 mls of ultrapure water; to make 1 litre of 0.5% SDS

Preparation of 1x TAE from 50x TAE

- To make 1 liter, draw 40 mls of 50X TAE stock, add upto 2000ml using ultrapure water.i.e. add 1960ml of ultrapure water

Preparation of DEPC treated water

- To make one liter, add 1 ml DEPC Into 1000ml of ultra-treated water
- Incubate overnight at room temperature
- Autoclave for 15 minutes

Preparation of 1 x loading dye

Reagent	**Amount**
50 mM Tris-HCL PH 7.6	0.5 ml (500ul)
0.25% Bromophenol blue	0.025g
Glycerol	6 ml
Deionized water	3.5ml

Store at 4℃

Preparation of 1.2% agarose gel electrophoresis

- Weigh 1.2 g of agarose into 100ml 1x TAE
- Boil in a microwave
- Add 2.5 µl gel red, mix and allow to cool
- Pour the gel into a casting tray with combs in place

Sterilization of gel apparatus

- Wash the gel tanks, and combs with 0.5% SDS (soak for 5 minutes in 0.5% SDS)
- Rinse with DEPC treated water
- Wipe out with RNAse Zap activation solution
- Allow to dry
- Wipe the bench with RNAse ZAP solution

Running denaturing gel electrophoresis

1. Mix 4µl RNA, 6µl Deionized formamide and 1µl Of 10x loading dye, 1µl gel red (1:100) all in single tubes corresponding to the number of samples
2. Do the same for the RNA marker (Riboruler)
3. Denature the samples by heating for 5 mins at 65℃ on a heater block, immediately chill on ice for 5 minutes and load onto the already cooled 1.2% gel. Load the RNA ladder as well
4. Run at 100 volts for 35 – 40 mins

Appendix 3: cDNA synthesis protocol

Reagent	**1x**
Enzyme mix	2µl
5x Reaction mix	4µl
Water	13µ
RNA sample	1.5µl (5µg)
Total volume	**20 µl**

-
- Dispense 19 µl of the mastermix into tubes for the number of samples, add 1.5µl of RNA, mix gently and spin down for 30 seconds
- Incubate for 1 mins at 25℃ followed by 15 minutes at 50℃, terminate the reaction by heating at 85℃ for 5 mins
- Store at -20℃ for up to one week. For longer storage,-70℃ is recommended
- Avoid free/thaw cycles of cDNA.

CONTROL REACTIONS

1. Reverse Transcriptase minus (RT-) negative control is important in RT-Qpcr Reactions to assess for genomic DNA contamination of the RNA sample. The control RT-reaction should contain every reagent for the reverse transcription except for the Maxima Enzyme Mix
2. No template control (NTC) is important to assess for reagent contamination. The NTC reaction should contain all the reagents for the reverse transcription reaction except for the RNA template.

Appendix 4: RT-PCR protocol for CABMV detection

Reagent	Concentration	Vol
Forward primer (F3)	1µg	2µl
Reverse primer (R2)	10pmol	0.8µl
RNase free water	10pmol	0.8µl
Cdna		16.4µl
Total Volume		**20µl**

PCR conditions

94℃ for 5 min
94℃ for 30 sec
55℃ for 30 sec } 35 Cycles
72℃ for 45 sec
72℃ for 7 mins
4℃ 8

Run the products on 2% agarose gel (2g of agarose in 100mls of 0.5x TBE)

for 35 mins

Use 1x TBE buffer
Load 1kb plus ladder (5µl)
Load 3µl of sample and 2µl of loading dye

Appendix 5: Purification of PCR products (GeneJET PCR purification kit)

1. Add approximately 17µl of binding buffer to 17µl PCR products
2. Transfer all the solution to the GeneJET Purification column
3. Centrifuge for 1 minute. Discard the flowthrough
4. Add 700µl of wash buffer (diluted with ethanol) to the purification column
5. Centrifuge for 1 minute. Discard the flowthrough and place the purification back to the collection tube
6. Centrifuge the empty GeneJET purification column for additional 1 minute to completely remove any residual wash buffer
7. Transfer the GeneJET Purification column to a clean 1.5ml microfuge tube
8. Add 15µl of elution buffer to the centre of the GeneJET Purification column membrane
9. Centrifuge for 1 minute at 1200 rpm
10. Discard the GeneJET Purification column
11. Read the nanodrop quantifications
12. Keep at -20℃

Run a gel (1%) gel

Preparation of Ladder

6x loading dye	100µL
1 kb plus	120µL
DdH20	380µL
TOTAL	**600µL**

Aliquot into small volumes and keep at -20℃

Appendix 6: Nanodrop readings for week 1 and 2 storage

Nanodrop readings for Week 1 and week 2 storage				
Week	**Method**	**Nucleic A.conc**	**Unit**	**260/280**
WEEK1	Freezer	1302.4	ng/µl	2.09
WEEK1	Freezer	1012	ng/µl	2.02
WEEK1	Freezer	1171.2	ng/µl	1.97
WEEK1	RNA later	264.8	ng/µl	1.89
WEEK1	RNA later	439	ng/µl	1.72
WEEK1	RNA later	109.2	ng/µl	1.43
WEEK1	FTA	653	ng/µl	1.85
WEEK1	FTA	982.1	ng/µl	1.79
WEEK1	FTA	855.7	ng/µl	1.62
WEEK1	Herbarium	1039.1	ng/µl	1.93
WEEK1	Herbarium	1335.1	ng/µl	1.96
WEEK1	Herbarium	1096.4	ng/µl	1.98
WEEK1	silica gel	1183.5	ng/µl	1.98
WEEK1	silica gel	1639.6	ng/µl	2
WEEK1	silica gel	1519.7	ng/µl	1.98
WEEK2	Freezer	4500.5	ng/µl	2
WEEK2	Freezer	4236.2	ng/µl	2.05
WEEK2	Freezer	3778.6	ng/µl	2.05
WEEK2	RNA later	1298.8	ng/µl	1.87
WEEK2	RNA later	855.8	ng/µl	1.97
WEEK2	RNA later	627.8	ng/µl	1.9
WEEK2	FTA	1250.8	ng/µl	1.77
WEEK2	FTA	1165.5	ng/µl	1.56
WEEK2	FTA	447.1	ng/µl	1.83
WEEK2	Herbarium	154.2	ng/µl	1.66
WEEK2	Herbarium	227.9	ng/µl	1.85
WEEK2	Herbarium	921.6	ng/µl	1.57
WEEK2	silica gel	2336.6	ng/µl	2.04
WEEK2	silica gel	2235.8	ng/µl	2.04
WEEK2	silica gel	2435.8	ng/µl	1.98

Appendix 7: Validation sample details

Sample ID	SAMPLING DATE	REGION	GPS coordinates
1	17/08/2014	Nakuru	S00.27303, E035.86749
2	18/08/2014	Uasin Gishu	N/A
3	18/08/2014	Uasin Gishu	N00.59130,E035.43787
4	18/08/2014	Keiyo marakwet	N00.64764, E 03549635
5	18/08/2014	Keiyo marakwet	N00.64761, E035.49679
6	18/08/2014	Keiyo marakwet	N00.64984,E035.50057
7	18/08/2014	Markwet county	N00.65711, E035.50057
8	18/08/2014	Keiyo	N00.6443, E035.46272
9	19/08/2014	Tranzoia	N00.97257,E034. 86390
10	19/08/2014	Tranzoia	N01.02963,E034.84140
11	19/08/2014	Tranzoia	N00.98287,E035.04226
12	21/08/2014	Mumias	N00.34077,E034.49106
14	25-11-2014	Nairobi	S 0118945,E 03648688
15	25-11-2014	Nairobi	S 0118945, E 03648688
16	25-11-2014	Nairobi	S 0118945, E 03648688
17	25-11-2014	Nairobi	S 0118232, E 036 44900
18	25-11-2014	Nairobi	S 0118232,E 036 44900
19	25-11-2014	Nairobi	S 0118232,E 036 44900
20	26-11-2014	Kiambu	S 0105879, E03700696
21	26-11-2014	Kiambu	S 0105879,E03700696
22	26-11-2014	Kiambu	S 0105879, E03700696
23	26-11-2014	Kiambu	S 0105879, E03700696
24	26-11-2014	Kiambu	S 0105879, E03700696
25	26-11-2014	Kiambu	S 0105879, E03700696
26	28-11-2014	Nyeri	S 0046747, E 03694269
27	28-11-2014	Nyeri	S 0046747, E 03694269
28	28-11-2014	Nyeri	S 0046747, E 03694269
29	28-11-2014	Muranga	S 0061510, E 03703553
30	28-11-2014	Muranga	S 0061510, E 03703553
31	28-11-2014	Muranga	S 0059720, E0370451
32	28-11-2014	Muranga	S 0100078, E03704810
33	28-11-2014	Muranga	S 0100078, E03704810
34	28-11-2014	Kirinyaga	S 0053740,E 03745.165
35	28-11-2014	Mwea	S 0069241,E 03739993
36	28-11-2014	Mwea	S 0069241,E 03739993
37	28-11-2014	Mwea	S 0069241,E 03739993
38	28-11-2014	Mwea	S 0069241,E 03739993