MOLECULAR DETECTION OF VIRUSES ASSOCIATED WITH PASSIONFRUIT (*PASSIFLORA EDULIS* SIMS) WOODINESS DISEASE, MONITORING AND MANAGEMENT OF APHID VECTORS IN KENYA

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Declaration

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

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Dedication

To my mother Constance and husband Joseph who have had faith in my abilities and have motivated me to learn and continue learning 'Your prayers and support have made me come this far"

Magnificat:

"My soul glorifies the Lord
  and my spirit rejoices in God my saviour,
for he has been mindful
    of the humble state of his servant.
From now on all generations will call me
      blessed,
  for the mighty One has done great things
    for me-
  holy is his name.
His mercy extends to those who fear him,
  from generation to generation.
He has performed mighty deeds with his
      arm;
  he has scattered those who are proud in
    their inmost thoughts.
He has brought down rulers from their
    thrones
  but has lifted up the humble.
He has filled the hungry with good things
  but has sent the rich away empty.
He has helped his servant Israel,
  remembering to be merciful
to Abraham and his descendants for ever,
  even as he said to our fathers" (Luke 2: 46-55)

Acknowledgement

Abbreviations and acronyms

aa	amino acids
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	Complimentary deoxyribonucleic acid
CP	Capsid/ coat protein
DAS	Double antibody sandwich
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ds	Double strand
dsDNA	Double strand deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and cell cultures)
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethyl alcohol
HC-Pro	Helper component proteinase
IgG	Immunoglobulin G
IgG-AP	IgG conjugated in Alkaline Phosphatase
L	Liter
mRNA	Messenger ribonucleic acid
M	Molar
mM	millimolar
Mm	millimeter
μg	microgram
ng	nanogram
mg	milligram
Kg	Kilograms
KCl	Potassium Chloride
KH_2PO4	Potassium di-Hydrogen Phosphate

min	minutes
μl	microliter
NaCl	Sodium Chloride
Na_2HPO_4	Sodium di-Hydrogen Phosphate
NaN_3	Sodium azide
NaOAc	Sodium acetate
NCBI	National Centre for Biotechnology Information
NIb	Nuclear inclusion protein b
nt (s)	Nucleotide (s)
ORF	Open reading frame
OD	Optical density
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	revolutions per minute
RT	Reverse Transcriptase
PBS	Phosphate buffered saline
PBS-T	PBS in Tween-20
PCR	Polymerase chain reaction
PNPP	p-nitrophenyl phosphate
RT-PCR	Reverse transcription-PCR
Rubisco	Ribulose-1, 5- biphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
Sec	second (s)
spp	species
Taq	*Thermus acquaticus*
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) l-aminomethane
TBE	Tris borate EDTA
T	Tween-20
U	Unit
V	volume

w/v	weight/volume
MEGA	Molecular Evolutionary Genetic Analysis
CABMV	*Cowpea aphid borne mosaic virus*
CMV	*Cucumber mosaic virus*
PWD	Passionfruit woodiness disease
PWV	*Passionfruit woodiness virus*
SAPV	*South African passiflora virus*
SeMV	*Sesame mosaic virus*
OYDV	*Onion yellow dwarf virus*
WMV-2	*Water melon virus-2*
SMV	*Soybean mosaic virus*

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ABSTRACT

Passionfruit (*Passiflora edulis* Sims) is an important economic crop in the world as an income earner and for food and nutrition security. Among the biotic stresses constraining production, woodiness disease is important causing economic losses wherever passionfruit is grown worldwide. In Kenya, passionfruit is a source of income to many small scale farmers. Although viral diseases are reported to be present in Kenya, there is limited information on the causal agents and the relationship with aphid species found in passionfruit agroecosystem. This study was undertaken to identify the causal agents of woodiness disease, incidence and distribution; monitor and identify the potential aphid vectors, determine virus transmission ability of the aphid species present in the orchards and to evaluate the effect of aphid repellant sources such as plastic reflective mulch to manage aphid vectors.

A survey was undertaken in major passionfruit growing areas in the Rift Valley, Central and Eastern provinces of Kenya in 2008 and 2010. The area was stratified according to administrative districts and agroecozones from which 130 fields were selected along rural roads. Suspected passionfruit leaf samples collected were assayed using ELISA and RT-PCR techniques for detection and identification of the viruses. Sequence diversity for *Cowpea aphid borne mosaic virus* (CABMV) was determined. Experiments for monitoring aphid vectors and evaluating aphid repellant sources were laid out in randomized complete block design in two sites. The presence and abundance of aphids were monitored using yellow water pan traps. The aphids captured were counted and identified under a stereomicroscope and using a dichotomous key.

The viruses were distributed widely with moderate to high incidence in all the passionfruit growing surveyed areas. The incidence was significantly ($p<0.05$) higher in the upper midland zones (82%) compared to the lower highland zones (50%). The most common virus had 45% incidence. Coat protein sequence analysis for the most common virus isolated from Kenya, was closely related to *South African passiflora virus* and CABMV. Based on sequence identity, the virus was identified as a strain of CABMV. There was a high homology of the amino acid sequences among the Kenyan isolates suggesting a low diversity with strain differences. Other viruses present were *Cucumber mosaic virus* (CMV) and an unidentified potyvirus.

On aphid monitoring, twelve aphid species were identified in Kabete and Embu sites. The most abundant species were *A. gosypii* Glover, *Ropalosiphum maidis* Fitch, *Acyrothosiphon pisum* Harris, *Brevicoryne brassicae* and *Aphis fabae* Scopoli. All are known vectors of many viruses including CMV and CABMV. Significantly ($p<0.05$) more aphids were collected in Kabete compared to Embu and more aphids were present during the long rains compared to the short rains season. The aphids were a reflection of the crop diversity within the cropping system. They peaked only once in June (2008 and 2009) coinciding with the food crop growing season. Disease transmission tests by *A. gosypii, R. maidis, B. brassicae, A. fabae* and *Sitobion avenae* (L) indicated a significant ($p<0.001$) difference in the ability of the aphid species to transmit the CABMV virus isolate from Kenya. *Aphis gosypii* and *R. maidis* were more efficient in transmitting the virus isolate compared to *B. brassicae* and *A. fabae. Sitobion avenae* did not transmit CABMV. Aphid repellant sources significantly ($p<0.05$) reduced aphid populations in passionfruit orchards compared to the untreated control. Plastic reflective mulch had the highest reduction of aphid numbers (60%) compared to treated yellow material and mineral oil. Reflective mulch also significantly ($p<0.05$) reduced individual aphid species numbers for *A. gosypii, R. maidis, A. pisum* and *M. euphorbiae* which were the most abundant.

These results have expanded our knowledge of the causal agents of the woodiness disease and its distribution in Kenya. Ability to detect passionfruit viruses particularly CABMV from leaf tissues provides a valuable diagnostic tool for certification programs and early stage screening of passionfruit planting materials in nurseries, studies on epidemiology and for breeding programs. The information generated from monitoring and evaluation of aphid repellant sources has implications for the development of virus disease and vector management strategies. Farmers can adopt the use of plastic reflective mulch and monitoring to delay entry and spread of viruses within passionfruit orchard

CHAPTER ONE

INTRODUCTION

1. 1. Background information

Passionfruit (*Passiflora edulis* Sims*)* is native to the tropical regions of Southern American rain forests, mainly in Brazil. The crop is cultivated widely throughout the tropics and sub tropics mainly by small-scale growers who constitute about 80% of all the growers (Vieira and Caneiro, 2005). The fruit is either eaten fresh, or processed for juice production. It is high in calories, rich in minerals and is a fairly good source of pro- vitamin A, vitamin C and vitamin D (Sema and Maiti, 2006). It has medicinal properties, used as a laxative and sedative in Brazil (Sema and Maiti, 2006). In Florida, United States of America (USA) it has been established that taking fresh passionfruit juice regularly significantly reduces the development of cancerous cells in the blood (Sema and Maiti, 2006; Marie de Neira, 2003). In Kenya, the crop is mainly grown for fruit and juice processing.

In Kenya, the passionfruit industry is based on lines of the purple passionfruit (*P. edulis f. purpla* Sims), the yellow passionfruit (*P. edulis f. flavicarpa*) and a hybrid of purple and yellow varieties (*P. edulis* Sims x *P. edulis* f. *flavicarpa*) (Annon., 2006). The crop has spread and become popular in areas with favorable ecological conditions stretching from the coastal belt to the highlands since its introduction in Kisii and Sotik (HCDA, 2005). It is grown on small land units of 0.5 to 2 acres (HCDA, 2008). Passionfruit offers a quick means of generating income within nine months of transplanting and produces an approximate yield of 60,000 tons annually from 5000 ha of land (HCDA, 2008). Only 20-30 % of the fruit produced meets export quality while the rest is sold and consumed in the local market

(Annon. 2008). About 2 % of the total production is exported annually earning approximately US $ 375,000 as foreign exchange (KHDP, 2006). It is a source of livelihood for a large part of the population in all areas where it is grown (KHDP, 2006; Munene, 2003).

Although there is a high potential to expand, passionfruit production is constrained by several biotic (Barros, 2007; Manicom *et al.*, 2003; Lima *et al.*, 2002) and abiotic factors (Gesimba, 2008; KHDP, 2006). The major constraints include pests and diseases, inadequate disease free planting materials, high costs of investments, nutrient deficient soils, erratic rainfall, poor storage and handling of fruits (Gaturuku *et al.*, 2012; Gesimba, 2008). These contribute to yield loss and poor quality of produce. According to Manicom *et al.* (2003) and Trevisan *et al.* (2006) pests and diseases impact negatively on the life of most plantations around the world limiting the productive life to 1.5 years from five years.

The Kenya Agricultural Research Institute (KARI) in 2001 estimated that 80% of passionfruit in Kenya was destroyed by a combination of pests and diseases which include brown spot (*Alternaria passiflorae*), fusarium wilt (*Fusarium spp*), passionfruit woodiness disease (PWD) and plant parasitic nematodes. Woodiness disease is a major limiting factor for passionfruit production worldwide (Moreira, 2008; Barros, 2007; Gioria *et al.*, 2000). It was first reported in Kenya in 1944 (Natras, 1944). Incidence and severity have reached epidemic proportions as reported by Njuguna *et al.* (2005). Its importance seems to have increased with the expansion of passionfruit cultivation and the use of grafted passionfruit seedlings to manage fusarium wilt. This study was conducted to identify major biotic

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constraints limiting passionfruit production and assess potential sustainable management methods for increased productivity.

1. 2. Problem statement

Passionfruit is a highly suitable crop in small-scale production systems. It avails to the farmers a diversification enterprise to secure income due to its high market value and short maturity period. However, there is low production and poor quality of passionfruit and significant reduction of orchard age in Kenya and the entire East African region. Production and expansion is limited by pests and diseases, limited germplasm pool, poor pre- and post harvest practices, inadequate water and declining soil fertility. Pests and diseases in particular, caused a shift in production from the Kisii and Sotik areas to Thika and Nakuru areas in the 1970's (Gachanja and Ochieng, 1988) and now current producing areas include Uasin Gishu, Meru, Embu and Trans Nzoia (Gesimba, 2008). Large plantations such as Kakuzi Ltd. have been abandoned in favour of small plantings for ease of crop protection (Annon., 2008). Among the pathogens limiting production, viruses are considered to be a major cause of low productivity in passionfruit affecting yield and quality and age of orchards (Moreira, 2008; Gioria *et al.*, 2000). There is inadequate information concerning the identity of causal agents of woodiness disease and the distribution pattern within Kenya. Information on pathogen transmission requires confirmation. Current management strategies of pests and diseases are unsuitable and are designed around the intensive use of pesticides. The pesticides are limited in efficacy and they do not control viral diseases. Use of pesticides is undesirable since consumers demand for a clean environment and safe and quality produce.

1. 3. Justification

Passion fruit production has the potential to reduce poverty levels through improved cash income. Even so, two key pest management problems are a limitation. The first is pests and diseases such as woodiness disease and the vectors whose information in Kenya is scarce. The second is unsuitable pest management practices that require evaluation. Accurate identification and timely detection of viruses is the cornerstone of viral disease management. Although woodiness disease has been reported in major passionfruit growing areas such as Australia, Brazil and Hawaii, no remedy has been developed. In Kenya, Natrass (1944), Ondiek (1973) and Njuguna *et al.* (2005) reported the disease as existing. Natrass (1944) even confirmed the ability to spread woodiness disease by grafting single leaves. Little work has been carried out to identify the pathogens responsible and to develop appropriate and sustainable measures for passionfruit woodiness disease control in Kenya.

Several management practices of virus diseases in passionfruit have been reported including selection of tolerant plants, pre-immunization with mild strains of the virus and adoption of cultural practices (Alfenas *et al.*, 2005; Novaes and Rezende, 2003). In Kenya, the efficacy of cultural practices as a component of integrated pest management has not been evaluated. Correct identity of the disease pathogens and their vectors and evaluation of ecological based pest management approaches were the subject of these studies.

1. 4. Objectives

1. 4.1 Overall objective

The overall objective of the study was to contribute to increased passionfruit productivity through sustainable management of biotic constraints.

1.4.2. Specific objectives

Specific objectives of the study were:

a. To identify the pathogens causing passionfruit woodiness disease, their distribution and incidence in the main passionfruit growing areas in Kenya

b. To characterize at molecular level the most common virus, *Cowpea aphid borne mosaic virus* (CABMV) and determine its diversity in Kenya

c. To identify potential vectors of CABMV and monitor the population abundance in passionfruit orchards

d. To determine efficacy of selected aphid repellants, attractants and mineral oil in the control of aphid vectors and aphid transmitted viruses.

1. 4.3 Hypotheses

(a) Viruses that cause passionfruit woodiness disease are not widely spread in Kenya

(b) Cowpea aphid borne mosaic virus isolates from Kenya are not related

(c) Potential Cowpea aphid borne mosaic virus (CABMV) aphid vectors are not present in large populations within passionfruit orchards

(d) Cultural practices are not effective in the management of the vectors that cause passionfruit woodiness disease

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution of passionfruit

The purple passion fruit (*Passiflora edulis* Sims) belongs to the family passifloraceae and in the genus passiflora (Ulmer *et al.*, 2004; MacDougal, 2001). The genus is the largest with about 500 species in the family (Souza *et al.*, 2008; Bellon *et al.*, 2007; Ulmer *et al.*, 2004). The purple passionfruit species originated from Brazil and have been naturalized beyond their native range into the tropical regions (Souza *et al.*, 2008; Viana *et al.*, 2003). Although, South America is the centre of diversity of the genus Passiflora with 95% of all species, other species in the genus are native to other places such as the USA, China, South Asia, New Guinea, Australia and New Zealand (Annon., 2006; Vanderplank, 1991). In 2005 and 2006 two species namely *Passiflora xishuangbannaensis* and *P. pardifolia* were identified (Vanderplank, 2006; Krosnick, 2005).

The passion fruit industry is based on the purple and yellow cultivars *P. edulis fp purpla* and *P. edulis fp flarvicarpa*. The purple cultivar is able to withstand frost while the yellow cultivar is intolerant to frost (Souza *et al.*, 2008). Both need protection from strong winds and require annual rainfall of about 900mm (Bellon *et al.*, 2007). In Kenya passionfruit was introduced in 1920 in Kisii and Sotik and have since spread to all areas that favour their growth (HCDA, 2005).

2.2 Description of passionfruit

The passionfruit is a woody, perennial vigorous vine which climbs by means of tendrils. It has shallow roots with about 87% rooting system found within 0- 45cm soil depth (Borges

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and Lima, 2002). The alternate, evergreen leaves, are deeply 3-lobed and finely toothed when mature with a length of 7.5-20 cm. The plant is mainly pollinated by the carpenter bees and other pollinators include hamming birds, bumble bees, wasps and bats (Alexandra-Maria *et al.*, 2007, Kasina, 2007; Thapa, 2006; Stefan- Dewinter *et al.*, 2005).

2.3 Production trends and economic importance of passionfruit

Brazil is the world's main producer of passionfruit ahead of Ecuador, Hawaii, Australia, New Zealand and South Africa (Perry *et al.*, 1991). In Kenya, the industry is based on the purple type (HCDA, 2008). Table 1 shows an increasing production trend in Kenya from 2005 to 2007. The passionfruit industry in Kenya is worth US $ 24 million (HCDA, 2008). However, most of the produce is wasted through poor storage and post harvest handling techniques (Annon., 2008). A sizeable proportion is wasted due to poor quality especially the hard fruits that have no pulp (Annon., 2008). Approximately 1000 tons worth US $ 375,000 are exported annually, while the rest are utilized locally for processing or as fresh fruit (HCDA, 2008).

Table 2.1 Area under passionfruit cultivation and yields per province, 2005-2007 in Kenya

Province	Hectare (Ha)			Production (MT)			Value (Kshs'000')		
	2005	2006	2007	2005	2006	2007	2005	2006	2007
Eastern	550	1,098	1,311	7,425	14,823	16,388	222,750	444,690	491,625
Central	1,018	967	1,317	13,743	13,054	16,463	412,290	391,635	493,875
Western	218	211	235	2,725	2,638	2,938	81,750	79,125	88,125
Rift Valley	988	1,027	1,180	13,338	13,864	14,750	400,140	415,935	442,500
Nyanza	1,109	1,054	1,054	13,863	13,175	13,175	415,875	395,250	395,250
Coast	61	63	80	763	788	1,000	22,875	23,625	30,000
Total	**3,944**	**4,420**	**5,177**	**51,857**	**58,342**	**64,714**	**1,555,680**	**1,750,260**	**1,941,375**

Source: HCDA export figures rationalized, 2008.

2.4 Passionfruit production constraints in Kenya

Several constraints limit passionfruit production in Kenya. These are pests and diseases, high establishment costs, inadequate planting materials, drought, declining soil fertility, poor post harvest handling in decreasing order of importance. Important diseases of passionfruit are *caused by several fungi such as Fusarium* wilt, brown spot, crown rot, Phytophthora blight, *Septoria*, root rot and bacteriosis caused by *Xanthomonas campestris pv. passiflorae* (Amata *et al.*, 2009; Gesimba, 2008; Mbora *et al.*, 2008). Woodiness disease, caused by viruses such as *Passionfruit woodiness virus, Cowpea aphid borne mosaic virus, East asian passiflora virus, Passiflora Chlorosis virus* and the Carlavirus *Passiflora latent virus*, is economically significant (Moreira, 2008; Baker and Jones, 2007; Nascimento *et al.*, 2006). In Kenya, viruses causing woodiness, fungi of the genera *Fusarium, Alternaria passiflorae* and the bacterium, *Xanthomonas passiflorae* are among the major biotic factors that constrain passionfruit production (Amata *et al.*, 2009; Gesimba, 2008).

2. 5 Importance of viral diseases in passionfruit

Viral-induced crop damage is a significant problem in agricultural production especially in the developing countries where they hinder efforts of reducing poverty levels in communities (Garzo *et al.*, 2004; Rybicki and Pieterson, 1999; Waterworth and Hadidi, 1998). Productivity of passionfruit is reduced by infection with CABMV (Cerqueira-Silva *et al.*, 2008). Gioria *et al.* (2000) evaluated damage and yield loss of passionfruit by CABMV under greenhouse conditions and reported significant losses in crop yield. In the study passionfruit infected within the first two months of planting yielded 2.4 kg/plant (80% reduction) compared to 12 kg/plant for the plant infected at the eighth or ninth month after transplanting, bbesides reducing the orchard life from 36 months to 18months. Apart from

yield reduction, quality of the infected crop is affected (Annon., 2008). Losses from viral diseases depend largely on when plants become infected, the variety, virulence of the virus strain, and the environment (Mathews, 2002; Gioria *et al.*, 2000). Yield and quality loss of passionfruit due to viruses has not been estimated in Kenya.

2. 6 History of passionfruit woodiness disease

Passionfruit woodiness disease is considered an important disease due to its association with economic losses wherever passionfruit is cultivated (Junqueira *et al.*, 2003). Shukla and Ward (1988) reported that characterization of the causal agent of woodiness disease started in Australia, where the virus was named *Passion woodiness virus* (PWV). However, studies conducted over the last two decades have demonstrated that other potyviruses can cause woodiness in passionfruit. These are *Cowpea aphid borne mosaic virus* (CABMV) in Africa and Brazil (Nascimento *et al.*, 2006; Van Regenmortel *et al.*, 2000; Sithole-Niang *et al.*, 1996), *East Asian passiflora virus* (EAPV) in Asia (Iwai *et al.*, 2006) and *South African Passiflora Virus* (SAPV) in South Africa (Brand and Wechmar, 1993). Using molecular techniques to identify viruses in passionfruit tissue, Sithole-Niang *et al.* (1996), Nascimento *et al.* (2006) and Moreira (2008) suggested the renaming of PWV isolates found in South Africa and in Brazil as CABMV strains. The International Committee of Taxonomy for Viruses (ICTV) in 2009 accepted *South African passiflora virus* (SAPV) and *Sesame mosaic virus* (SeMV) as synonyms of CABMV (Carstens, 2009). *Cucumber mosaic virus* (CMV) and Tobacco necrovirus (TNV) are other viruses reported as co-infections, which enhance the severity of woodiness disease and its damage on passionfruits (Taylor and Kimble, 1964; Brand and Wechmar, 1993).

2.6.1. Other viruses that affect passionfruit

Other viruses that infect passionfruit but do not necessarily cause woodiness disease are *Passion fruit ring spot virus*, *Passionfruit latent carlavirus*, yellow mosaic tymovirus, *purple granadilla mosaic virus*, vein clearing rhabdovirus, *Tomato ring spot virus* and Maracuja mosaic tobamovirus (Manicom *et al.*, 2003; Inoue *et al.*, 1995; Wijs, 1974). *Passionfruit mottle potyvirus*, *Uganda passiflora virus*, *Passionfruit virus Y* and *Malaysian passiflora virus* are also known to infect passionfruit (Abdullah *et al.*, 2009; Ochwo-Ssemakula, 2004, Parry *et al.*, 2004; Chang, 1992). Similarly, *Soybean mosaic potyvirus* and *Passionfruit chlorosis virus* have been reported to infect Passiflora species (Baker and Jones, 2007; Castillo *et al.*, 2001). Several of the viruses named belong to the potyviridae family of plant infecting viruses which also affect many agricultural crops causing significant yield losses. Knowledge on the identity, incidence and distribution of viruses infecting passionfruit in Kenya is scarce. The impact of these viral pathogens on passionfruit yield and quality has not also been assessed.

2.6.2 Host range and symptomatology

Potyviruses such as PWV, CABMV, EAPV and SeMV have been reported to infect several plant families including legumes (Omatsu *et al.*, 2004; Nascimento *et al.*, 2004; Pappu *et al.* 1997). They affect some solanaceae species such as *Nicotiana benthamiana* used as a diagnostic plant for many viruses, passiflora species, chenopodiaceae and some cucurbitaceae species. They generally cause mosaics, mottles, hard (rugose) leaves and hard fruits that are misshaped and reduced in size hence the 'woodiness' term (Sithole-Niang *et al.*, 1996; Nascimento *et al.*, 2006; Iwai *et al.*, 2006). Symptom expression is however,

dependent on the conditions of the host, environment and the species/ strain of the virus and maybe obscured when the plants are healthy and vigorously growing (Trevisan *et al.*, 2006)

2. 7 Methods for detecting passionfruit viruses

Isolation of different strains of a virus can be achieved using various methods based on comparison of type and severity of symptoms on a range of test plants, by serology, immuno-electophoresis or by Enzyme-linked immunosorbent assays (ELISA) tests (Akinjogunla *et al.*, 2008; Guartelli *et al.* 1998). Following progress in biology, biochemistry and immunology, immunosorbent assays are now widely applied in the detection of many plant viruses because of their sensitivity. These include precipitation/agglutination tests and immunosorbent electron microscopy based on proteins (Hampton *et al.*, 1997). Other techniques are molecular diagnostics such as polymerase chain reaction, nucleic acid hybridization and DNA/RNA probes ((Duncan and Torrance, 1992; Hampton *et al.*, 1997).

Polymerase chain reaction (PCR) provides the most sensitive method of detecting a number of plant viruses including CABMV (Guartelli *et al.*, 1998). The method utilizes a pair of markers (synthetic oligonucleotide primers) each hybridizing to one strand of the dsDNA target with the pair spanning a region that multiplies exponentially (Mackay *et al.*, 2002). The hybridization primer is a substrate for DNA polymerase which creates a complimentary strand via sequence addition of deoxyribonucleic acid (Mackay *et al.*, 2002). The PCR process occurs in 3 cycles, dsDNA separation at >90°C, primer annealing at 48-75°Cand optional extension at 72-78°C (Mackay *et al.*, 2002). Comparing to ELISA, PCR is not

laborious and the method is 10^{x5} more sensitive to ELISA and is able to detect viruses in very low concentrations in plant samples (Gillapsie *et al.*, 2001).

While ELISA can be used to screen many samples, it relies on antibodies that have to be prepared from test animals such as rabbits (Shukla and Ward, 1988). Antibody production is expensive and the procedure takes a long period of time. Polymerase chain reaction on the other hand is sensitive but may not be able to distinguish between virus species and strains for cross reacting viruses such as those in the potyviridae family. This raises the need for sequencing to elucidate the genome (s) for the virus (es) concerned. In this study, some of these techniques were used in combination to confirm and identify viruses associated with passionfruit in Kenya. They include test plants biological assay, ELISA and PCR for screening samples collected in the field followed by sequencing.

2.8 Arthropods pests of passionfruit

Passionfruit is affected by several arthropods. Mites, fruit flies, soft scales, whiteflies, beetles, mealybugs, stinkbugs, thrips, leaf miners and aphids are some of the pests that are associated with passionfruit all over the world (Wyckhuys *et al.*, 2012, Wyckhuys *et al.*, 2010; Kitajima *et al.*, 2003; Dominiguez-Gil and McPheron, 1992; Chacon and Rojas, 1984). Mealybugs and mites are important during the dry period where the populations increase in large numbers making the plants weak and vulnerable to other infections. Stinkbugs and thrips are found on the flower and the young fruits. Their damage can create local hard parts on the fruit leading to misshaped and sometimes reduced fruit sizes (Shylesha and Rao, 2004). Aphids are not important for the direct damage they cause on passionfruit vines but

they are important as vectors of viral pathogens which cause diseases that are difficult to manage. The arthropod fauna associated with passionfruit in Kenya is not well known, only a few phytophagous pests and pollinators have been reported (Kasina, 2007; Alexandra-Maria *et al.*, 2007; Thapa, 2006). The information is necessary for developing pest management strategies.

2.8.1 Aphid vectors of passionfruit viruses

Nearly all plant viruses that cause extensive agricultural damage use specific vectors to spread them between hosts. Aphids are the main transport devices for plant virus spread (Brault *et al.*, 2010). They cause direct and indirect damage to a wide range of crops leading to considerable crop losses (Dedryver *et al*, 2010). Aphids transmit nearly two-thirds of all insect-transmitted plant viruses and about 30% of all known plant viruses (Brault *et al.*, 2010; Macdonald *et al.*, 2003). The aphid species are in the genera *Aphis*, *Myzus* and *Macrosiphum* (Murant *et al.*, 1988). *Myzus persicae* (Sulzer) and *Aphis gosypii* Glover transmit PWV and CABMV in the field in a non-persistent manner (Bashir *et al.*, 2002). As major vectors of plant viruses, aphids have evolved a number of transmission patterns, non-persistent, semi-persistent and persistent modes of transmission and non-persistent transmission is the most transient of the vector virus relationships (Brault *et al.*, 2010).

Efficiency of virus transmission varies among species and biotypes and some viruses need a helper factor to be acquired and transmitted (Ammar *et al.*, 1994). Helper components (HC) are virus-encoded amorphous molecules that accumulate within the cytoplasm of virus infected plant cells (Pirone and Blanc, 1996). Wang *et al.* (1998) illustrated how some aphid

vectors can only use a few specific HCs and how the presence of certain HCs will determine the ability of a vector to transmit a particular virus. On the other hand, population density of vectors, presence of inoculum and the period over which plants are exposed to insect vectors may influence plant infection (Difonzo *et al.*, 1997). There is limited iinformation on aphid species associated with passionfruit orchards in Kenya.

2.9 Virus disease epidemiology and vector relationships

2. 9. 1 Virus transmission in passionfruit

Viruses can be transmitted by mechanical means, pollen, seeds, fungi, nematodes, mites, and insects (Green and Kim, 1991). Known insect vectors of passionfruit viruses are beetles and aphids (Manicom *et al.*, 2003). *Brevipalpus* species of mites have been reported to transmit viruses (Manicom *et al.*, 2003). However, there is considerable specificity (Brunt *et al.*, 1996; Jones *et al.*, 2001). According to Baker (1974) aphids are vectors of viral pathogens infecting passionfruit in Kenya. The non-persistent mode of transmission and transient feeding behaviour makes aphids' efficient transmitters of potyviruses from one host to another (Omatsu *et al.*, 2004).

2. 9. 2 Virus-vector relationships

According to Raccah (1986), presence of inoculum and weather conditions are essential factors in virus transmission. Therefore, environmental factors and insect pest population dynamics are important to the understanding of the relationship between the virus and the vector. Weather conditions directly influence the rate at which viruses are spread until that level when there are no more plants to infect (100% infection). Thresh *et al.* (1998) indicated

that spread is most effective from the local inoculum, which already exists on a few plants, outwards to the nearest hosts; and that from the initial foci, spread can be either monocyclic or polycyclic where the upper limit is determined by host maturity, weather or lack of susceptible hosts.

The epidemiology of each virus varies with locality, time and is a factor of local source of innoculum, vector complex involved and how the presence of vectors synchronizes with phenology of the crop (Difonzo *et al.*, 1997). According to Irwin and Ruensink (1986) the severity and incidence of a vector-borne disease is determined by the interaction between the pathogen, host, environmental conditions and the behaviour of its vector. Seasonal contrasts in rainfall and temperature influence pest populations (Difonzo *et al.*, 1997). Rainfall provides adequate moisture for growth of plant hosts, but may also affect the success or failure of oviposition and egg development of the vector (Duffus, 1992). Aphid vector population development may also be influenced by the intensity of crop cultivation (Summers *et al.*, 2004).

Aphid species that have been implicated in the spread of passionfruit woodiness disease are known to occur in Kenya (Nyaga, 2008; Machangi, 2004). The aphids are reported to transmit passionfruit viruses to crops in a range of other countries like Australia, Brazil and Japan (Bashir *et al.*, 2002) and a similar situation is expected in Kenya. However, vector seasonality and ability to spread passionfruit virus diseases in Kenya, in space and time, is not known. Absence of data about aphid species and their behaviour in passionfruit orchards,

like the unknown identity of the viral pathogen(s) infecting the orchards, has affected development of appropriate management strategies for passionfruit viruses in Kenya.

2.10 Management of passionfruit virus diseases

Viral plant diseases have few or no direct means of control like in bacteria and fungal diseases. Two categories of methods for controlling plant viruses are adopted, that is the use of host plant resistance and the control of vector populations (Brault *et al.*, 2010).

2.10.1 Vector management

Vector management options include cultural practices like the use of mulch, manipulation of planting and population density of plants and inoculum source elimination or phytosanitation (Murphy *et al.*, 2009; Summers and Stapleton, 2002). Cultural and biological pest management tactics are used to control homopteran pests and mites to overcome pesticide resistance (Palumbo *et al.*, 2001). Being a perennial crop, passionfruit seems to have a natural way of sustaining predators like ants that are attracted to the sugar exuded at the base of the leaf nodes (Sema and Maiti, 2006). Aphids, such as *Aphis gosypii, Myzus persicae, Toxoptera citricidus, Uroleucon ambrosiae, A. cracivora, Brevicoryne brassicae* are major vectors of virus diseases on passionfruit (Brunt *et al.*, 1996; Inoue *et al.*, 1995). However, passionfruits are unsuitable hosts for colonization by aphids and most of the aphids are immigrating alates (Omatsu *et al.*, 2004; Novaes and Rezende, 2003).

Cultural practices that are the agronomic practices manipulated by farmers to make the environment unfavorable for pest development are used. Some of these cultural practices are

mulching and intercropping with nitrogen fixing cover crops like *Lablab purpureus* L. used to reduce pest infestation (Lima *et al.*, 2002). Beans and maize have been recommended for intercropping in the first year of the passionfruit cycle to manage weeds and aphids in Brazil (Lima *et al.*, 2002). Difonzo *et al.* (1997) and Muthomi *et al.* (2009) have demonstrated that border crops reduce aphid landing on Irish potato crop thereby reducing virus disease incidence. In Kenya, passionfruit orchards are intercropped with crops such as Artemisia, cucurbits, kale, potato and beans not for pest management but for efficient use of land that is limiting in size (KHDP, 2006). Use of mulches, particularly reflective mulches intended to reduce landing rates of flying insects such as aphids and to delay incidence of viruses has been extensively evaluated (Kumar and Poehling, 2006; Greer and Dole, 2003; Cradock *et al.*, 2001). Reflective mulches reduce the incidence of vector-borne virus diseases in crops by repelling the insects (Murphy *et al.*, 2009; Greer and Dole, 2003; Cradock *et al.*, 2001).

Removal of diseased plants from within a crop stand is a basic approach for controlling viral diseases in crops (Thresh *et al.*, 1998; Holt *et al.*, 1999; Jeger *et al.*, 2004). In Sri Lanka, management of passionfruit viruses is done by removal of infected plants as a way of reducing innoculum and this is enforced through the help of legislation. Other effective methods reported are debris removal from the field, disinfection of support trellises before re-use, crop quarantine measures executed for incoming seeds, crop rotation, the use of skimmed milk foliar sprays and application of antiviral agents like cytovirin (Green and Kim, 1991; Simmons, 1959). In Kenya, rogueing is not a popular practice among passionfruit farmers neither is debris removal, disinfection of support trellises or quarantine measures for incoming seeds/seedlings.

Pesticide application is another option for managing vectors that transmit passionfruit virus disease pathogens but vector population control using insecticides is difficult (Perring *et al.*, 1999; Satapathy, 1998). According to Shukla *et al.* (1994) insecticide use for vector control is unsuitable for a non-persistent, non-cumulative transmission. This is because the rapid acquisition of non persistent transmitted viruses and the behaviour of probing alates (vectors) offers an ideal combination for the spread of the disease before the vector dies (Thottappily, 1992). Use of the pesticides eliminates beneficial insects and also disturbs the vectors thereby increasing their activity which translates to increased rate of virus spread within the field (Cradock *et al.*, 2001). Concentrating on vector control alone is not always effective in reducing virus incidence for those that are transmitted in a non- persistent manner (Nault *et al.*, 2004).

2.10. 2 Host plant resistance

Cross- protection can be described as the resistance of a plant infected by a mild virus to infection by other severe strains of the same virus. This method has been used in Australia to protect passionfruit vines (Simmons, 1959). However, the mild virus strain from Australia failed to protect healthy plants against severe strains in Brazil (Novaes and Rezende, 2003). Synergistic effects between PWV and CMV were reported to hinder cross protection use (Pares *et al.*, 1985). Similarly, uneven distribution of the virus in plant tissues limited cross protection use for virus management in passionfruit plants (Novaes and Rezende, 2003).

Host plant resistance, deploys the plants ability to withstand infection and infestation, and is the most desirable method of protection against viral diseases. Breeding programmes however, take long to avail the desired cultivars for farmers. In Australia, planted

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passionfruit hybrids (flarvicarpa x purple) demonstrated tolerance for woodiness disease but mixed infections of CMV and PWV reportedly overcame this tolerance (Taylor and Kimble, 1964). Breeding work for resistance has and is taking place utilizing both conventional and molecular techniques in Brazil (Fonseca, 2008, Cerqueira-Silva *et al.*, 2008). Alfenas *et al.* (2005) and Trevisan *et al.* (2006) attempted genetic modification of passionfruit using CABMV nuclear inclusion protein b (NIb) and PWV coat protein (CP) genes with little success.

Currently, there are no passionfruit woodiness virus resistant varieties in the world. Resistant crop varieties are convenient and cost-effective as a control measure for farmers where the small scale producer cannot afford costly management practices (Nono-Womdim *et al.*, 2001; Yang and Speed, 2004). Nevertheless, it is known that virus resistance only slows down but does not prevent virus spread in vegetatively propagated crops such as passionfruit (Fargette and Vie, 1995). Passionfruit resistance to both virus and aphids may be of great contribution to the management of field spread of non-persistently transmitted viruses. While transgenic or traditionally improved virus resistant varieties may be promising, no single resistant line is a panacea for all localities. The performance of a resistant line is dependent on the strain of the virus to which it is exposed, the weather and soil conditions of the locality in which it is grown and the type of farming practice employed (Mathews, 2002). An integrated approach to virus and vector management is still the best and most sustainable solution.

2.10. 3 Integrated disease and vector management

Integrated vector management (IVM) is the application of a number of vector control options at the same time. Integrated vector management plays a significant role in the control of many viral diseases. Several vector management tools such as the use of pesticides, mineral oils, barriers, insect traps/ repellants have been evaluated (Simmons and Zitter, 1980; Palumbo *et al.*, 2001; Greer and Dole, 2003). All these do not eliminate the vectors completely. Even at very low densities, some vectors can be effective in transmitting viruses. Since no one method of control is likely to keep a crop free of vectors and virus infections, there is need for evaluating several management tactics combined in an integrated management strategy.

CHAPTER THREE

IDENTIFICATION AND DISTRIBUTION OF VIRUSES ASSOCIATED WITH PASSIONFRUIT IN MAJOR GROWING AREAS OF KENYA

Abstract

A survey on passionfruit virus diseases was conducted in major passionfruit growing areas in Kenya to determine the prevalence, incidence and severity in the field. Three hundred and forty symptomatic and asymptomatic leaf samples were collected in 2008 and 105 samples in 2010. The samples were assayed for the presence of viruses using virus specific polyclonal antisera for *Cucumber mosaic virus* (CMV), *Cowpea aphid-borne mosaic virus* (CABMV) and the broad spectrum potyvirus test. All fields (100%) visited had plants that were infected by viruses. Virus incidence was significantly ($p<0.05$) higher in the upper midlands compared to the lower highlands. There was a high incidence of virus symptoms (70-100%) particularly in Thika/Maragua and Meru areas. Serological tests revealed moderate incidence of viruses in 2008 and 2010, respectively. The viruses detected were CABMV, CMV and an unidentified potyvirus. There were variations in incidences of 4% to 70% which were observed and these depended on the virus and location. The least detected virus was CMV (4%-7%) while the most detected was CABMV (25% - 46%) in the samples collected in 2008 and 2010. The incidence of CABMV was highest in Embu (25%) and least in Uasin Gishu (>10%). Twenty five percent and 21% passionfruit leaf samples in 2008 and 2010 respectively were infected by the unidentified potyvirus. Only 6% and 4% of the samples collected in 2008 and 2010 were infected with CMV, respectively. The most common virus (CABMV) was confirmed by RT- PCR. This information is useful for developing a diagnostic tool and strategies for management of the viral diseases.

3.1 Introduction

Passionfruit (*Passiflora edulis* Sims) is one of the major fruit crops grown in Kenya. The extent of passionfruit cultivation is about 5000 ha whose annual production is 60,000 tons (HCDA, 2008). The crop demand is high in the domestic and export markets, where the crop is used for processing or as fresh fruit (Njuguna *et al.*, 2005). Viral diseases limit production of passionfruit and have been reported to reduce orchard age, yield and quality of fruits significantly (Abdullah *et al.*, 2009; Gioria *et al.*, 2000). Following promotion of passionfruit by the Government of Kenya (GOK) and the Germany Agriculture team (GTZ) through private sector development support in Agriculture (PSDA), passionfruit viral diseases have been reported as the second most important limitation after the fungal problems (MOA, 2008, Njuguna *et al.*, 2005). According to Njuguna *et al.* (2005) virus-like symptoms were reported in all passionfruit growing areas, with 70% farmers in Thika District reporting passionfruit woodiness disease complex as the most important constraint in production of passionfruit.

This complex was reported in Kenya as early as 1944 (Natrass, 1944). The viruses causing this complex are well documented in other parts of the world (Moreira, 2008; Iwai *et al.*, 2006; Mckern *et al.*, 1994). Based on the evaluation of the biological, serological and molecular properties, the viruses named as causative agents of woodiness complex include PWV (Taylor and Kimberley, 1964; Iwai *et al.*, 1997), *South African passiflora virus* (SAPV) (Brand and Wechmar, 1993), CABMV (Moreira, 2008, Nascimento *et al.*, 2006; Mckern *et al.*, 1994) and *East asian passiflora virus* (EAPV) (Iwai *et al.*, 2006). Besides the individual infections, Taylor and Kimble (1964) reported a severe form of PWD expressed as

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tip necrosis when PWV and CMV exist as a mixed infection in the crop. Following the initial epidemics of PWD in Australia in 1930's, techniques for diagnosis have improved hence the identification of the named viruses (Akinjonkula *et al.*, 2008; Cerqueira-Silva *et al.*, 2008; Adams *et al.*, 2005).

Despite reports of PWD complex in Kenya since 1944, few attempts have been made to identify the viruses responsible for its epidemics. Woodiness disease incidence and distribution have not been established and it is not known whether the virus (es) that cause 'woodiness' are more than one species or strain. With the expansion of passionfruit cultivation to different agroecological conditions and management, these virus diseases have affected production reducing the quality and yield of produce. The objectives of the study were to identify the viruses causing passionfruit woodiness epidemics, assess the spread of passionfruit virus diseases and estimate the severity in the main passionfruit growing districts.

3.2 Materials and methods

3.2.1 Passionfruit orchard selection for sample collection

Passionfruit orchards were surveyed in ten major growing locations in western and eastern Kenya in 2008 and 2010. These areas were Uasin Gishu, Trans Nzoia, Bungoma, Nakuru, Kiambu (Kabete and Gatundu), Maragua/ Thika/ Yatta, Nyeri, Kirinyaga, Embu and Meru. The locations of the survey were selected after consulting with agriculture extension staff of the Department of Agriculture in different districts and regions. The survey areas comprised administrative districts which were further divided into locations where clusters of

passionfruit farmers were found. These districts were located in two main agro ecological zones (AEZ) with farms located over 2000m above sea level (Lower highlands) and those below 2000 m (Upper midlands). The (AEZ) formed a major stratification of the farms from which the samples were picked. The selection of orchards was based on the criterion that the area typically represented passionfruit growing areas and that farmers had at least 100 plants aged between 6 months and 3 years. The orchards used for sampling were located along the rural access roads 3-5 km apart. Where the farms were located on both sides of the road, the two farms were sampled.

3.2.2 Sample collection, virus incidence and severity assessment

During September 2008 and April 2010, 100 passionfruit orchards were surveyed. On a 50 x 50m area, the orchards were examined diagonally at random and plants suspected to be diseased counted along the 2 diagonals. Disease incidence was determined by considering the ratio of the number of plants with symptoms to the number of plants examined expressed as a percentage (James, 1974). In all the fields visited, 1000 plants were examined and disease severity determined for viral like symptoms using the scale 1 to 5 where; 1= no symptoms, 2= mild symptoms, leaf distortion and stunting; 3 = moderate symptoms, leaf distortion and stunting; 4 = severe symptoms and 5 = very severe symptoms. Disease prevalence was estimated as the proportion of fields having disease symptoms expressed as a percentage of the total number of fields visited (James, 1974).

The suspected diseased plants showed mosaic symptoms, had rugose leaves, or were carrying small size hardened cracked fruits. Each sample was collected from a different plant targeting

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the first three expanding leaves from five different growing points of the vine. The collected samples were bagged, stored on ice and transported in cold boxes to the BecA- ILRI laboratories where they were refrigerated at -80°C to await processing.

3.2.3. Virus identification

3.2.3.1. Enzyme linked immunosorbent assays

Virus detection in passionfruit was performed with double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA) according to the general protocol described by Clarks and Adams (1977). The antisera were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) in Germany. The criteria for purchasing the antisera were the specific viruses reported to infect passionfruit in other parts of the world and the symptoms observed in the orchards over time. The polyclonal antibodies used included CMV (AS-0929), CABMV (AS-0417) and the broadspectrum test for potyviruses (AS-0573/1). In the test, all buffers were prepared according to the manufacturer's instructions. Passionfruit leaf samples were homogenized in extraction buffer at 300mg of leaf per 1 ml of extraction buffer (PBS-T containing 2% polyvinyl pyrolidone) using a pestle and mortar. Sample extracts (200μl per well) were incubated overnight at 4°C in microtitre plate wells (Nunc Maxisorb, Denmark) previously treated with 200μl of 1:1000 dilution of specific immunoglobulin G (CABMV-IgG and CMV-IgG) in carbonate coating buffer.

In the microtitre plates, positive and negative control tests were included. These were positive tests purchased from DSMZ, Germany (PV-0396, PV-929 and PV-0573 for CABMV, CMV and broadspectrum potyvirus test, respectively), negative buffer and healthy

plant extract controls. Each step of ELISA was followed by 4 hours incubation at 37°C. The plates were washed with a PBS-T washing buffer (8g NaCl, 0.2g KH_2PO_4, 1.15g Na_2HPO_4, 0.2g NaN_3, 0.2g KCl L-1 containing 0.05% Tween-20, pH 7.4) three times allowing an interval of 5 minutes between each wash. The plates were then treated with 200 µl/well of the relevant conjugate solution (IgG in Alkaline phosphatase) and incubated. After washing with PBST buffer from conjugate solution, p-nitrophenyl phosphate (p-NPP) substrate solution (10 mg p-NPP dissolved in 10ml substrate buffer) was added. The plates were incubated for 1 hour at room temperature and the absorbance determined using a microtitre plate reader (Titertek Multiskan MCC/340) at 405 nm. All samples were assayed in duplicate and results judged to be positive if the absorbance was greater than or equal to twice the average reading of the negative (healthy) controls.

The broad spectrum potyvirus test was slightly different from DAS ELISA as recommended by the manufacturer. The samples were extracted in coating buffer and after incubation, 2 % skimmed milk in PBS-T (blocking solution) was added to the plates and incubated at 37°C for 30mins. The blocking solution was removed and IgG in conjugate buffer at a dilution of 1:1000 added to the wells. After incubating for 4 hours at 37°C, the plates were washed and the Rabbit anti-mouse conjugate in alkaline phosphatase (RAM-AP), provided by the manufacturer, added. Incubation for 2 hours followed after which washing was done. Subsequently, the substrate was added and the absorbance judged similarly to the specific viruses. Some of the samples that were CMV, broad spectrum potyvirus and all CABMV ELISA positives were amplified and sequenced for virus identification.

The virus was isolated from leaf samples that tested CABMV ELISA positive and the sap mechanically inoculated on various plants. That is tomatoes, Tobacco (*Nicotiana tabacum* and *N. benthamiana*), Kales, Courgettes, Cowpea, Beans and Passionfruit. The symptomatic leaves from *N. benthamiana* were used to prepare sap that was used to inoculate clean passionfruit samples. In addition the symptomatic passionfruit samples were assayed, amplified and sequenced for CABMV virus presence.

3.2.3.2 Total RNA extraction and RT-PCR

The Total RNA was extracted from infected leaf tissue by the Phenol SDS extraction method and by means of a commercial kit, ZR RNA miniprep plant RNA extraction kit (Firmentas, Lithuania). Infected leaf material (300mg) was ground in liquid nitrogen and homogenized. Phenol and RNA extraction buffer (2% SDS, 0.1M Glycine, 10mM EDTA and 0.1M NaCl) at equal volumes of 500µl were added to the homogenized leaf material in a 2ml eppendorf tube. After vortexing for 10 s, the extract was centrifuged for 10 min in a refrigerated centrifuge at 13000 rpm. The upper supernatant was transferred to a new eppendorf tube containing 250µl phenol and 250µl chloroform: Isoamyl alcohol (24:1) on ice. The solution was vortexed and centrifuged for 5 min at 13000 rpm. This step was repeated twice and each time the supernant transferred into a new eppendorf tube with phenol and chloroform: isoamyl alcohol on ice.

The supernatant was later transferred into tubes containing 400µl Isomyl alcohol. The mixture was vortexed and centrifuged for 10 min. The RNA was precipitated by transferring the supernant into a new tube containing 40µl of 3 M Sodium acetate. To the mixture 800µl

of 96% ethanol were added and the tubes incubated at -80C for 20mins or overnight. The RNA was recovered by centrifuging the contents in the tube at 13000 rpm for 5 min and discarding the supernatant. This was followed by addition of 800µl of 80 % ethanol and centrifuging for 10 min. The supernatant was poured and the RNA pellet left to dry for 15 min at room temperature. The RNA was then dissolved in 50µl of double distilled water. The Total RNA amount and integrity was checked using 0.8% agarose gel electrophoresis stained with ethidium and a spectrophotometer (Nanodrop Thermo- Scientific, South Africa).

3.2.3.2.1 First strand (cDNA) synthesis

Specific primer pairs for each virus were synthesized to amplify the coat protein gene using sequences already deposited in the Genbank (Tables 3.1 and 3.2). The primers were synthesized by Bioneer Company, Korea and were used for first strand synthesis, PCR reactions and in sequencing the specific viruses. Using Moloney Murine Leukemia virus (MMLV) reverse transcription system (Promega, Madison, CA) and the manufacturers' protocol the complimentary strands (cDNA) were synthesized from 1-5µg of the total RNA used as templates for each sample. The relevant reverse primers (1µl of 2 pmol/µL) and 1µl MMLV Reverse Transcriptase (200 U) were added to a master mix containing 1µl dNTP (10 mM), 4 µl MMLV buffer (5x), 0.5µl DTT (0.1M) and 1µl RNAseOUT (40 U). The contents were incubated for 1 hour at 37°C after which the reaction was stopped at 70°C for 15 mins. The ready to use cDNA in a PCR system were stored at -20°C. The cDNA were further amplified in a polymerase chain reaction and the products viewed under an ultra violet illuminator and photographs taken.

Table 3.1 A list of nucleotide sequences of oligonucleotide primers used in molecular testing for diagnosis of specific viral and internal control genes

Primer	Sequence 5' to 3'	Expected product size
CABMV JF1	5'- TAGTTCTTCAGGACAACTAGTTCCACG -3'	
CABMV JR1	5'- AAGCCTTTACTGCCCATGCGTCAT -3'	567bp
CABMV JF2	5'- CACCAGAGCATCAAAGACACAGCTCA -3'	
CABMV JR2	5'- CAGTGTTCTCACTAGTTGTTGCCAC -3'	628bp
CABMV JF3	5'- CAGCTCAGTAAATGGTTTGAGGCCA -3'	
CABMV JR3	5'- TTCATTTGCGCTATTGCTTCCCTTGC -3'	453bp
CABMV F4	5' - CGCTCAAACCCATTGAAC - 3'	
CABMV R4	5' - TATTGCTTCCCTTGCTCTTTC -3'	221 bp
CMV JF1	5'- ATGGACAAATCTGAATCAACCAGTGC -3'	
CMV JR1	5'- TAAGCTGGATGGACAACCCGTTCAC -3'	1000bp
CMV JF2	5'- CCGCGTCGTGGTTCCCGCTCCGC -3'	
CMV JR2	5'- CACCGCATAGCGTTTAGTGACTTCAG -3'	500bp
CMV JF3	5' - GTCGTCCAACTATTAACCACCCAAC -3'	
CMV JR3	5'- AGATGTGGGAATGCGTTGGTGCTC -3'	500bp
M4D F	5'- GTTTTCCCAGTCACGACTCG – 3'	300bp
M4D R	5'- GGTAATAATAGTGGACAACC -3'	
PWV JF1	5' – GACAAGGACGTCATGCAGGC - 3'	
PWV JR1	5'- CATCACTTGCAGTGTGCCTTTCA -3'	800bp
PNADH F 1	5'- TGAGATATTGGGCCCTTATGGTATC- 3'	231bp
PNADH R 1	5'- TCCGGTCTTAACGCCCTTACTATA– 3'	
PRUB F1	5' -CACTGCAAATACTAGCTTGGCTCAT - 3'	226bp
PRUB R1	5' -CAGTAAATCAACAAAGCCCAAAGTG- 3'	

Table 3.2 A list of Genbank deposited virus sequences aligned and used to design the oligonucleotide primers used in RT-PCR assays

Virus name	origin	Accession number
CABMV-Br	Brazil	AF 241233
CABMV (SAPV)	South Africa	D 10053
CABMV- DF Brs	Brazil	DQ 397532
CABMV	Morocco	Y 18634
CABMV	Zimbabwe	AF 348210
CABMV- F101	Brazil	AY 433951
PWV	Australia	P 32574
PWV	Japan	D 85849
PWV	Taiwan	AF 208662
CMV	India	AF 281864
CMV	Japan	AF 103992
CMV	Korea	AB 369269
CMV	Australia	AF 198103
Rubisco		L01940
Rubisco		EF 590556

CABMV-*Cowpea aphid borne mosaic virus*, PWV-*Passionfruit woodiness virus*, CMV-*Cucumber mosaic virus*, SAPV- *South African pasiflora virus*

3.2.3.2.2 Polymerase Chain Reaction amplification of CABMV coat protein gene

Six samples for CMV, 6 samples for PWV and 48 for CABMV that reacted positive in ELISA were tested by RT-PCR technique using specific primers designed to amplify the coat protein gene (Table 3.1). The virus specific cDNA were .amplified by PCR with the use of *Taq* DNA polymerase (Promega, Madison, CA).   A 2µl cDNA sample was used for PCR amplification by adding 1µl each of the forward and reverse primers (10 pmol) specific to the viruses and   2.5 U of *Taq* DNA polymerase (0.25 µL) in a master mix. The master mix contained 5µl standard PCR buffer (10 xs), 3 ul $MgCl_2$ (25mM),   and 1µl dNTP (10mM) to synthesize the PCR product. The cycling parameters for the PCR reactions (25 ul) were 5 min of denaturing at 94°C followed by 35 cycles at 94°C for 30 s, 55°C for 45 s and 72°C for 45 s for CABMV coat protein gene and Rubisco, 5 min of denaturing at 94°C followed by 35 cycles at 94°C for 30 s, 48°C for 45 s and 72°C for 1 min for CMV and general potyvirus primers followed by final extension at 72°C for 7 minutes. Water, no cDNA and Rubisco controls were included. Cowpea aphid borne mosaic virus and Rubisco annealed at 55°C, while CMV and the general potyvirus annealed at 48°C.

3.2.3.2.3 Agarose gel electrophoresis

Standard agarose gel was used to analyze the PCR products. The preparation of the gel was done by mixing 1g of agarose and 100ml of 1x TBE buffer (89 mM Tris, 89mM boric acid and 2.5mM EDTA, pH8.3). The mixture was boiled using a microwave oven until the agarose dissolved completely in the buffer. The mixture was cooled and supplemented with 1µl ethidium bromide (0.5µg mL^{-1}) and subsequently poured onto a gel tray and a comb placed to create wells. The gel was later placed in an electrophoresis unit filled with 1x TBE

and the comb carefully removed. The nucleic acid containing samples were mixed with loading dye and nuclease free water in equal proportions of 3µl and carefully loaded onto each well. One well was preserved for loading the standard DNA marker (Firmentas, Lithuania) to estimate the sizes of the PCR fragments being analyzed. Electrophoresis was performed at 100 volts for 45-60 minutes. The nucleic acids were visualized under the ultra violet transilluminator and photographs taken.

3.2.3.2.4 Sequence analysis of RT-PCR products

The sequencing was done at BecA-ILRI laboratories sequencing unit. The PCR products (25µl) were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturers' protocol. After elution, 50µl products were saved in the tubes and preserved at -80°C for DNA estimation and sequencing. The DNA concentration in the purified PCR products was estimated using a spectrophotometer (Nanodrop, ThermoScientific, South Africa) and 2µl of each sample used for sequencing. Sequencing of the purified PCR product was done using the BigDye 3.1 terminator protocol modified after Sanger *et al.* (1977) procedure for sequencing in an automatic ABI 3130 DNA analyzer (Perkin Elmer, USA). Sequencing reactions consisted of 1µl purified PCR products, 4µl of BigDye terminator (PE Biosystems, CA), 4µl of 2.5x dilution buffer (containing 200 mM Tris HCl, 5 mM $MgCl_2$) and 6 pmol of reverse or forward primers specific to the virus being sequenced to a final reaction volume of 20µl. The PCR cycling parameters followed closely those of the PCR reactions during DNA fragment amplification. Excess dye terminators were removed using Armesia Pharmacia Autoseq G 50 columns according to the manufacturer's protocol. Included were the standard M13 primer and the pGEM T vector as

controls for the reaction. Data were analyzed using the ABI sequence analysis software. The sequences obtained were compared with other sequences available in the Genbank using Basic Local alignment Search Tool at the National Centre for Biotecnology information (Altschul *et al.*, 1997).

3.2.5 Statistical analysis

Data on disease prevalence, virus incidence and severity were subjected to one way Analysis of variance (ANOVA) using Genstat discovery edition software (2005) to test for significant differences. Mean comparisons of the incidence, severity were done using student t-test at 95% confidence level.

3.3 Results

3.3.1 Virus symptom distribution, severity and incidence

Characteristic symptoms of woodiness diseases of passionfruit were observed in all the fields in 2008 and 2010. These were leaf mosaic, leaf mottles with dark green blister like areas (localized and raised parts of the leaf) against a background of yellow leaf tissue. Leaves were often rugose, misshapen and reduced in size and in some cases had stunted growth accompanied by a bunchiness of hard textured terminal leaves. The fruits were small, hard and misshapen with abnormally thickened rinds, reduced pulp cavity and fruit pulp. Severely affected plants showed distorted and reduced size leaves, stunting and poor fruit development (Plates 3. 1 to 3.6).

Tomatoes, Courgettes, Tobacco (*N. tabacum*) and Kales did not develop symptoms after inoculation with plant extracts from symptomatic passionfruit samples. Cowpeas, beans,

passionfruit and *N. benthamiana* developed symptoms after inoculation. Further plant extracts from *N. benthamiana* inoculated onto clean passionfruit resulted in the development of mosaics and blister like symptoms characteristic of woodiness disease. The passionfruit leaf samples tested CABMV ELISA positive and had nucleic acid sequences amplifieand identifie as reported in Chapter 4 of this thesis.

Plate 3.1 Leaf distortion and puckering

Plate 3.2 Leaf mosaic on passionfruit leaf

Plate 3.3 Mild mosaic and leaf roll,
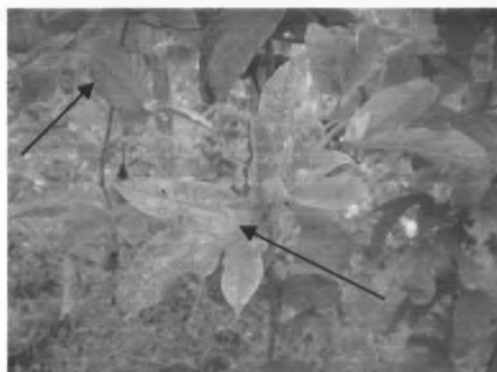
Plate 3.4 Mild vein banding and mosaic

Plate 3.5 Hard cracked misshapen fruits
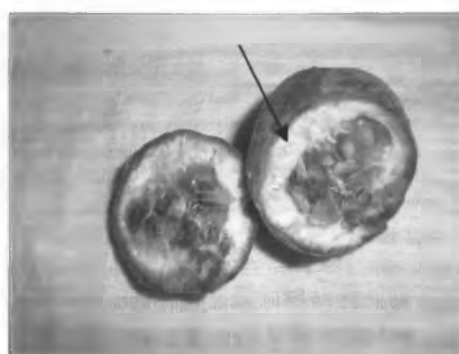
Plate 3.6 Thick rind and reduced pulp

All the surveyed fields showed symptoms of virus infection at different levels of severity. A range of management practices were observed. Farmers also practiced migration into new areas where passionfruit has not been planted before. For example, Kiplombe area in Uasin Gishu where passionfruit was being introduced had fields with no virus like symptoms. In all the growing areas, most farmers planted low growing crops within the orchards such as artemisia, kales, courgettes, pumpkins, beans and millet. In July to September 2008, every farm had plants with virus symptoms (100%), severity varied within a range of 2.3 to 3.7 while incidence based on visual observation ranged from 70-95% in the growing areas (Table 3.3). Virus symptom incidence was highest in Thika/Maragua area (95%) while symptom severity was intense in Uasin Gishu area (Table 3.3). A repeat in fewer localities in April 2010, including Nakuru which was not surveyed in 2008, revealed that all farms (100%) had plants with virus symptoms whose severity range was 2.3 to 3.55. The incidence range was 28 to 100% (Table 3.4). Virus symptom incidence was highest in Meru (100%) while the least was observed in Uasin Gishu (28%). Symptom severity was most intense in Meru (3.55) while the least intensity was observed in Uasin Gishu (2.37). There was a negative, non significant correlation between altitude and the visually observed virus incidence (- 0.902).

Table 3.3 Passionfruit virus symptom incidence, prevalence and severity in the major passionfruit growing areas in Kenya July to September, 2008

Location	No of samples	No. of fields	Prevalence (%)	Incidence (%)	Severity
Uasin Gishu (LH)	90	20	100	80	3.7
Trans Nzoia / Bungoma (LH)	50	15	100	80	2.6
Thika /Maragua (UM)	40	12	100	95	2.9
Nyeri (UM)	50	10	100	70	3.1
Kirinyaga (UM)	24	7	100	90	3.4
Embu (UM)	70	15	100	80	3.3
Meru (UM)	60	15	100	75	3.0
Gatundu (UM)	28	7	100	85	2.4

LH- low highland zone (>2000m), UM- Upper midland zone (< 1800m)

Table 3.4 Passionfruit virus symptom incidence, prevalence and severity in the major passionfruit growing areas in Kenya during April 2010

Location	No of samples	No. of fields	Prevalence (%)	Incidence (%)	Severity
Uasin Gishu (LH)	32	7	100	28	2.4
Nakuru (LH)	22	5	100	85	3.4
Embu (UM)	32	7	100	70.3	2.8
Meru (UM)	22	5	100	100	3.6

LH- low highland zone (>2000m), UM- Upper midland zone (< 1800m)

There was no significant difference in virus symptom severity between the Lower high land zones and upper highland zones. The upper midlands had high ($p<0.05$) viral symptom incidence compared to the lower highlands (Table 3.5).

Table 3.5 Virus symptom incidence and severity in the lower highlands and upper midlands agrocozones

Agro ecological zone	Incidence (%)	Severity
Lower highlands (LH)	50.5	2.8
Upper midlands (UM)	82.4	3.1
Mean	**66.5**	**3.0**
t-test ($p<0.05$)	- 4.83 *	- 1.87 (ns)
s.e	0.07	0.17

*Mean incidence and severity from the farms visited; *significance level at $p<0.05$*

3.3.2 Serology results

Based on ELISA, the relative frequencies of viruses infecting passionfruit are reported in Tables 3.6 and 3.7. In 2008, CMV was the least detected compared to the other viruses. Meru and Thika/Maragua areas had the highest mean virus incidence while Kirinyaga had the least. The highest incidence for CMV was in Embu and Meru while Uasin Gishu and Kirinyaga had no CMV detected in the leaf samples. There were no significant differences for the mean CMV and the unidentified potyvirus incidences between the districts (Table 3.6). However, there was a significant ($p< 0.01$) difference of CABMV incidence between the districts.

Thika/Maragua area had high CABMV incidence (30%) followed closely by Gatundu, Meru and Embu at slightly above 20%. No CABMV was detected in Trans Nzoia (Table 3.6). The unknown potyvirus had the highest incidence among the detected viruses (>20%) but the difference did not vary between the districts. The highest incidence was recorded in Uasin Gishu (41%) followed by Thika/ Maragua at (38%) and the least incidence was recorded in Embu (Table 3.6). Only two samples had CMV and CABMV detected together.

Table 3.6 Reaction of specific virus antibodies with passionfruit samples collected from major growing areas of Kenya in July to September 2008 (%)

Location	No. of samples	CMV	CABMV	Unknown potyvirus	Non-reactive
Uasin Gishu (LH)	90	0	7.1	41.4	51.5
Trans Nzoia / Bungoma (LH)	50	14	0	20	66.0
Thika / Maragua (UM)	40	2	30	38	30.0
Nyeri (UM)	50	2.5	2.5	22.5	72.5
Kirinyaga (UM)	24	0	4.2	12.5	83.3
Embu (UM)	70	20	22.5	7.5	50.0
Meru (UM)	60	20	27.5	20	32.5
Gatundu (UM)	28	0	28.6	17.9	53.5
Mean		**7.3**	**15.3**	**22.5**	
Significance		ns	*	ns	
Lsd p<0.05		21.2	18.1	38.6	

Siginificance level at p<0.05; ns: no significant difference

In 2010, CMV had the least incidence while CABMV had the highest incidence (46%). Meru area had the highest incidence of viruses followed closely by Embu and the least virus incidence was recorded in Uasin Gishu. Embu and Meru had high CABMV incidences while Nakuru and Meru had the highest unknown potyvirus incidence (Table 3.7). The unknown potyvirus incidence remained at the same level, which was slightly above 20% in 2008 and in 2010 (Tables 3.6 and 3.7).

Table 3.7 Reaction of specific antibodies with passionfruit samples collected from the major growing areas of Kenya in April 2010 (%)

Location	No. of samples	CMV	CABMV	Unknown potyvirus	Non-reactive
Uasin Gishu (LH)	32	10	23.3	13.3	53.4
Nakuru (LH)	22	0	22.7	27.3	50.0
Embu (UM)	32	0	70.0	16.7	13.3
Meru (UM)	22	4.5	68.1	27.3	0.0
Mean		3.6	46.1	21.2	
Significance		ns	*	ns	
Lsd p<0.05		18.2	19.1	35.6	

3.3.3 RT-PCR amplification and nucleotide sequence analysis of the CP gene

The presence of the three viruses CMV, CABMV and the unknown potyvirus in the ELISA positive samples was confirmed using the designed primers shown in Table 3.1. The passionfruit woodiness virus primers were also used to amplify PCR products in the positive broad spectrum potyvirus ELISA samples. The unknown potyvirus was amplified using general potyvirus primers designed by Pappu *et al.* (1993). The PCR products of expected sizes for CMV, PWV and general potyvirus of approximately 500, 800 and 1000 bp, respectively, were amplified. On sequencing, the nucleotide sequences obtained did not compare with plant virus sequences deposited in the Genbank.

The most abundant virus, CABMV, PCR products gave sequences that were comparable to CABMV sequences deposited in the Genbank (Plate 3.7). Subsequently, the primer pair that was most efficient was used to amplify all the samples that were CABMV ELISA positive (Plate 3.7). The primer used was CABMV F2 5'-caccagagcatcaaagacacagctca -3' and CABMV R3, 5'-ttcatttgcgctattgcttcccttgc -3' with an expected DNA fragment of size 626bp. Nucleotide sequence analysis of the amplified DNA fragments with Basic Local alignment

Search Tool (BLAST) revealed a high identity (86-98%) to the available CABMV sequences deposited in the Genbank. The accession numbers of the sequences are *South African passiflora virus* (SAPV) D10053, *Sesame mosaic virus* (SeMV) U90326, Brazilian CABMV isolates AY 433951 and DQ 397527. The obtained 42 sequences of CABMV were used to determine the similarity among the isolates from Kenya.
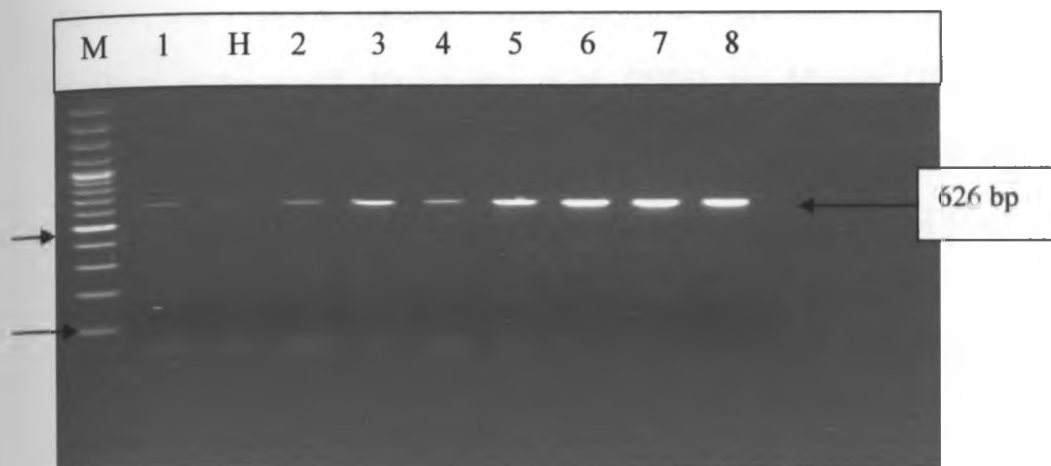
Plate 3.7 Cowpea aphid borne mosaic virus RT-PCR products (626 bp) from infected passionfruit leaf samples total RNA. Lane M – 100bp DNA marker; H –healthy control and lanes (1, 2 to 8) passionfruit leaf samples from Uasin Gishu (1, 2), Nakuru (3-5) and Embu (6-8).

3.4 Discussion

Viral diseases affect passionfruit in the field causing losses of quality and yield of fruits and degeneration of planting materials. Determination of the extent of occurrence of the virus complex infecting passionfruit orchards would enable the identification of the viruses present and the development of strategies to manage them. In this study, the occurrence and distribution of viruses infecting passionfruit in Kenya was determined.

The observed foliar symptoms were characteristic of passionfruit woodiness disease indicating the presence of the disease in major passionfruit growing areas. Serological assays

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confirmed the presence of CMV and CABMV infecting passionfruit in Kenya. The findings are consistent with the reports by Njuguna *et al.* (2005) who reported that viral diseases, particularly woodiness symptoms were a major limitation to passionfruit production. These results compare with those by Ochwo-Ssemakula (2004) in Uganda, where the occurrence of CABMV, PWV and a Ugandan novel potyvirus were reported infecting passionfruit. Elsewhere in the world, Nascimento *et al.* (2006) and Moreira (2008) have identified CABMV as the main pathogen causing passionfruit woodiness disease in Brazil. Woodiness disease severity is increased by mixed infections with CMV (Taylor and Kimble, 1964).

The presence of viruses in all the passionfruit growing areas indicates how widely the disease is distributed in the country. The high incidence of CABMV in Embu and Meru which stand at a lower altitude (< 2000m) compared to Uasin Gishu and Nakuru (> 2100m) suggests that the virus may be more serious in lower altitudes which have slightly higher temperatures that favour virus vector multiplication and survival. The negative correlation between virus symptom incidence and altitude indicates no contribution of altitude in the spread of the viruses. The moderate to high incidence could be due to the prevailing weather conditions that vary with altitude, seasons and with the perennial nature of the crop that encourages new infections. It may also be attributed to grafting as a method of propagation recommended by KARI and is especially used in Meru and Embu where grafted seedlings were adopted earlier than in Uasin Gishu. Machangi (2004) while monitoring aphid vectors for potato viruses reported that lower altitudes supported high vector populations that were responsible for higher virus incidence in such areas. According to Mathews (2002) abiotic factors play a role in disease development and spread.

Lack of a certification scheme for planting materials, recycling, free movement of infected material from one area to another, lack of a method to clean up the infected material in the field and establishing orchards near the old ones may have a role to play in disease spread and the high incidence which was observed in this study. According to Takaichi *et al.* (2001) garlic in Japan was infected by several viruses for lack of a certification program of planting material. Similar results were reported by Fajardo *et al.* (2001) and Mahmoud *et al.* (2007) for garlic in Brazil and Egypt, respectively. According to them, recycling of own seed and the perennial nature of the cloves was the reason for widespread occurrence of *Onion yellow dwarf virus* (OYDV) in garlic. Intensive cropping systems such as intercropping with crops that could be potential hosts of virus and vectors may be contributing to the prevalence and incidence of viruses infecting passionfruit in Kenya. There is a need to emphasize on local quarantine to minimize pathogen spread and disease incidences.

The RT-PCR test done on total RNA extracted from leaf samples collected from farmer's fields confirmed the presence of viral diseases in the major passionfruit growing areas in Kenya. One of the causal agents is CABMV which has a wide host range and vectors. This reveals a threat to the passionfruit industry reinforcing the need to control passionfruit woodiness disease.

The study has shown that viral diseases are present and widespread in passionfruit in Kenya. The viruses detected are CMV, CABMV and an unidentified potyvirus. Of these, CABMV is the most abundant and maybe associated with woodiness disease in Kenya. The information

will help in improving virus detection systems and control strategies. It is necessary to identify the unknown potyvirus and the role of these viruses in disease development.

CHAPTER FOUR

DIVERSITY OF THE COAT PROTEIN GENE AMONG KENYAN ISOLATES OF *COWPEA APHID BORNE MOSAIC VIRUS* (CABMV)

Abstract

Passionfruit woodiness symptoms are commonly observed in orchards throughout Kenya indicating the presence of viruses responsible for loss in yield and quality. This study aimed at understanding the diversity of the most frequent virus, CABMV infecting passionfruit. Specific primers designed for CABMV coat protein (CP) amplification were used and the resultant PCR products purified and sequenced. Comparison with sequences available at the Genbank database using BlastN showed that the passionfruit infecting virus was an isolate of CABMV, displaying an overall sequence identity of 88%-98% with *South African passiflora virus* (SAPV), *Sesame mosaic virus* (SeMV) and CABMV isolates from Brazil and Zimbabwe. A phylogenetic tree based on the partial CP gene generated using the neighbor-joining method with a 1000 boostrap replications indicated a close relationship between the Kenyan isolates. The Kenyan CABMV isolates were grouped together with SAPV, SeMV and UPV while PVY, used as an out-group, was alone in another group. However, Kenya CABMV isolates were more closely related to SAPV and SeMV than CABMV isolates from Brazil and the UPV from Uganda. The analysis revealed a high degree of similarity among the Kenyan CABMV isolates with a low degree of variation. The low variation may indicate potential strain differences that may need further study. These results have implications in the development of a PCR-based diagnostic system that is specific and sensitive for use in a virus free planting material certification programme and design of appropriate management strategies.

4.1 Introduction

Passionfruit (*Passiflora edulis* Sims) is an important horticultural crop in Kenya. It is grown by small scale farmers for employment and income generation. Its cultivation covers about 5,000 hectares with production concentrated in the high altitude areas above 1500m like Uasin Gishu, Nakuru, Meru and Embu areas (HCDA, 2008). Decline in passionfruit production is attributed to a great extent to woodiness disease, which is caused by PWV, CABMV, EAPV members of the family potyviridae and genus potyvirus (Iwai *et al.*, 2006; Nascimento *et al.*, 2006; McKern *et al.*, 1994; Taylor and Kimble, 1964). The disease reduces orchard life, yield and quality of fruits (Trevisan *et al.*, 2006). In Brazil and NewZealand, the losses are estimated at 80% (Trevisan *et al.*, 2006). The loss of passionfruit due to viral diseases has not been estimated here in Kenya.

Natras (1944) reported the presence of woodiness disease in passionfruit growing highlands, Sotik and Kisii in Kenya. Since then, passionfruit woodiness disease has become important following the promotion of passionfruit production as a diversification venture and the use of grafted seedlings to reduce the incidence of fusarium wilt (Njuguna *et al.*, 2005). The disease is more pronounced in the highlands where rainfall amounts are high and the purple cultivar is predominant (Njuguna *et al.*, 2005). In chapter three of this thesis, a study to identify the causal agent of passiofruit woodiness disease and to determine the incidence of virus diseases, revealed that CABMV was the most abundant virus affecting passionfruit in Kenya. Continued cultivation has resulted in variations of expressed symptoms of virus diseases in Kenya and woodiness disease has become critical. As a result some farmers have abandoned passionfruit production and others continue to migrate into new areas as a strategy to reduce

losses due to the passionfruit viral diseases (Gachanja and Ochieng, 1988; Gesimba, 2008). Since CABMV is one of the common viruses identified, it was necessary to study its diversity in order to gain knowledge and inform the process of developing a diagnostic tool and strategies for the management of passionfruit virus diseases. The study was undertaken to analyze the coat protein gene of the isolates from Kenya in comparison with other CABMV isolates reported and whose sequences are deposited in the Genbank. The information gathered would be used for the development of a diagnostic system (PCR-based) for detection of viruses to differentiate diseased and healthy passionfruit plants and for the vector and pathogen derived resistance (PDR) breeding.

4.2 Materials and methods

4.2.1 Virus isolates

The CABMV isolates used in chapter three of this thesis for identification were amplified through RT-PCR using specific CABMV primer pair designed to amplify the CP region of the virus. The primer pair was CABMV F2, 5'-caccagagcatcaaagacacagctca -3' and CABMV R3, 5'-ttcatttgcgctattgcttcccttgc -3' with an expected DNA fragment of size 626 bp. The PCR products were purified using Qiaquick PCR kit (Qiagen, United Kingdom) and subsequently sequenced in both directions using the same pair of primer following the method described in chapter three sections 3.2.3 of this thesis. The 42 sequences obtained were used for this diversity study.

4.2.2. Sequence analysis of CABMV isolates from Kenya

The National Centre for Biotechnology and information ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/) was used to find the open reading frame (ORF) in the 42 CABMV CP sequences obtained. These sequences were aligned and compared with the corresponding region of specific viruses available in the Genbank. Percent sequence identity was determined using Bioedit program ver 5.09. Pairwise comparison and multiple alignments were done using CLUSTALW (Thompson *et al.*, 1994) and MegAlign program version 4 from DNASTAR package (DNASTAR, USA). These sequences were compared with other virus isolates whose sequences have been deposited with the Genbank including PVY as an out-group virus. The Genbank virus accession numbers for the isolates used for comparison are shown in Table 4.1. A neighbour-joining method was used to construct a phylogenetic tree based on partial CABMV CP gene amplified with 1000 boostrap replications using MEGA 4 and pairwise evolutionary distances calculated with maximum likelihood nucleotide substitution model (Saitou and Nei, 1987, Tamura *et al.*, 2004). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Disparity index, Tajima's and Fischer's neutralty test statistics were calculated for sequence comparison.

4.3 Results

4.3.1. RT-PCR analysis of CABMV isolates from Kenya

Reverse transcription polymerase chain reaction products were successfully amplified from Total RNA from infected passionfruit leaf tissues using specific oligonucleotide primers pairs for CABMV and Rubisco, a housekeeping gene. Plate 4.1 shows PCR product bands

obtained from CABMV infected passionfruit leaf tissues with an expected size 626bp. Plate 4.2 shows PCR product bands from CABMV infected passionfruit leaf tissues and those from Rubisco (expected size 230bp) used as an internal house keeping gene . All samples used had a Rubisco DNA band amplified thus confirming the presence of RNA in all the tested samples. Subsequent sequencing yielded coding sequences of the capsid protein of CABMV which compared to those of the GenBank NCBI database.
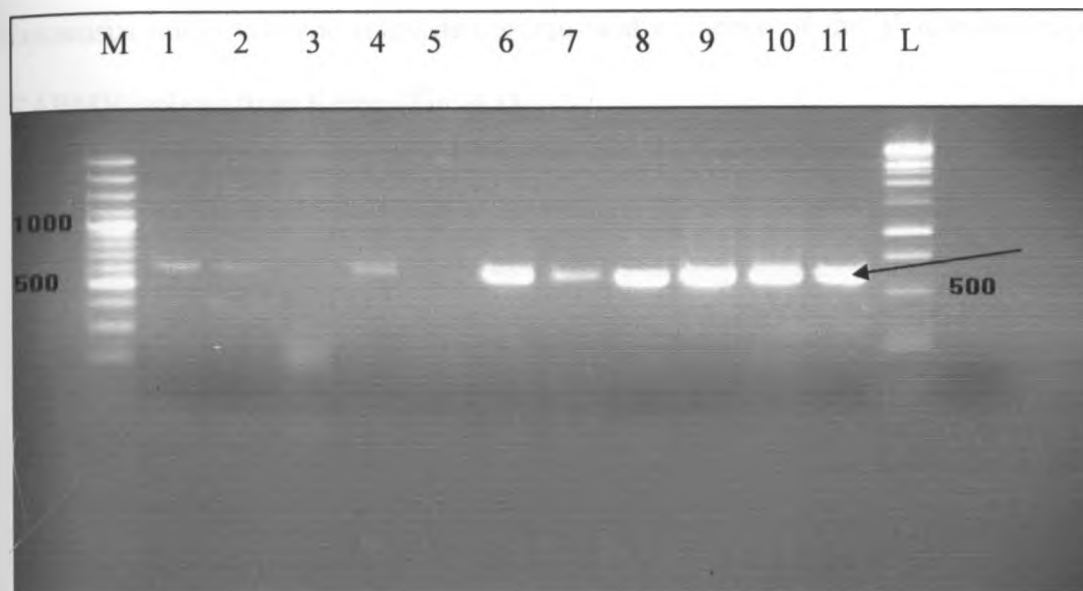
Plate 4.1 RT-PCR amplified products of CABMV/CP using a specific primer pair. The expected product was 626 bp as indicated by arrow. Lanes 1-4 and 6-11 are suspected passionfruit samples, Lane 5 healthy plant sample and M- the DNA marker in 100bp
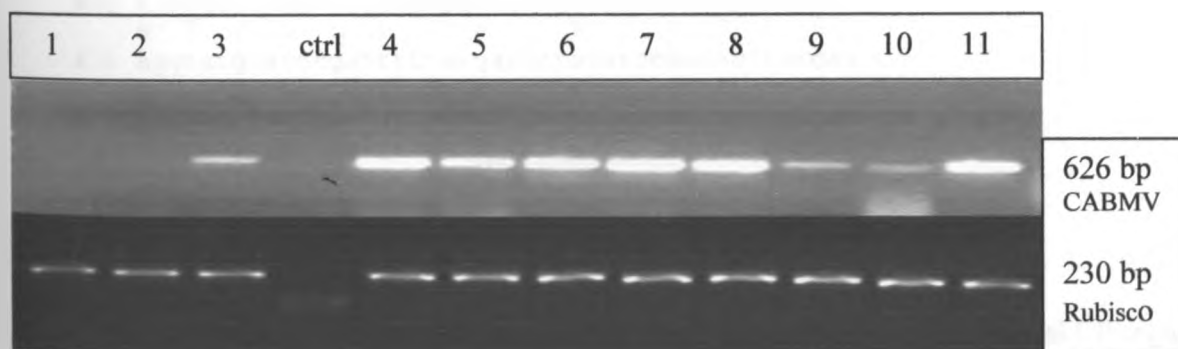
Plate 4.2 RT-PCR amplified products of CABMV/CP and Rubisco gene from Total RNA extracted from suspected passionfruit samples. Lanes 1-3 and 4-11 are suspected passionfruit leaf samples, Lane 5- negative control without cDNA

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4.3.2 CABMV diversity and coat protein sequence analysis

Polymerase chain reaction resulted in the amplification of a DNA fragment of approximately 606 Kb length for all the amplified samples. The coat protein sequences had an approximate length of 800 nucleotides representing a single open reading frame (ORF). On the basis of sequence relationships with other potyviruses, the product encoded partial coding sequence of 200 ammino acids for the coat protein gene. The coding sequence is represented by a consensus nucleotide and translated amino acid sequence of the 3' terminal region of the CABMV isolates from Kenya (Fig. 4.1)

```
 28  ctgcaaaagatcagcaagaagatgaatcttcccatggttgctggc
     L  Q  K  I  S  K  K  M  N  L  P  M  V  A  G
 73  aagattattcttaatgtggatcatttaatagagtataaaccagca
     K  I  I  L  N  V  D  H  L  I  E  Y  K  P  A
118  cagagtgatttgttcaacacaagggcatcaaagacacagtttaat
     Q  S  D  L  F  N  T  R  A  S  K  T  Q  F  N
163  aaatggtttgaggctatcaaagaggaatatgagttggatgatgat
     K  W  F  E  A  I  K  E  E  Y  E  L  D  D  D
208  aagatgggtgtggttatgaatggttttatggtttggtgcattgag
     K  M  G  V  V  M  N  G  F  M  V  W  C  I  E
253  aatggaacctcacccgatgtgaatggagtgtggaagatgatggat
     N  G  T  S  P  D  V  N  G  V  W  K  M  M  D
298  ggggatgaacaggttgaattcccactgaagcccattgtagagaat
     G  D  E  Q  V  E  F  P  L  K  P  I  V  E  N
343  gcaaaacccacactcagacaaattatgcaccacttgtcagacgca
     A  K  P  T  L  R  Q  I  M  H  H  L  S  D  A
388  gctgaagcgtatattgagatgagaaattccgaagggttctacatg
     A  E  A  Y  I  E  M  R  N  S  E  G  F  Y  M
433  tccaggtatggattactgaggaatttgagggacaagagcttggca
     S  R  Y  G  L  L  R  N  L  R  D  K  S  L  A
478  aggtatgcatttgatttttatgaagttacatccaaaacttatgaa
     R  Y  A  F  D  F  Y  E  V  T  S  K  T  Y  E
523  agagcaagagaagcaatagcacaaatgaaggccgcagctctcgcc
     R  A  R  E  A  I  A  Q  M  K  A  A  A  L  A
568  aacgttaacaccaggatgtttggcctggatggcaacgtggcaaca
     N  V  N  T  R  M  F  G  L  D  G  N  V  A  T
613  actagtgag 621
     T  S  E
```

Fig 4.1 Consensus nucleotide and deduced amino acid sequence of the 3' terminal CP region of the CABMV isolates from Kenya

All Kenyan CABMV isolates shared a high degree of sequence identity when compared to each other (82% to 100%) except for isolates 27, 50 and 64 which displayed differences at the amino acid level to a low of 62% and at nucleotide level a low of 32%, respectively (Tables 4.1a and 4.1b). Multiple alignments of amino acid sequence of the coat protein of the 42 CABMV isolates from Kenya showed a high level of similarity in the CP gene. Sequence comparison with other viruses whose sequences are available in the Genbank indicated a high degree of homology. In particular, the virus was most closely related to CABMV where the nucleotide sequence identity ranged from 86% to 98% with the isolates reported from South Africa (SAPV), Georgia (SeMV), Brazil (CABMV-Br Jgr, CABMV-F101) and Zimbabwe (CABMV-Z). Comparing the CABMV isolates from Kenya and other potyviruses such as the *Potato Virus Y* (AB 270705), *Onion Yellow Dwarf Virus* (AJ 293278), *Dasheen mosaic virus* (NC_003537) and *Sweet potato feathery mottle virus* (AJ 010702), the sequence identity was 79%, 80%, 70% and 84%, respectively indicating a difference in similarity or distance in relationship between the viruses and the isolates.

Table 4.1a *Percent identities between the different isolates of CABMV from Kenya; Coat protein amino acid sequence below diagonal and coat protein nucleotide above diagonal*

Isolate	47	14	15	27	32	33	39	50	56	57	58	63	64	65	66	68	69	70	71	73	74
47		86	86	73	94	96	94	97	86	85	86	86	86	85	85	86	85	85	86	85	86
14	88		98	70	79	89	87	89	98	98	98	97	97	98	98	97	97	97	98	97	98
15	89	96		62	75	88	87	87	98	98	98	97	97	98	98	97	97	97	98	98	98
27	83	83	70		72	73	74	72	76	75	75	83	83	85	84	86	83	84	86	84	86
32	88	92	79	83		98	96	96	83	83	83	87	87	87	87	87	87	87	87	87	87
33	91	95	95	93	98		98	98	89	88	89	88	89	88	89	88	88	88	89	88	88
39	94	92	92	89	93	95		98	87	86	87	87	87	86	86	87	86	86	86	86	86
50	89	81	71	86	87	91	92		87	87	87	87	87	86	86	87	87	87	87	86	87
56	88	98	96	89	92	96	91	85		99	100	99	98	99	99	98	98	98	99	98	99
57	88	99	96	89	92	95	91	85	100		99	98	97	98	98	98	98	97	99	98	94
58	88	98	96	89	92	96	91	85	100	100		99	98	99	99	99	98	98	99	99	99
63	78	90	89	83	87	86	81	79	92	91	92		99	82	99	99	99	99	99	99	99
64	68	70	58	36	60	76	72	32	76	76	76	79		99	99	99	99	99	99	98	99
65	81	90	89	97	95	89	85	89	92	91	92	82	70		99	99	99	99	99	99	99
66	88	97	96	97	95	96	92	89	99	98	99	89	80	93		98	99	99	99	98	99
68	82	90	89	97	95	90	86	89	92	91	92	83	70	98	93		98	99	99	98	99
69	82	90	89	91	95	89	85	89	92	91	92	86	70	99	93	97		99	99	98	99
70	82	90	89	97	95	90	86	89	92	91	92	83	70	100	93	98	98		99	99	100
71	87	97	96	97	95	96	91	89	99	98	99	88	80	92	100	93	93	93		99	99
73	88	97	96	97	95	96	92	89	99	98	99	89	80	93	100	93	93	93	100		99
74	81	90	89	97	95	89	84	89	92	91	92	82	70	99	93	98	99	100	92	93	

Samples 14-27 from Uasin Gishu; 32-50 from Nakuru; 63-74 from Embu; 27, 50 and 64 are different displaying differences at the amino acid level to a low of 32% and nucleotide level to a low of 62%, respectively.

Table 4.1b Percent identities between the different isolates of CABMV from Kenya; coat protein amino acid sequence below diagonal and coat protein nucleotide above diagonal

Isolate	47	76	80	86	87	88	89	90	91	92	93	94	95	96	98	100	103	104	105	106	108
47		86	85	85	86	86	86	85	85	86	85	85	85	86	85	85	85	86	86	86	85
76	87		98	98	98	99	98	99	99	98	99	99	98	99	97	98	98	97	97	99	98
80	76	90		98	98	98	97	98	98	98	99	98	99	98	97	99	97	97	97	99	99
86	87	95	92		98	98	98	98	98	98	98	97	98	99	98	99	99	99	99	98	98
87	87	93	89	99		99	99	99	99	99	98	98	98	98	99	99	99	98	99	98	98
88	88	99	86	99	99		99	99	99	99	99	99	99	98	98	99	99	99	99	98	99
89	84	84	86	89	97	96		97	97	99	98	97	98	98	97	99	98	98	98	99	97
90	86	94	91	100	99	99	92		99	99	99	100	100	99	92	99	92	92	92	99	92
91	87	93	89	99	100	99	97	99		98	99	99	99	98	92	99	92	92	92	99	93
92	86	99	88	94	91	98	92	94	91		98	98	99	99	98	99	98	99	97	98	98
93	88	98	87	98	99	99	96	98	99	98		99	99	98	98	99	98	97	98	99	99
94	88	94	89	99	100	99	97	99	100	92	99		99	98	97	99	97	98	98	99	98
95	88	100	89	100	92	99	89	100	92	99	98	93		98	98	99	98	98	98	99	98
96	88	92	88	92	87	95	89	100	87	92	94	89	95		98	99	99	98	98	98	98
98	83	83	87	88	95	96	82	84	95	82	94	95	88	81		99	99	98	98	98	98
100	81	93	84	99	100	92	97	99	100	91	93	98	92	87	95		99	99	99	99	99
103	88	91	89	91	92	100	89	90	92	91	98	93	92	87	90	92		99	99	98	98
104	88	91	89	91	92	100	89	90	92	91	98	93	92	87	90	92	100		98	98	98
105	88	91	89	91	92	88	89	90	92	91	98	93	92	87	90	92	100	100		98	98
106	82	93	87	96	96	92	97	99	100	91	93	98	87	87	87	100	92	92	92		99
108	88	91	89	91	92	100	89	90	92	91	98	93	92	87	90	92	100	100	100	92	

47 from Nakuru; 76-80 and 103 from Embu; 86-106 and 108 from Meru; a lot of planting material changes hands between these areas, and the isolates show a high identity level above 80% at the nucleotide level and above 85 % at the amino acid level, indicating the same virus strain

Gene diversity for the 42 sequences was analyzed and the following observed. The average nucleotide composition for the sequences analyzed was 24% Thymine, 19% Cytosine, 32% Arginine and 25% Guanine. While testing for the homogeneity of substitution patterns between sequences, Disparity index that judges differences of base pair composition bias between sequences was 0. This indicates the probability that the sequences have evolved with the same pattern of substitution. Results from Fisher's exact test of neutrality for sequence pairs testing the hypothesis of strict-neutrality in favor of the alternative hypothesis of positive selection was accepted for all the sequence pairs except for sequence 14 pairing with 22 other sequences where $p < 0.05$, disproving the hypothesis of strict- neutrality while accepting the alternative for positive selection of sites frequency spectrum (Table 4.2). Tajima's neutrality test had a negative value while the nucleotide diversity was 0.164 indicating long-term purifying selection or a selective sweep that may have occurred when too many rare polymorphisms were taking place in the population (Table 4.3).

Phylogenetic analysis, based on CP sequence, grouped together all the isolates from Kenya and other CABMV isolates from the Genbank including the Uganda Passiflora virus confirming the relationship with CABMV. Potato virus Y used as an out-group virus was placed alone in another group (Fig 4.2). Among the isolates from Kenya, some were more closely related to each other. The CABMV isolates displayed five subgroups within the major clustering group (Fig 4.2).

Table 4.2 Synonymous and non synonymous differences between sequence 14 and other sequences using Fischers exact test for neutrality

Sequence 1	Sequence 2	P- value
66_CABMV	88_CABMV	1.000
57_CABMV	14_CABMV	0.033
58_CABMV	14_CABMV	0.033
98_CABMV	14_CABMV	0.021
102_CABMV	14_CABMV	0.050
103_CABMV	14_CABMV	0.386
104_CABMV	14_CABMV	0.050
105_CABMV	14_CABMV	0.050
108_CABMV	14_CABMV	0.038
80_CABMV	14_CABMV	0.056
105_CABMV	14_CABMV	0.033
106_CABMV	14_CABMV	0.056
63_CABMV	14_CABMV	0.033
64_CABMV	14_CABMV	0.033
65_CABMV	14_CABMV	0.033
68_CABMV	14_CABMV	0.056
70_CABMV	14_CABMV	0.033
71_CABMV	14_CABMV	0.033
74_CABMV	14_CABMV	0.033
75_CABMV	14_CABMV	0.021
76_CABMV	14_CABMV	0.038
86_CABMV	14_CABMV	0.033
87_CABMV	14_CABMV	0.033
89_CABMV	14_CABMV	0.050
92_CABMV	14_CABMV	0.050
93_CABMV	14_CABMV	0.038
94_CABMV	14_CABMV	0.050
95_CABMV	14_CABMV	0.038
96_CABMV	14_CABMV	0.033
73_CABMV	14_CABMV	0.033
33_CABMV	14_CABMV	0.053
66_CABMV	14_CABMV	0.013
88_CABMV	14_CABMV	0.038
57_CABMV	15_CABMV	0.549
58_CABMV	15_CABMV	1.000
98_CABMV	15_CABMV	0.549
102_CABMV	15_CABMV	0.549
103_CABMV	15_CABMV	1.000
104_CABMV	15_CABMV	0.549
105_CABMV	15_CABMV	0.549
108_CABMV	15_CABMV	0.496
80_CABMV	15_CABMV	0.442
14_CABMV	56_CABMV	0.033

P values smaller than 0.05 are considered significant at the 5% level and are highlighted. The numbers of synonymous and nonsynonymous differences between sequences were estimated using the Nei-Gojobori method (Nei and Gojobori, 1986). The analysis involved 39 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 151 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2007)

Table 4.3 Kenyan CABMV isolate sequence diversity index using Tajima's Neutrality Test

m	S	ps	T	p	D
39	542	0.981550	0.232160	0.164530	-1.095361

Abbreviations: m = number of sequences, S = Number of segregating sites, ps = S/m, T = ps/a1, p = nucleotide diversity, and D is the Tajima test statistic (Nei and Kumar, 2000). NOTE: The analysis involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 542 positions in the final dataset. Evolutionary analyses were conducted in MEGA 4 (Tamura *et al.*, 2007)

Further comparison of the isolates deduced amino acid sequences showed that all contained WCIEN, QMKAAA motifs and NTGS the N-glycosylation motif (Fig 4.3 and 4.4). The isolates had a gap (deletion) of 11 ammino acids in the CP region (between 101 and119 positions) similar to that of SAPV, SeMV and CABMV isolates from Brazil and Zimbabwe (Figs 4.2 and 4.3). Two sequences (63 and 80) had an insertion instead in the same position.
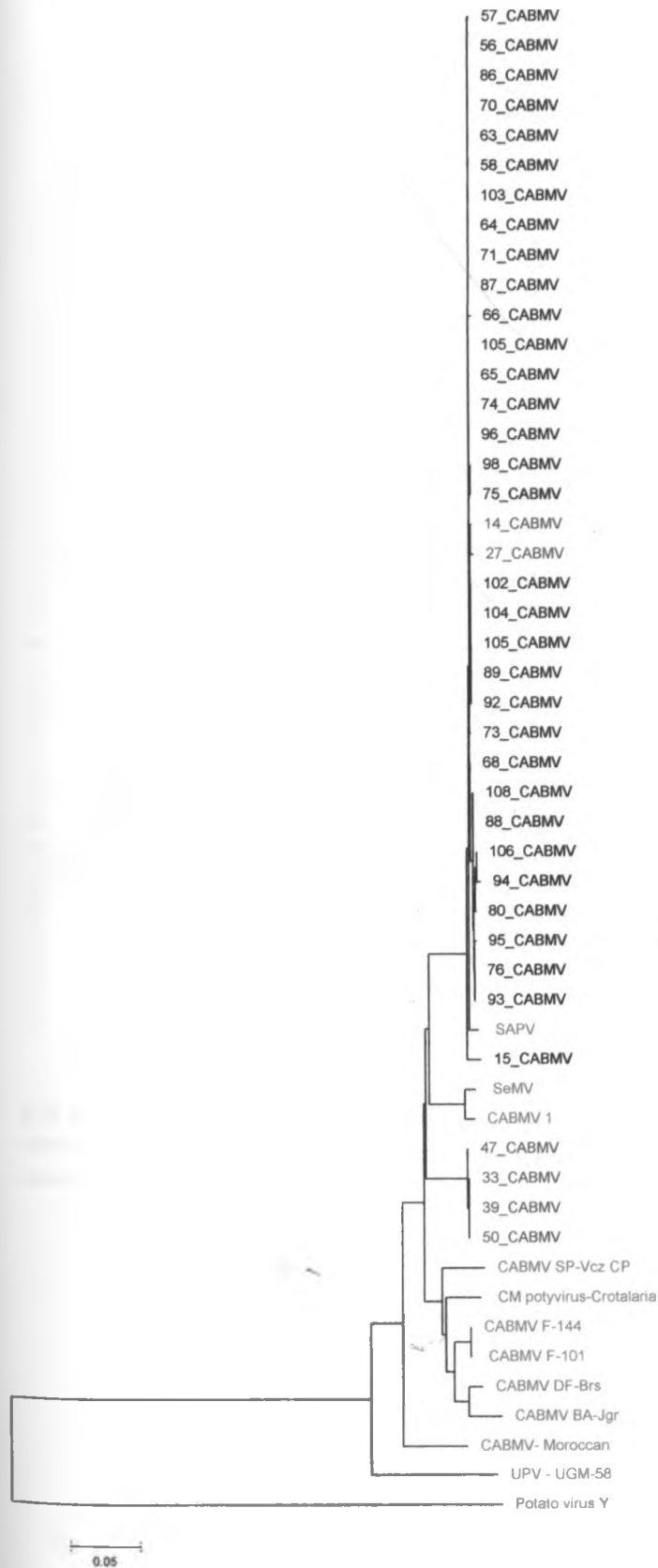
Fig 4.2 A phylogenetic tree of CABMV based on the partial coat protein sequences. The evolutionary tree was inferred using the neighbour joining method (Saitou and Nei, 1987).The distance is in units of the number of base substitution per site. Red and Black represents Kenyan isolates with the red ones from areas >2000m while black ones were from areas < 2000m. Green represents other CABMV isolates and UPV obtained from the Genbank. Potato Virus Y was used as an out-group for comparison.

```
80   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVNFHLSPSWRTQNP-FDRLCTIFQTQ
63   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVNFHLSPSWRTQNPHFDRLCTIFQTQ
14   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
15   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
103  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
105  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
108  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
104  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
57   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
56   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
58   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
65   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
86   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
69   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
89*  1  KMGVIMNGFMVWCMK-----------------KVEFPLKPIVENAKP-----------T
106  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
74   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
70   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
100  1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
91   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
27   1  KMGVIMNGFMVWCIENGTSPDVNGVWNMMDGDEQVEFPLKPIVENAKP-----------P
75   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
68   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
87   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
94   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
98   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
47   1  KMGVVMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPMVENAKP-----------T
50   1  KMGVVMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
39   1  KMGVVMNGFMVWCIENGTSPDVNGVWKMMDGDEQVEFPLKPIVENAKP-----------T
33   1  KMGVVMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
32   1  KMGVVMNGFMVWCIENGTSPDV-----MMDGDEQVEFPLKPIVENAKP-----------T
73   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
93   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
66   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
90   1  KMGVIMNGFMVWCIE------------MMDGDEQVEFPLKPIVENAKP-----------T
96   1  KMGVIMNGFMVWCIE------------MMDGDEQVEFPLKPIVENAKP-----------T
71   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
76   1  -MGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
92   1  -MGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
95   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
88   1  KMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP-----------T
```

Fig 4.3 Multiple alignment of deduced amino acid sequences of the partial capsid protein amplified from passionfruit leaf samples Kenya. A conspicuous gap is common to all except two isolates 80 and 63

```
64              44 SRRS---------------MNWMMTR---------WE-------------------------------------
80              44 IKEEYELDDDKMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVNFHLSPSWRTQNPFDRLCTIFQTQLKR
47              51 IKEEYELDDDKMGVVMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPMVENAKP----------TLRQ
gi|1899218SeMV  161 VKEEYELDDDKMSVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP----------TLRQ
gi|1542875Zimb  158 VKEEYELDDDKMSVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP----------TLRQ
gi|222583SAPV   161 IKEEYELDDDKMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP----------TLRQ
gi|89145895BAJg 104 IKGEYELDDDKMGVIMNGFMVWCIENGTSPDVNGCGTMMDGDEQVEFPLKPIVENAKP----------TLRQ
gi|38046104DFBr 104 IKEEYELDEDKMGVIMNGFMVWCIENGTSPDVNGVWTMMDGDEQVEFPLKPIVENAKP----------TLRQ
consensus       161 ikeeyeldddkmgvimngfMvWciengtspdvngvwtmmdgdeqvefplkpivenakp          tlrq


64               64 AEAYIEMRNSEGFYMPRYGLLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVKTRMFGLDGN
80              124 AEAYIEMRNSEGFYMPRYGLLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVKTRMFGLDGK
47              121 AEAYIEMRNSEGFSLSRYGFLRNLRDKSLARYAFDFYEVTSKTYERAREAIAQMKAAALANVNTRMFGLDGN
gi|1899218SeMV  231 AEAYIEMRNSEGFYMPRYGLLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVNTRMFGLDGN
gi|1542875 Zimb 228 AEAYIEMRNSEGFYMPRYGPLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVNTRMFGLDGN
gi|222583SAPV   231 AEAYIEMRNSEGFYMPRYGLLRNLRDKSLARYAFDFYEVTSKTPDRAREAIAQMKAARLANVNTRMFGLDGN
gi|89145895 BAJg 174 LEAYIEMRNSEGFNMPRYGLLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVNTRMFGLDGN
gi|38046104DFBr 174 AEAYIEMRNSEGFYMPRYGLLRNLRDKSLARYAFDFYEVTSKTSDRAREAIAQMKAAALANVNTRMFGLDGN
consensus       241 aEAYIEMRNSEGFympRYGlLRNLRDKSLARYAFDFYEVTSKTsdRAREAIAQMKAAaLANVnTRMFGLDGn
```

Fig 4.4 Multiple alignment of deduced amino acid sequences of the partial capsid protein amplified from Kenya 64, 80 and 47 from different subgroups compared to the *South African Passiflora Virus* (Brand *et* *Virus* (Pappu *et al.,* 1997), CABMV isolate from Zimbabwe (Sithole-Niang *et al.,* 1996) and CABMV is (Nascimento *et al.,* 2006).

4.4 Discussion

In this study, molecular characterization of CABMV isolated from passionfruit in Kenya is described. The isolates displayed 90-98% nucleotide sequence identity with CABMV isolated from South Africa (D10053), Georgia (U90326), Brazil (AF 433951, DQ 397532, and DQ 397527) and Zimbabwe (AF 348210) which are SAPV, SeMV, Brazilian and Zimbabwean CABMV isolate, respectively. *South African passiflora virus* (SAPV) and SeMV are now accepted synonyms of CABMV by the International Committee of Taxonomy for viruses (Carstens, 2009). According to Frenkel *et al.* (1992) and Shukla *et al.* (1994) the coat protein and 3'UTR sequences can be used as markers of genetic relatedness of potyviruses. Distinct potyviruses are reported to be 38-71% identical in the CP region and 90-99% identity among virus strains (Shukla *et al.*, 1994; Ward *et al.*, 1994; Adams *et al.*, 2005). In terms of species demarcation, the Kenyan virus isolate is a strain of CABMV.

The Kenyan isolates were clustered into one group with other CABMV isolates and Uganda passiflora potyvirus. Brazilian isolates were all in one subgroup in the phylogenic tree. The results compare with those of Pappu *et al.* (1997) who identified SeMV which clustered with the passionfruit woodiness potyvirus group of viruses and most closely to CABMV including SAPV and the Zimbabwean CABMV isolate. They also compare with those of Nascimento *et al.* (2006) which clustered former Brazilian PWV isolates with CABMV. In addition, the results serve to reinforce Sithole-Niang (1996), Nascimento *et al.* (2006) and Moreira (2008) who suggested that the virus affecting passionfruits in Africa (SAPV) and Brazilian PWV isolates were a CABMV strain. Uganda Passiflora virus clustered together with the CABMV isolates suggesting a close relationship with CABMV.

These results indicate that all the Kenyan isolates have a high degree of similarity among themselves and with CABMV. The sub grouping within the main cluster suggests some variation among the isolates, possibly strain differences. The variation is low and could be taking place as is indicated by sequence 14 and the Tajima test of neutrality where evidence of possible selection was demonstrated. While characterizing the passionfruit virus isolates in Brazil, Nascimento *et al.* (2006) reported that Brazilian isolates of CABMV were varied but within the same species. In Uganda four CABMV strains, which cause variable infection in cowpeas have also been isolated (Orawu, 2008).

The variability observed maybe a result of slow base substitution due to mutation or intraspecific recombination occasioned by grafting as a method of propagation or the unrestricted movement of seedlings for planting from one region to another and the perennial nature of the crop availing innoculum for two different viruses or strains to recombine or mutate. The claim that such evolution/changes appear to be going on is supported by the two sequences (80 and 63) that did not match the others by having amino acids in the conspicuous gap common to other isolates. Despite the variation, the isolates revealed important conserved amino acid motifs described for all potyviruses. The conserved motifs are WCIEN, QMKAAA and NTGS (Pappu *et al.* 1993; Kwon *et al.*, 2002; Yoon and Ryu, 2002). According to Pappu *et al.*, (1993) the conserved motifs are unique to the potyviruses. Hence the virus isolate from Kenya is a potyvirus.

The variation of the two isolates from Kenya to the level of 62% falls within the species demarcation range (38-71%) reported by Shukla *et al.* (1994) and Ward *et al.* (1994) separating distinct viruses among potyviruses in the CP sequence. This indicates that there could be more than one strain or species among the Kenyan isolates amplified by the specific primer used. More research is necessary to validate the PCR detection method for sensitivity and specificity and to determine whether the virus isolates are different and whether the symptom expression varies as in CABMV strains isolated from cowpea in Uganda (Orawu, 2008.

The evidence gathered in this study indicates that the virus isolated from passionfruit in Kenya is a potyvirus comprising a strain of CABMV. The presence of CABMV in passionfruit orchards in Kenya may be the primary cause of woodiness disease. *Cowpea aphid borne mosaic virus* isolates from Kenya have low variability. There is a possibility for potential distinct CABMV strains which should be investigated and considered while making decision on detection methods and management strategies.

CHAPTER FIVE

MONITORING OF APHID FAUNA IN PASSIONFRUIT ORCHARDS IN KENYA

Abstract

Passionfruit woodiness disease viral pathogens limit passionfruit production and are non-persistently transmitted by aphid vectors. The study was conducted to identify aphid species and assess the population dynamics of potential vectors in the orchards for purposes of developing viral disease management tactics. Field trials laid out in a randomized complete block design with four replicates, were conducted in Kabete and Embu and aphid populations monitored weekly in passionfruit orchards for a year using yellow water pan traps under natural conditions. Aphid transmission tests using commonly found aphids *Aphis gosypii, A. fabae, Brevicoryne brassicae, Ropalosiphum maidis*, and *Sitobion avenae* and a CABMV isolate from the field were also carried out in the greenhouse. These tests would establish the ability for the aphid species to transmit CABMV.

Twelve species of aphids were captured but the most abundant were *Aphis gosypii, Ropalosiphum maidis, Acyrthosiphon pisum* and *Brevicoryne brassicae* accounting for 97% and 95 % of the total aphids collected in Embu and Kabete, respectively. The species diversity was rich and abundant at 0.79 and 0.7 for Kabete and Embu, respectively. The aphid population in Kabete was significantly ($p < 0.001$) higher than that collected in Embu whereas the population collected during the long rains season was significantly ($p<0.001$) higher than that which was collected in the short rains. Individual species populations were higher in Kabete than in Embu and only *A. gosypii, Macrosiphum euphorbiae, Myzus persicae* and *R. maidis* had significantly ($p<0.05$) higher populations in Kabete.

Transmission tests indicated that *Aphis gosypii and R. maidis* had a higher ($p < 0.001$) ability to transmit the CABMV isolate at 71% and 63%, respectively compared to *B. brassicae, A. fabae* and *Sitobion avenae* and the control. *Sitobion avenae* did not transmit CABMV isolate used. The aphids

were present in the orchards throughout the year with one major seasonal peak in June, a time period when food crops and other vegetation such as weeds grow vigorously. About 70% of the total aphids were collected during the peak period in both sites indicating greatest aphid dispersal and flight activity. The occurrence of aphids in the orchards throughout the year with the peak population density coinciding with the cropping season has serious implications in the management of the pest and spread of viral diseases of passionfruit.

5.1 Introduction

Viral diseases are a major limiting factor to passionfruit production worldwide (Moreira, 2008). In Kenya, CABMV, CMV and an unknown potyvirus are affecting passionfruit orchards causing woodiness disease as reported in Chapter 3 of this thesis. These are a potential threat to the passionfruit industry. In areas where PWD is prevalent, the disease can reduce the orchard life span to only a year resulting to 100% yield loss (Trevisan *et al.*, 2006). Crop susceptibility, virus strain and environmental conditions do influence the extent of loss incurred (Bashir *et al.*, 2002). Typical symptoms include strong mosaic, stunted growth, leaf rugose, size reduction and distortion, inhibition of fruiting, hard fruits of reduced size with thick pericap and little or no pulp (Novaes and Rezende, 2003; Pappu *et al.*, 1997).

Despite the reports of the presence of the disease in Kenya, no quarantine measures have been undertaken to contain the spread. To date the disease is widely spread in all regions where passionfruit is grown reducing fruit yield and quality. Viral diseases have no remedy and can be spread long distances by germplasm, rootstocks or grafted seedlings. Once established natural spread within and between orchards occurs by aphids in a non-persistent manner (Shukla *et al.*, 1994). Immigrating aphids that do not feed on or colonize the host

plant can effectively transmit the viruses (Zeger *et al.*, 1990). Omatsu *et al.* (2004) reported three main vectors of PWV in passionfruit orchards which are the sow thistle aphid (*Hyperomyzus lactucae*), green peach aphid (*Myzus persicae*) and Cotton aphid (*A. gosypii*). Currently, limited information exists on aphid species composition, population dynamics and potential aphid vectors in passionfruit orchards in Kenya. Knowledge of aphid population and their flight activity within and around the orchards is necessary to assess the potential aphid species in the spread of viruses infecting passionfruit in Kenya.

The aphids cause more harm by transmitting viruses other than by feeding on the plants hence the need to control them. A monitoring system of aphids that is low cost and simple needs to be developed to avail information to farmers to take informed and appropriate management actions. Many methods for trapping aphid species have been reported and compared for studies on diversity and population changes in the environment (Boiteau, 1990). They include suction traps, coloured water pan traps and sticky traps. These methods are capable of affecting population size estimates and the species collected. Sticky traps are non-selective and capture similar aphid species to those of the suction trap (Boiteau, 1990). Water pan traps located within the crop more often indicate the true picture of the species landing on the crop, however, the colour of pans may influence the aphid species trapped (Difonzo *et al.*, 1997).

Since trap characteristics influence species composition and richness, more than one type of monitoring technique maybe necessary to be able to understand the aphids alighting on a crop. In this case, the yellow water pan traps were preferred for their attractiveness and the

fact that they are within the crop to capture flight activity of aphid species indicating peak presence within agro ecosystem and periods of dispersal. The study was undertaken to determine the species prevalent and to describe their population.

5.2 Materials and methods

5.2 1 Site description and experimental layout

Two experiments were conducted in Kabete field station, 1940m high, latitude 1°15'S and longitude 36° 45'E and in Embu- Manyatta area, 1545m, latitude 0° 53S and 37° 45'E. In Kabete, a one acre plot was divided into three equal parts while in Embu; four farms belonging to small scale farmers were selected for monitoring of aphids. Each plot/farm had four water pan traps placed in a zig zag transect along the length to trap the flying aphids. The traps standing on a representative area within the plot or farm served as replicates. The study was carried out over two seasons in each site from April 2009 to February, 2010. Prior to this monitoring work, preliminary monitoring was conducted in Kabete from May 2008 to November, 2008. Data collected was mainly aphid populations in water pan traps and on vines and aphid species identified.

5.2.2 Assessment of aphid populations in the field

Aphids were assessed using yellow water pan traps and direct counting on five growing points of the vine from different directions of the wind. In each plot/farm four traps were placed equidistantly. Water pan traps used were yellow round basins 30 cm diameter and 20 cm deep, covered with a sunshine yellow plastic paper. The traps were placed on wooden frames 1.5m high above the ground. They were half filled with clean water and a few drops

of liquid detergent (monoethylene glycol) added to break the surface tension to allow the insects to sink to the bottom and to preserve the specimens. Samples were collected weekly for 35 continuous weeks. The specimens were preserved in 70% ethyl alcohol for identification and counting. These were later combined /merged to one cumulative sample to give the total sample collected per month per field. Surrounding vegetation and crops were recorded for the two growing seasons. The aphids collected were separated, identified and counted in the laboratory using a stereomicroscope. Apart from the traps, direct sampling was done on the vines. Four vines randomly selected per field were examined for aphid presence and quantification on five different growing points per plant. These observations were made on a weekly basis to count nymphs or alates on leaves, while collecting aphids in the traps.

5.2 3 Aphid species identification

Aphids collected once a week from the field were preserved in 70% ethyl alcohol and taken to the College of Agriculture and Veterinary Science Entomology laboratory for identification. Voucher specimens were selected on the basis of shared morphological characteristics used to identify similar aphids to species level. The species were identified with the help of existing laboratory collection and entomological keys based on morphological features as described by Martin (1983) and Blackman and Eastop (2000) (Table 5.1). These features include body colour, length of antennae relative to the body, antennal tubercles development and placement, cornicles length and colour, siphunculi shape, number of caudal hairs and length relative to the cauda and dorsal abdominal pigmentation.

Table 5.1 Features used to identify different aphid species collected in traps

Species	Body colour	Antennal tubercles	Siphunculi	Dorsal abdominal pigmentation
Myzus persicae	Green or olive	Well developed and inner sides converging	clavate	Has a dorsal black patch
Macrosiphum euphorbiae	Green or olive or yellow/ orange	Well developed and inner sides diverging	Cylindrical or tapering	No pigment completely green
Aphis gosypii	Black or green	Less developed or absent		Black transverse bars on abdominal side
Aphis fabae	black	Less developed	Short and same length with cauda	No abdominal marks all dark
Ropalosiphum maidis	Blue-green or grey	Less developed		Dark strip in the middle

Source Martin (1983); Blackman and Eastop (2000)

Specific diversity of aphids was determined by Simpson diversity index (Margurran, 1988) using the following equation: $D = 1 - (P_i)^2$; $P_i = n_i /N$ where:

D = species diversity; N = total number of individuals; P_i = proportion of sample that contributes to the total population; n_i = Number of individuals of the ith species. The value of Simpson diversity index (1-D) ranges between 0 and 1. The greater the value of the diversity index the greater is the richness and abundance of the species (Margurran, 1988).

5.2.4 Aphid transmission tests

Aphid species which included Maize aphid (*Rhopalosiphum maidis*), Wheat grain aphid (*Sitobion avenae*), Cabbage aphid (*Brevicoryne brassicae*), Bean aphid (*Aphis fabae*) and cotton aphid (*Aphis gosypii*) were reared on their respective hosts in a greenhouse. These were chosen because of their abundance in the main passionfruit growing areas, since their preferred hosts are grown as food crops in the same areas. The aphid species are present in different agro ecosystems and will fly into orchards which are within close range because of

the farming systems prevailing in the passionfruit growing areas. The aphids were captured in the field and reared in green houses on preferred hosts. They were then removed gently from the hosts using camel hair/paint brush and placed on petri-dishes with moist filter papers. The aphids were starved for 1 hour. After the hour, leaf discs from infected passionfruit plants, maintained in a separate green house, were given to the aphids in the same □etri-dishes to allow acquisition of the virus for 10mins. Thereafter, the aphids were collected in groups of 10 and transferred onto healthy/clean passionfruit plants in three replicates (Walkey, 1991) The aphids were allowed an inoculation period of 24 hours on the plants and were then killed with an insecticide (cypermethrin). This experiment was repeated three times using 10 plants /aphid and replicated 3 times each time. Once the virus symptoms developed plants were tested serologically with DAS ELISA protocol.

5.2.5 Data analysis

Experimental data collected was analyzed by one way analysis of variance (ANOVA) using Genstat Discovery Edition software (Rothamsted, UK) to determine aphid population density and species differences between sites and seasons. The means were compared using Fischer's protected least significant difference (LSD) procedure at $p<0.05$ (Steele and Torrie, 1980)

5.3 Results

The year 2009 and part of 2010 was relatively warm, the lowest temperatures were experienced in July (11°C) and the highest in March (27°C). The rains were poorly distributed and insufficient in Kabete averaging up to five rainy days per month (Fig 5.1). In Embu, the rains were enough but poorly distributed in the year 2009 (Fig, 5.1). During the rainy period, May to July the passionfruit plants exhibited spectacular virus symptoms especially mosaics following the rainfall received.
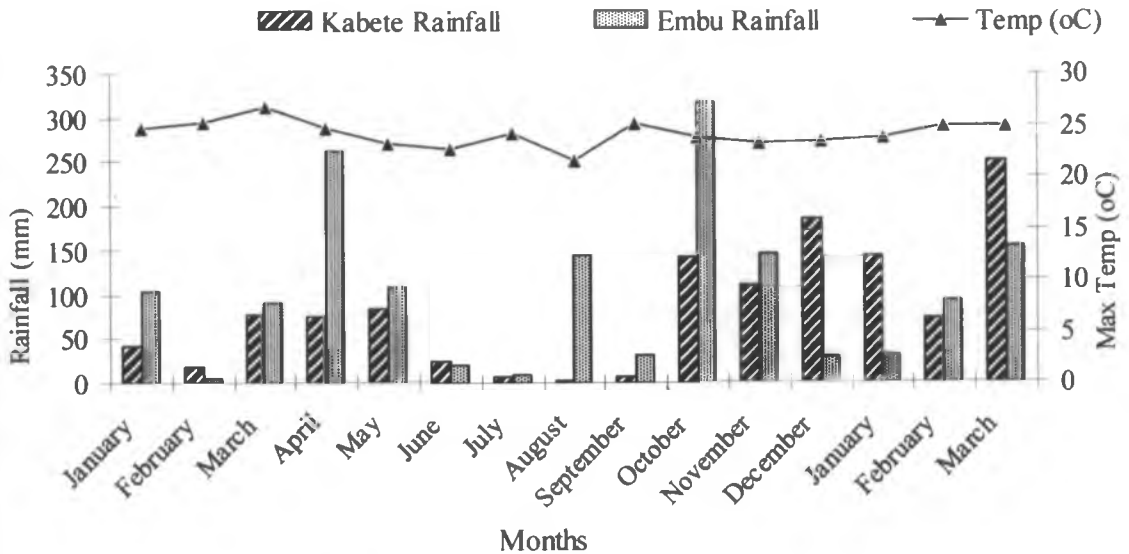
Fig 5.1 Mean rainfall (mm) received and Temperature (oC) in Embu and Kabete 2009 to March 2010

5.3.1 Aphid abundance and fluctuation trends

A total of 10,900 aphids were collected, 8000 and 2900 from Kabete and Embu, respectively. The aphid population in Kabete was significantly (p<0.001) higher compared to that collected in Embu (Table 5.2). Similar aphid species were observed in both sites. These species were *Aphis gosypii* Glover, *Ropalosiphum maidis* Fitch, *Acyrthosiphon pisum* (Harris), *Aphis fabae* Scopoli, *Brevicoryne brassicae* Linnaeus, *Cavariella aegopodii*

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Linnaeus *Macrosiphum euphorbiae* Thomas, *Myzus persicae* Sulzer and *Lipaphis erysimi* Linnaeus, *Uleurocon spp*, *Hyperomyzus lactucae* L *and Therioaphis trifolii* L. Some individual species such as A. *gosypii, M. euphorbiae, Myzus persicae* and *R. maidis* had significantly ($p<0.05$) higher populations in Kabete compared to the same species populations in Embu (Table 5.2). *Aphis fabae, Acyrthosiphon pisum, B. brassicae and M. pesicae* had higher populations in Kabete compared to Embu but the differences were not significant. *Aphis gosypii, R. maidis, B. brassicae* and *A. pisum* were the most abundant in both sites. No aphids were observed developing or feeding directly on the vines throughout the sampling period.

Table 5.2 Mean number of aphids captured in passionfruit orchards in Embu and Kabete from May, 2009 to February 2010

Sites	*A. fabae*	*A. gosypii*	*A. pisum*	*B. brassicae*	*M. euphorbiae*	*M. persicae*	*R. maidis*	Total* aphids
Kabete	61	310	56	37	49	56	208	792
Embu	16	142	35	23	1	32	62	290
Mean	**39**	**226**	**46**	**30**	**25**	**30**	**135**	**541**
F-test	**ns**	**	**ns**	**ns**	**	**ns**	**	**
LSD (p=0.05)	45.8	79.8	85	36.2	42.9	32.6	62.5	197.5

* Total aphids = overall mean of all aphid species; ** significance level at $p<0.05$, ns: No significant difference

Aphid population trends were different in the two sites. In Kabete the population peaked in June continued on into July followed by a sharp drop in August. Two smaller peaks were observed in September and October followed by another drop before peaking again in December and February, 2010. In Embu, the population peaked only in June followed by a sharp drop and it remained low for the rest of the sampling period (Fig 5.2). The aphid population peaks followed the rainfall which was received 4-6 weeks before (Fig 5.1). The

least aphid population was observed in November (Fig 5.2). The aphid population in the short rains season (November, 2009 to February, 2010) was significantly (p<0.001) lower than that which was collected in the long rains season.
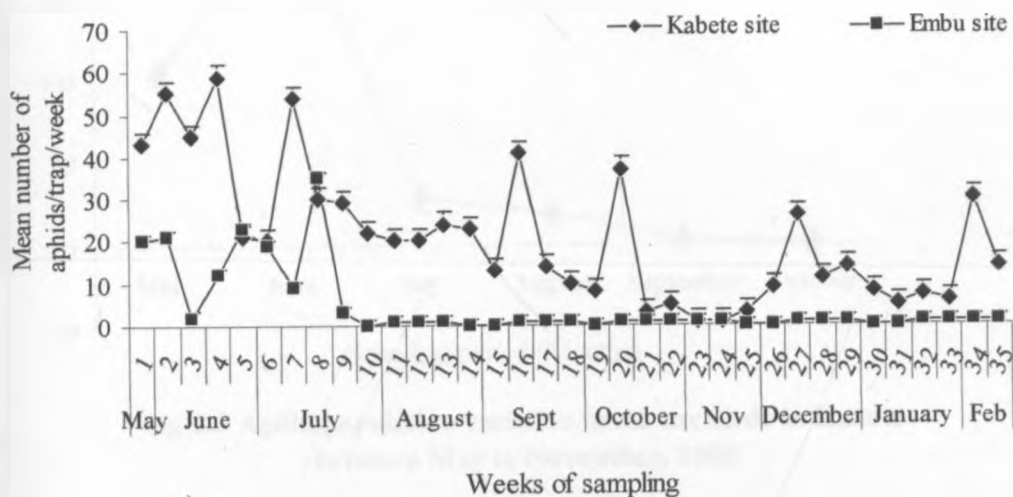
Fig 5.2 Mean number of aphids collected in Embu and Kabete sites
May 2009 to February 2010

In 2008, preliminary work done in Kabete revealed only one peak activity of aphids in June, 2008. The peak was followed by a sudden drop in population density which remained low for the rest of the sampling period (year) (Fig 5.3). The total aphid population was significantly affected by the prevailing conditions over time (p< 0.001). Figure 5.4 shows the monthly rainfall amount received during the period (2008).
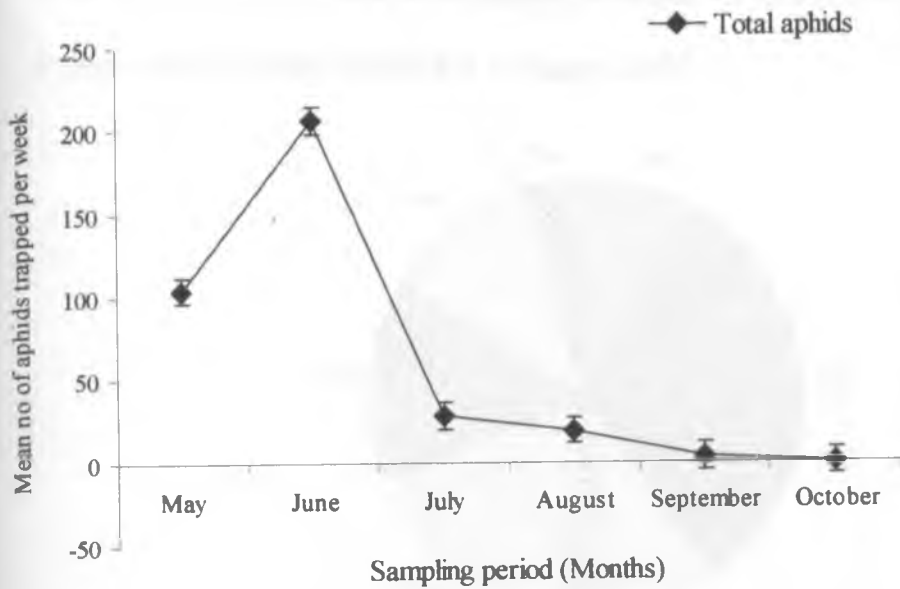
Fig 5.3 Aphid population variation in the orchards in Kabete between May to November, 2008
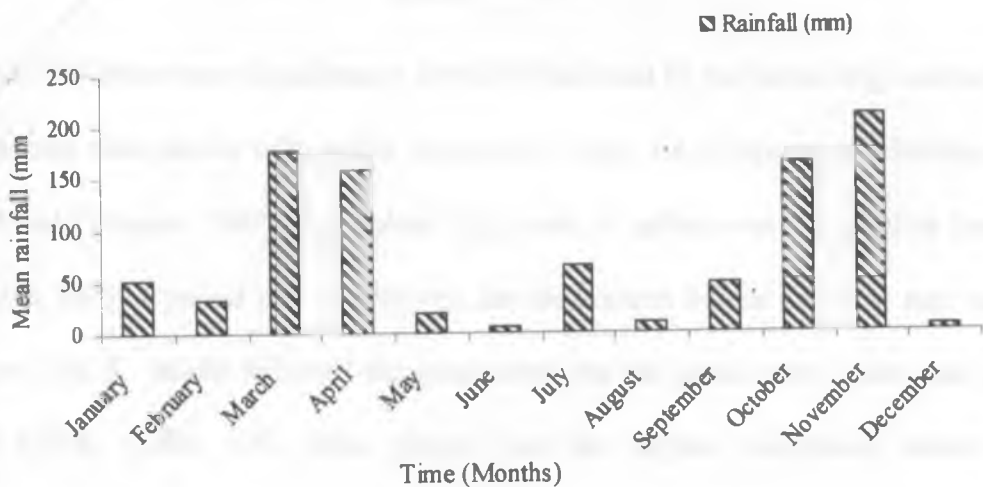
Fig 5.4 Mean monthly rainfall received in Kabete, 2008

In 2009, six aphid species were considered abundant in Kabete. They exceeded 5% of the relative abundance during the year (Fig 5.5). The species diversity index in Kabete was 0.79, indicating a species richness and abundance of aphids. The abundant species were *A. gosypii* Gloverii *(31%) R. maidis* Fitch *(18%)*, *B. brassicae* Linnaeus *(15%)*, *M. persicae (*Sulzer*)*

(9%), *A. fabae* Scopoli *(8%)* and *M. euphorbiae* Thomas *(5%)* and *A. pisum (3%)* making

97% of the total aphid population trapped in Kabete. Sixty eight percent of all the aphids in

Kabete were collected during May to August, 2009.
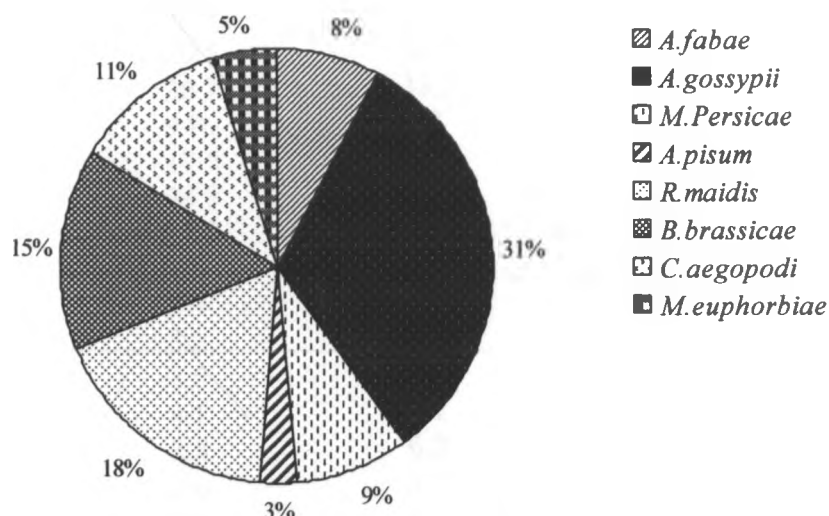
Fig 5.5 Proportion (%) of total aphids sampled in Kabete and categorized into individual species from May 2009 to February 2010. A total of 8000 aphids collected

Aphids in Kabete were significantly (p<0.001) affected by the prevailing weather conditions.

All aphids were active with peaks observed in June, July, September, October, December.

2009 and February, 2010. An unusual high peak of aphids was observed in September and

October 2009, a period that is relatively dry that comes before the short rain season. *Aphis*

gosypii and *R. maidis* followed the same trend but the peaks were lower than those of the

total aphids (Table 5.3). *Aphis gosypii* had the highest population density above the

population of the other species (p<0.001) while *Lypaphis erysimi* had the least population

density.

Table 5.3 Mean number of alate aphids captured per trap per week in Kabete from May, 2009 to February, 2010

Month	Sampling week	A. gosypii	R. maidis	Total aphids*
May	1	16.7	5.1	43.4
June	2	20.3	16.1	55.4
	3	20.5	19.2	44.8
	4	36.7	19.5	58.5
	5	9.9	4.9	21.4
July	6	12.5	5.1	20
	7	24.9	17.7	53.8
	8	12.7	10.3	29.8
	9	6.8	6.3	28.5
August	10	7.9	4.6	22.2
	11	5.2	3.8	19.8
	12	4.6	40	20.2
	13	4.1	3.1	24.4
September	14	3.8	6.2	22.9
	15	2.6	1.9	12.8
	16	8.7	5.1	40.5
	17	2.7	13	14.0
October	18	2.8	1.1	10.3
	19	2.8	1.0	8.3
	20	14.2	7.3	37.3
	21	1.6	1	2.8
November	22	0	0	5.1
	23	0	0	1.3
	24	0	0	1.4
	25	2.4	0	3.3
December	26	5.6	2	9.0
	27	13.5	5.3	2.2
	28	4.3	5.5	11.8
	29	6.8	4.8	13.6
January	30	7.2	0	7.8
	31	2.6	1	47
	32	3.8	1	7.1
	33	4.3	0	5.8
February	34	14.5	8.4	30.4
	35	6.8	5.7	13.9
mean		8.4	5.12	21
Significance		**	**	**
Lsd $_{p<0.05}$		4.71	4.07	10.51

Mean no. of aphids captured per trap weekly, * Total aphids = overall mean for all aphid species;
** Significance at p<0.05

Four aphid species were considered dominant in Embu, because they exceeded 5% of the relative abundance during the year (Fig 5.6). The species diversity index was 0.70 in Embu indicating a richness and abundance of aphid species. The most abundant species were *Aphis gosypii (48%)*, *R. maidis (23%)*, *Acyrthosiphon pisum* (12%), *Brevicoryne brassicae (8%) and Aphis fabae (5%)* making 96% of the total aphids collected (Fig 5.6). Seventy two percent of all the aphids in Embu were collected during the wet season that coincided with the peak activity of the aphids May to July 2009. Peak activity of the aphids was observed in June 2009 (Table 5.4). Thereafter, there was a sharp drop in aphid population density which remained low for the rest of the sampling period. The least density was collected in November 2009 (Table 5.4). The increase of aphid populations followed a rainfall received 4 weeks before (Fig. 5.1). The total aphid population and all aphid species were significantly (p< 0.001) affected by the prevailing weather conditions over time. Like in Kabete, the aphids were always present in the orchards.
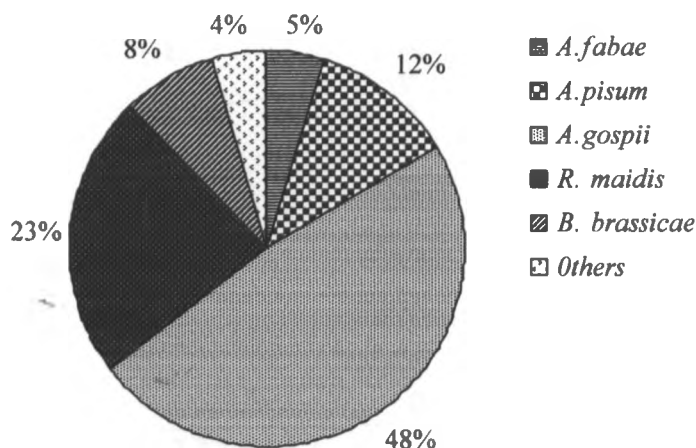
**Fig 5.6 Proportion (%) of total aphids sampled in Embu and categorized
by individual species from May 2009 to February 2010
A total of 2900 aphids collected**

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Table 5.4 Mean number of alate aphids captured per trap/week in Embu from May, 2009 to February 2010

Month	Sampling week	A. gosypii	R. maidis	Total aphids*
May 2009	1	8.6	0	20
June	2	5.4	0	21.3
	3	1.2	0	1.7
	4	68	2.4	12.2
	5	13.9	6.5	22.6
July	6	7.9	9.0	19.3
	7	4.1	3.6	8.8
	8	18	12.4	34.6
	9	2.3	0	3.1
August	10	0	0	0
	11	0	0	1.6
	12	1	0	1.5
	13	0	0	1
September	14	0	0	1
	15	0	0	0
	16	0	0	0
	17	0	1	1
October	18	0	1	1
	19	0	0	0
	20	0	0	0
	21	1	0	0
November	22	1	1	1
	23	1	0	0
	24	0	0	0
	25	1	0	1
December	26	1	0	1
	27	1	0	0
	28	1	0	1
	29	0	0	0
January 2010	30	0	0	0
	31	1	0	1
	32	0	1	1
	33	0	0	0
February	34	0	0	0
	35	1	0	0
mean		3.23	1.6	6.7
Significance		**	**	**
Lsd		6.62	4.43	13.23

Mean no. of aphids captured per trap weekly; * Total aphids = overall mean for all aphid species; ** Significance at $p < 0.05$

5.3 2 Transmission of the Cowpea aphid borne mosaic virus (CABMV)

The CABMV isolate used was transmitted by aphid species after 10 min of acquisition period. ELISA tests indicated presence of the virus protein in the leaf extracts. *Aphis gosypii, Ropalosiphum maidis, Aphis fabae, Brevicoryne brassicae* transmitted the virus with variable ability. The aphids ability to transmit CABMV isolate was significantly ($p<0.001$) different from control. *Aphis gosypii* had the highest transmission ability of CABMV isolate at 71% but was not different with that of *R. maidis* at 62.8%. *Aphis fabae* and *B. brassicae* were not different from each other but differed with *A. gosypii* and *R. maidis* in the ability to transmit CABMV isolate. *Sitobion avenae* did not transmit the CABMV isolate. It was not significantly different from control (Table 5.5).

Table 5.5 Transmission ability of CABMV from passionfruit by five different aphid species

Aphid species	% plants with virus symptoms	Mean ELISA values (10 ELISA positives)
Aphis gosypii Gloverii	71.0	3.5
Ropalosiphum maidis (Fitch)	62.8	3.4
Aphis fabae Scopoli	30.3	3.3
Brevicoryne brassicae L.	25.5	3.3
Sitobion avenae Fabricius	11.4	3.2
Control**	0.00	0.2
F-test $_{(5, 66)}$	*	
LSD $_{(p<0.05)}$	11.4	
cv %	37.9	

* Significance at $p<0.05$,
** Control *Aphis gosypii* allowed to feed on a plant with no CABMV and used for transmission

5.4 Discussion

Aphids are prevalent in small scale farmers' fields. Twelve aphid species were trapped in passionfruit orchards and the most abundant in both sites were *A. gosypii, R. maidis, B. brassicae* and *A. pisum*. These were consistently identified and were prevalent throughout the year. The composition is similar to that which was observed by Atsebeha *et al.* (2009) and

Nault *et al.* (2004) in pepper fields and by Iwai *et al.* (2006) and Garcez *et al.* (2011) in passionfruit orchards in Japan and Brazil, respectively. The aphid composition reflected the diverse cropping system in the adjacent landscapes. The main crops surrounding or intercropped within the orchards consisted of Napier grass, maize, dry beans, potatoes, kales, tomatoes, pigeon pea, bananas, tea and coffee. Hence, the aphid species had the preferred hosts represented within the mix of crops listed.

The aphid species were non-colonizers and were able to alight on passionfruit vines throughout the year. Several of these species are well known vectors of CMV and CABMV (Bashir *et al.*, 2002). It is hypothesized that the aphids were immigrating from their preferred hosts into the passionfruit orchards. The peak aphid population activity especially for the most abundant aphids, namely *A. gosypii and R. maidis*, was in June as observed in 2008 and 2009 in Kabete and Embu and maybe linked to the cultivation of food crops (Maize, wheat, beans, potatoes) on a large scale during the wet season. The cropping season favoured aphid population increase as preferred hosts for food, shelter and breeding. Similar findings were observed by Khalleshwarraswamy *et al.* (2007) and Khalleshwarraswamy and Krishankumar (2008) while studying the efficiency of transmission of Papaya ring spot virus by three aphid species and when monitoring aphid vectors responsible for the spread of *Papaya ring spot virus*, respectively. The absence of aphids on vines confirms non-colonization of passionfruit by aphids but the non-colonizing aphid species have a role as virus vectors in passionfruit orchards.

The aphid populations were high during the long rains season and were more abundant in Kabete than in Embu; unlike in Irish potatoe fields where it is reported that the aphids are abundant in the short rains and in warmer conditions (Olubayo *et al.*, 2004; Nyaga, 2008). According to Radcliffe (1982) temperatures below 17.8°C restrict aphid population growth. The prevailing temperatures during the experiments, were above 22°C hence favoured the survival of aphids in Kabete and Embu. Aphids prefer warm conditions as opposed to cold conditions as long as food is available (Hanafi, 2000). The temperatures may not have been a limiting factor for the increase in aphid population density but the rainfall. Heavy rainfalls received in Embu above 150 mm/day in April and May and in October, November and December, compared to approximately 80mm/day in Kabete could have confounded the increase of aphid populations. The aphids were probably washed away from the hosts and the populations did not peak as expected. This explains why the aphid populations were much lower in Embu than in Kabete.

Aphid population peaked in June when there was plenty of food resource for the aphids after the rainfall hence the increase in numbers. The crops and vegetation vigorously growing around the orchards could have acted as reservoirs of aphids that were immigrating into the orchards. This observation is consistent with the findings by Handizi and Legorbou (2002) who reported that the first vegetation around a target crop such as seed potato plays a critical and important role in aphid population dynamics. Rainfall promotes growth of weeds and pasture plants which aphids utilize to increase populations and acquire virus pathogens that are later transmitted to target crops (Thackray *et al.*, 2002). The populations peaked after

rainfall events such as in March, April, May, October and November 2009 thus explaining the unusual peaks of aphids in September and October in Kabete.

The aphid species found immigrating passionfruit orchards are confirmed vectors of viruses. *Aphis gosypii* and *M. persicae* are efficient vectors of woodiness disease pathogens according to Baker (1974) and Omatsu *et al.* (2004). *Ropalosiphum maidis, A. gosypii, M. euphorbiae, M. persicae* and *A. pisum* are vectors of CABMV in cowpeas (Bashir *et al.,* 2002). These aphid species are also vectors of CMV and other virus pathogens in a wide host range of crops (Diaz-Perez *et al.,* 2003; Ng and Falk, 2006). The most abundant aphid species *A. gosypii and R. maidis*, in Embu and Kabete are efficient vectors of CABMV. These aphid species and others are the cause of viral infections observed in the passionfruit orchards.

The abundance of *A. gosypii, R. maidis* and *A. pisum* which are polyphagous insects, coincided with the wet season depicting peak activity of aphids in the orchards. The occurrence of aphids in the orchards throughout the year with the peak population density coinciding with the cropping season and the time passionfruit vines are lush, favours probing and feeding by insect pests. The kind of mixed cropping systems present and the species richness and abundance observed within the passionfruit growing areas, have serious implications on the epidemiology and management of the viral diseases. Careful monitoring of the aphid species activity is necessary to initiate preventive measures. Farmers would be advised to take management actions to reduce aphid activity and spread of viral diseases during this period.

This study showed that the yellow water traps used for sampling vectors in passionfruit orchards could be used to indicate the aphid species entering the agro-ecosystem and the greatest dispersal period. The results compare with those of Omatsu *et al.* (2004) and Garcez *et al.* (2011) who used yellow water traps to monitor aphid species immigrating passionfruit orchards in Japan and Brazil, respectively. Khaleshwaraswamy *et al.* (2007) used similar traps to study the role of transient aphid vectors in the spread of *Papaya ring spot virus* in India. In their studies, Demirel and Yildrim (2008) and Garzo *et al.* (2004) reported that yellow water traps were better indicators of peaks of aphid flight activity representing periods of greatest dispersal of an aphid infestation in addition to attracting some aphid species more often.

In this study aphid species alighting in the yellow water traps located at different sites varied with the cropping systems within the vicinity of the orchards. This implies that the crop plants adjacent to the passionfruit orchards had a role in the composition and abundance of aphid species. In their studies, Ban *et al.* (2009) and Summers *et al.* (2004) reported that aphid species alighting in a field crop were influenced by the type and colour of the trap used and major crops and weeds growing around the sampled areas. The traps used in this study are simple and low cost and could be adopted by farmers for monitoring vector presence and activity. An understanding of aphid-transmitted viral disease epidemics requires an appropriate method of monitoring vector activity.

Aphid transmission results indicate ability of several aphid species; *A. gosypii, A. fabae, R. maidis and B. brassicae* to transmit CABMV in varying degrees and that transmission can

take place from a passionfruit plant to another. The CABMV isolate is aphid transmissible in a non-persistent manner. According to Omatsu *et al.* (2004) and Iwai *et al.* (2006) aphid vectors in passionfruit orchards in Japan transmitted viruses in a non-persistent manner. *Aphis gosypii* is an efficient vector of PWV in passionfruit according to Taylor and Kimble (1964). *Aphis gosypii, Toxoptera citricidus, R. maidis, M. euphorbiae, A. pisum and A. fabae* are CABMV vectors in peanuts and cowpeas (Pio-Ribeiro *et al.* 2000; Bashir *et al.*, 2002). In this study, results obtained indicated differences in the ability to transmit CABMV among aphid species (*A. gosypii, A. fabae, R. maidis, B. brassicae* and *S. avenae*). *Aphis gosypii* had the highest ability (71%) while *Sitobion avenae* was unable to transmit the virus isolate. It shows that vector species differ in virus pathogen transmission. This implies that the aphid species were influenced by insect biotype, host plant, virus strain and weather conditions which are reported to influence pathogen transmission by aphids (Rosell and Thotapilly, 1985; Nono-Womdim *et al.*, 2001).

Aphis gosypii; *R. maidis* and other aphid species such as *B. brassicae*, *A. fabae* and *S. avenae* are present in most agro ecosystems in Kenya. They are vectors of several viruses and are able to transmit CABMV from passionfruit to passionfruit (Brault *et al.*, 2010). Considering the characteristic mixed cropping systems, particularly during the maize, bean and wheat growing seasons these results have implications on the distribution pattern and management of the virus disease in the passionfruit orchards. *S. avenae*, a wheat grain aphid, did not transmit CABMV, a characteristic of most grain aphids. Neverthless, inefficient vectors cannot be underestimated in their ability to transmit viruses when the population densities are

high in a field. There is need to consider all other aphids in the field when deciding virus management actions.

The study has confirmed that non-colonizer aphid species are prevalent in passionfruit orchards which were not captured on plants but in traps. The main aphid vectors were *A. gosypii, R. maidis, A. pisum and B. brassica*. Most flight activity characterized by large aphid populations concided with the dominance of other food crops in the vicinity of the the orchards in June and the vigorously growing lush passionfruit that attracted aphids encouraging feeding and possible spread of virus pathogens. Transmission studies have confirmed the ability of the most predominant species, *A. gosypii, R. maidis, B. brassicae and A. fabae* in transmitting CABMV that infects passionfruit orchards. Based on these observations then the most likely vectors of passionfruit viruses are *A. gosypii, R. maidis, A. fabae, A. pisum* in addition to *M. persicae* which appears in low abundance playing a role in disease spread. The influence of cropping patterns and rainfall on seasonal aphid population abundance cannot be ignored.

Many vectors present in the orchards throughout the year favour woodiness disease spread emphasizing the need to take management actions on the vectors. An aphid monitoring system that is low cost, not time consuming and possibly acceptable to farmers has been used. This aphid monitoring procedure can be used to initiate measures to reduce aphid activity and viral disease spread.

CHAPTER SIX

UTILIZATION OF REFLECTIVE MULCH FOR THE MANAGEMENT OF APHID VECTORS IN PASSIONFRUIT ORCHARDS IN KENYA

Abstract

Three methods were evaluated for their effectiveness in reducing aphid populations in passionfruit orchards. These were white reflective polythene mulch, straw mulch as repellants; yellow treated clothing material as a trap and refined mineral oil (DC Tron) for pest management. These were compared with an untreated/unmulched control. The repellants achieved high aphid population reduction when the densities were high as opposed to low densities. The reflective mulch significantly ($p<0.05$) reduced total aphid population density. In Kabete, the reflective mulch significantly ($p<0.05$) reduced aphid populations to 31 and 10 in both the long and the short rains seasons, respectively while in Embu, the reflective mulch significantly ($p<0.05$) reduced the aphid populations to 4 during the long rains season. The methods did not affect individual aphid species. The polythene mulch was consistently better than the other treatments in reducing total aphids population. The treated yellow trap material followed second and mineral oil the third with the population captured not different from that of unmulched control. Reflective mulch can decrease aphid population density immigrating passionfruit orchards and can be used to reduce vector activity in passionfruit orchards. Yellow material trap and mineral oil have the potential for use in repelling aphids and delaying entry and spread of viral disease in passionfruit orchards but require more evaluation.

6.1 Introduction

Passionfruit (*Passiflora edulis* Sims) is susceptible to several aphid borne viruses that cause the woodiness disease complex with typical symptoms such as mosaics, rugose leaves, vein clearing/ banding, small hard and misshaped fruits with little or no pulp (Novaes and Rezende, 2003). Passionfruit woodiness disease severely limits production of passionfruit (Nascimento *et al.*, 2006) and was first reported in Kenya by Natrass (1944). The viruses involved are PWV, CABMV, EAPV, and SAPV which have been reported in Australia, Brazil, Japan and South Africa (Nascimento *et al.*, 2006; Iwai *et al.*, 2006; Iwai *et al.*, 1997; Brand *et al.*, 1993; Taylor and Kimble, 1964). Other viruses affecting passionfruit include Malaysian Passiflora virus, Uganda passiflora virus, CMV and Passionfruit ring spot virus (Abdullah *et al.*, 2009; Ochow-Ssemakula, 2004; Wijs, 1974). These viruses are transmitted in a non persistent manner by several aphid species with alates being the principal vectors. Plants may be infected with one or more viruses during their growth.

A number of aphids have been recorded as feeding on members of passifloracee. They are an important factor in the secondary spread of viruses within a field and over long distances. In some cases it has been reported that diseased plants seem to be more favorable to rapid vector development than healthy plants (Difonzo *et al.*, 1997). In Japan, *Hyperomyzus lactucae*, *Aphis gosypii* and *Myzus persicae* have been reported vectors of PWV (Omatsu *et al.*, 2004) while *Aphis gosypii* and *M. persicae, Toxoptera citridus* and *A. spireacola* have been implicated as vectors of CABMV in Brazil (Gillapsie *et al.*, 2001, Shukla *et al.*, 1994). Non-colonizing aphids, that probe then reject host, can be important in the spread of viruses infecting crops (Wallis *et al.*, 2005; Omatsu *et al.*, 2004). Sometimes, vector efficiency, large

populations of transient immigrating vectors and geographical influences on the vector plays a role in the spread of virus pathogens. In virus epidemiology, the period that the virus infects or is introduced to a field is equally important because the earlier (longer) the virus is introduced and established in the host, the greater the damage/loss to the crop (Agrios, 2005).

Various practices of virus disease management have been evaluated by a range of authors. Reflective mulches have been used to delay colonization by aphid vectors and to reduce viral disease incidences such as in courgettes (Cradock *et al.*, 2001; Summers *et al.*, 1995). These reflect shortwave ultraviolet light that interferes with the ability of the vector to seek out hosts and reduce the frequency of alates alighting on the plants (Lobenstein and Racah, 1980). Wheat straw reduces aphid landing, virus incidence and retards aphid development by masking the attraction of bear land to aphids (Summers and Stapleton, 2002; Cradock *et al.*, 2001). However, wheat straw reflectance is not strong compared to aluminium coated plastic mulch (Summers and Stapleton, 2002; Cradock *et al.*, 2001).

Insecticides are not effective in controlling virus diseases because of the non-persistent nature of transmission of some viruses. Other practices tested include mild protection biological control, and yellow traps (Wallis *et al.*, 2005; Novaes and Rezende 2003; Desbiez *et al.*, 2002). This study was undertaken to determine the effect of ultra violet reflective plastic mulch and treated yellow material trap within passionfruit on alate aphid management for aphids geographically present in Kenya.

6.2. Materials and methods

6.2 1 Site description

Trials were conducted in two sites, namely Embu (Manyatta area) in farmers' fields and Kabete (University of Nairobi, Agriculture field station). These areas experience a bimodal rainfall pattern with the long rains received in March-June and the short rains in November to December. Temperatures range from 16 to 25°C on average through out the year. The farming systems are such that there is continuous cropping of various fields on small 0.5-2 acre plots in Embu with a lot of intercropping practiced. In Kabete, there is continuous cropping of various crops in separate rotation and commercial fields. There is a prolonged challenge of the crop by aphid presence. The trials were conducted for two growing seasons of other food crops, including the dry season since passionfruit is a perennial crop.

6.2.2 Experimental layout

In Embu, the crop was already established about 6 months to 1year old. Hence, the experiment was designed and the treatments laid out within the established crop which in some cases was already having woodiness disease symptoms. In Kabete, the crop was established by transplanting seedlings on a ploughed land that had planting holes prepared at a spacing of 2 by 3m. The treatment plot sizes were 15 by 8m separated by two rows (4m) of passionfruit from block to block and 6m from plot to plot within the blocks. Diammonium phosphate fertilizer (DAP {18: 46: 0}) was used at planting at a rate of 75g per planting hole mixed with 10kg of manure. This was followed by application of 30g of Calcium ammonium nitrate (CAN) every month. Another 75g of DAP (19.19.19) was applied before the beginning of the rains during the following seasons. A foliar fertilizer was also applied every

three months to correct any nutritional deficiencies as per agronomic recommendations. At the farmers field only manure was applied before the rains.

Treatments were arranged in a randomized complete block design with three replications in Kabete and four replications (farms) in Embu. The treatments consisted of the straw mulch (wheat straw) spread in the plot to a depth of 15cm deep and extended 0.2m on either edge during the preliminary period in 2008. Reflectance of straw and presence of natural enemies was expected to influence the number of aphids alighting on the crop. In 2009, the white reflective plastic mulch was stretched over the inter-row spacing to cover the whole plot. This plastic mulch was secured with pegs and soil at the edges. Holes were made around the plant base to allow for irrigation and application of fertilizers. The plastic mulch was expected to reflect UV light and discourage aphids landing on the crop. The petroleum/mineral oil (DC tron) was used as an insecticide sprayed once a month on the plots. Its smell was expected to affect the insects and the oil to form a thin film layer on the leaves to reduce virus entry during probing/ feeding by aphids. Yellow clothing material treated with an insecticide, Deltamethrin with a binder was another treatment. The insecticide was added on the cloth every two months in the field. The trap was to attract and kill the aphids with the chemical on the material. A control that consisted of untreated plants on unmulched area was used for comparison. In Embu, the farmers were allowed to continue with their management practices in the control plot (management include spraying with fungicides during the dry periods and intercrops such as dry beans and pumpkin vines).

Fluctuations of aphid numbers were monitored using yellow water pan traps with liquid detergent to reduce surface tension. Yellow traps were used since they are attractive to aphid species present in the field which indicates periods of dispersal. Two traps were placed in each replication plot (8 per treatment). The traps were emptied once a week collecting the aphids trapped and preserving them in 70% alcohol for identification and to estimate the populations.

6.2.3 Statistical analysis

Data on aphid populations was subjected to the general analysis of variance (ANOVA) using Genstat statistical software (2005) to assess the effects of treatments on the aphid population. Fishers' protected least significant difference (LSD) test was used to compare significant differences among treatment means ($p < 0.05$). The aphid populations were transformed, (using the formulae: square root + 1) to normalize the distribution for analysis and the non transformed data used for presentation (Binns *et al.*, 2000)

6.3 Results

6.3.1 Aphid abundance and fluctuation trends

Twelve aphid species were trapped and identified in the laboratory using the morphological features of the aphid (Table 5.1 of Chapter 5 of this thesis). The aphids commonly trapped during the experimental period were Cotton aphid (*Aphis gosypii* Glover), Maize aphid (*Ropalosiphum maidis* Fitch), green peach aphid (*Myzus persicae* Sulzer), potato aphid (*Macrosiphum euphorbiae* Thomas), pea aphid (*Acythrosiphon pisum* Harris), cabbage aphid (*Brevicoryne brassicae* L), sowthistle aphid (*Hyperomyzus lactucae*), false cabbage aphid

(*Lypaphis erysimi* Hille Ris Lambers), (*C. aegopodi (Scopoli)*, bean aphid (*Aphis fabae* Scopoli), (*Aphis nusturtii*), Alfalfa aphid (*Therioaphis trifolii*) and *Uleurecon* species complex. These were common in both sites and seasons except the Alfalfa aphid which was identified in Kabete only once in 2009 and the sow thistle aphid that was observed in Embu in the first season only. The total aphid populations were significantly higher in Kabete (6000) than in Embu (1700); and there was a general decline of the aphid population density as the seasons progressed from the beginning to the end of the experiments (Fig 6.1).
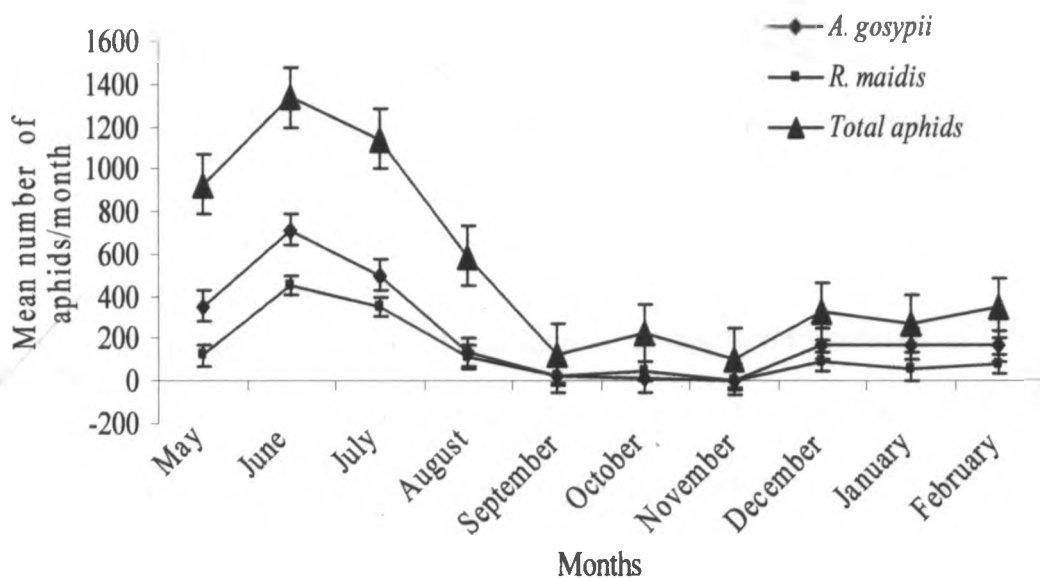
**Fig 6.1 Aphid population dynamics during the sampling period
May 2009 to February 2010**

The most abundant aphid species were *A. gosypii* and *R. maidis* followed by *B. brassicae* and *A. fabae* among others. There was variable effect of different aphid repellant sources on aphid populations present in the passionfruit orchards both in Embu and Kabete. The reflective mulch achieved greater reduction in aphid numbers in all the seasons except in Embu during the long rains season in 2009 (Fig 6.1). High aphid populations were

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consistently recorded in the untreated/ unmulched control plots while the lowest numbers were recorded in the reflective mulch except in the second season at Embu where the yellow material had the least population (Fig 6.1).

6.3.2 Treatment effects on the total aphid populations and aphid species

In 2008, preliminary work in Kabete, showed that aphids were present in the passionfruit orchards but there were no significant treatment effects on the total aphid population during the season (May to November, 2008) (Table 6.1). The population reduced from a peak in June and sharply dropped by August. It remained low for the rest of the experimental period (Fig. 6.2)

In 2009 long rain season, the total aphid population was significantly reduced by the aphid repellant sources (p<0.04) in Kabete. Reflective mulch and the yellow trap had the lowest mean aphid population compared to the untreated control but the two were not different from each another. In the dry season, the reflective mulch was significantly differently (p< 0.01) from the unmulched control on the ovearall aphid population occurring in the passionfruit orchard in Kabete. Reflective mulch had the lowest mean of 10 aphids per trap per week. During the short rains season (December 2009 to February 2010), no treatment effects on the overall aphid population were observed (p= 0.11) but the reflective mulch achieved the lowest population mean, of 8.9 aphids per trap per week compared to control but was not from the yellow treated trap and mineral oil treatments (Table 6.1). Mineral oil (DC Tron) did not differ with the control in all the seasons. However, it had lower aphid numbers compared to control.

Table 6. 1 Effect of different aphid repellant sources on the mean number of total aphid populations May to November, 2008, and May 2009 to February 2010 in Kabete.

Treatment	Trial 1 (LRWS) 2008 Mean no. of aphids/trap/week	Trial 2 (LRWS) 2009 Mean no. of aphids/trap/week	Trial 3 (DS) 2009 Mean no. of aphids/trap/week	Trial 4 (SRWS) 2009 Mean no. of aphids/trap/week
Control	1.7	42.3	25.7	15.2
Straw mulch	1.7	-	-	-
Reflective mulch	-	31.6	10.0	8.9
Yellow treated material	1.7	32.3	21.4	12.3
Mineral oil	1.6	37.6	18.8	13.1
Significance	ns	*	*	ns
Lsd (p< 0.05)	0.2	8.14	9.29	5.1
CV%	7%	6%	22%	30%

* Significance level at p<0.05; ns: not significant; LRWS: Long rain/wet season, DS: Dry season; SRWS: Short rain season/wet season; - treatment not evaluated in the season,
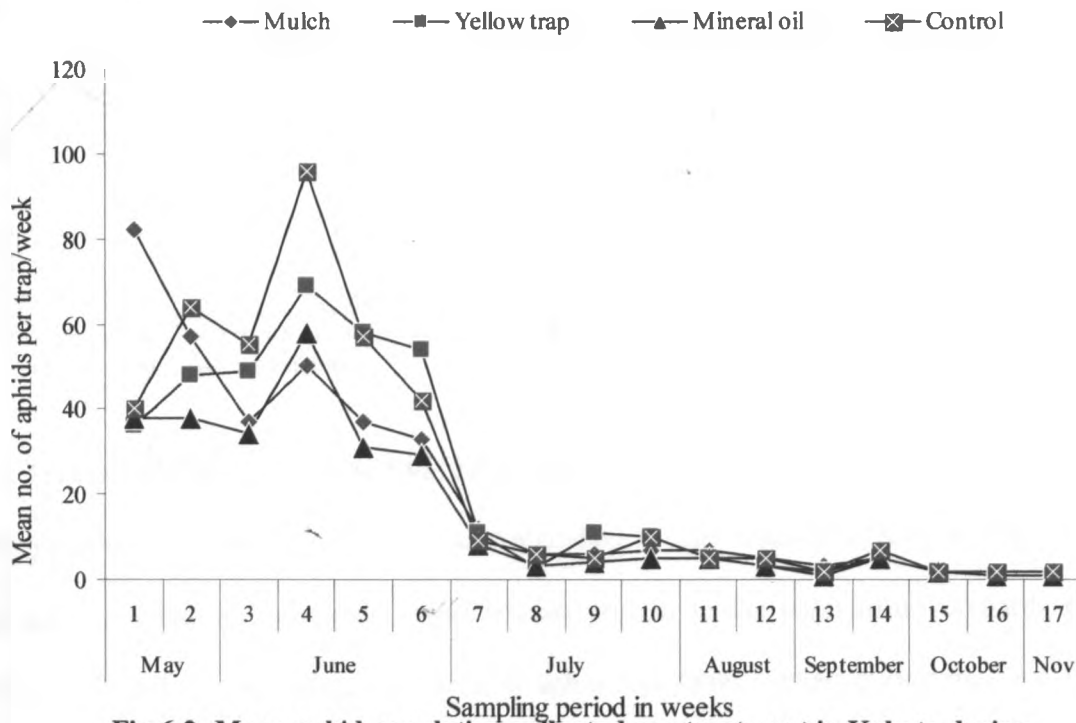
Fig 6.2 Mean aphid population collected per treatment in Kabete during May-November 2008

In Embu, the aphid repellant sources significantly (p<0.04) reduced the total aphid population present in passionfruit orchards during the long rains season. Reflective mulch had the least population of 4.1 aphids, compared to the untreated control with 14.4 aphids and was different from the other treatments. The yellow treated material trap and the mineral oil treatments were not different from control. In the following two seasons, the dry and the short rains seasons, the aphid populations were low and there were no treatments effects observed (Table 6.2).

Table 6.2 Effect of different aphid repellant sources on mean number of total aphid population in Embu from May 2009 to February 2010

Treatment	Trial 1 (LRWS) 2009 Mean no. of aphids/trap/week	Trial 2 (DS) 2009 Mean no. of aphids/trap/week	Trial 3 (SRWS) 2009 Mean no. of aphids/trap/week
Treated yellow material (trap)	13.2	0.9	0.5
Reflective mulch	4.1	0.5	0.7
Mineral oil	9.5	0.6	0.9
Control	14.3	0.5	0.7
significance	*	ns	ns
Lsd (p<0.05)	7.64	0.6	0.6

* Significance level at p<0.05, ns: not significant; LRWS: Long rains /wet season, DS: Dry season; SRWS; Short rains season/wet season

6.3.3 Treatment effects on the individual aphid species

Aphis gosypii and *R. maidis* were the most abundant species trapped in Kabete. Like in the total aphid population, individual aphid species present in the passionfruit orchards were variably affected by the different sources of aphid repellants applied during the long rains and dry seasons (Tables 6.3-6.6). During the long rains season, *Aphis gosypii* and *Acythrosiphon pisum* populations were significantly (p<0.05) reduced by the reflective mulch compared to control (Table 6.3). However, reflective mulch effect on *A. gosypii* was not

different from that by yellow treated trap and mineral oil treatments. Other aphid species populations were not affected by the treatments (Table 6.3).

Table 6.3 Effect of aphid repellant sources on mean number of individual aphid species population in Kabete during the long rain season (May to September 2009)

Treatments	A. gosypii	B. brassicae	C. aegopodii	M. persicae	M. euphorbiae	A. pisum	R. maidis
Yellow trap	13.0	1.2	0.6	1.5	1.8a	2.2	7.2
Reflective mulch	13.9	1.4	1.0	0.9	0.8a	0.9	10.4
Mineral oil	15.3	1.7	0.9	1.8	2.1a	2.6	11.1
Untreated /control	19.0	1.7	0.6	1.9	1.5a	1.7	12.0
significance	*	ns	ns	ns	ns	*	ns
Lsd	3.5	0.9	1.2	0.9	1.3	1.1	4.1
cv %	8	35	47	10	20	9	9

Means per trap/week; * significance at $p < 0.05$; ns: not significant

In the dry season, *A. gosypii*, Potato aphid (*Macrosiphum euphorbiae*), *A. pisum* and *Uleurecon spp* complex were the aphid species whose populations were significantly ($p < 0.05$) reduced by the reflective mulch compared to control. Reflective mulch had the least mean of Uleurocon spp population of 3.5. However, the reflective mulch did not differ from the yellow trap and mineral oil in effect on *A. gosypii*, *M. euphorbiae*, *A. pisum* and *Ulereucon* spp complex. The aphid repellant sources had no effect on other individual aphid species (Table 6.4).

Table 6.4 Effect of aphid repellant sources on mean number of individual aphid species population in Kabete during the dry season (October to December 2009)

Treat-ments	A. gosypii	B. brassicae	Ulereucon spp	M. persicae	M. euphorbiae	A. pisum	R. maidis
Yellow trap	1.7	1.4	8.0	2.5	2.6	2.0	2.4
Reflective mulch	0.7	0.6	3.5	1.3	1.0	1.2	1.4
Mineral oil	1.8	1.3	6.0	2.5	2.0	1.7	2.5
Untreated	1.8	1.4	10.9	2.5	2.5	3.5	2.3
Significance	*	ns	*	ns	*	*	ns
Lsd	0.8	1.0	5.9	1.5	0.9	1.8	1.1
cv %	33	34	31	20	11	41	8

Meansof aphids/ trap/week; * significance level at $p<0.05$; ns: not siginificant;

In the short rains season, only *R. maidis* population was affected ($p< 0.05$) by the reflective mulch and the treated yellow material trap whose mean populations were 2.2 and 2.4, respectively. The reflective mulch and the treated yellow material trap were not significantly different but were different from the unmulched control. Other individual aphid species were not affected by the treatments (Table 6.5).

Table 6.5 Effect of aphid repellant sources on the mean number of individual aphid species populations in Kabete during the short rains season (December 2009 to February 2010)

Treatments	A. gosypii	B. brassicae	A. fabae	M. persicae	M. euphorbiae	R. maidis
Yellow trap	7.2	0.6	0.9	0.5	0.3	2.4
Reflective mulch	4.8	0.5	0.9	0.2	0	2.2
Mineral oil	6.4	0.8	0.9	0.6	0.3	3.9
Untreated	7.8	0.7	1.8	0.6	0.3	4.0
Significance	ns	ns	ns	ns	ns	*
Lsd	2.6	0.5	2.0	0.5	0.31	1.5
cv %	30	31	50	39	38	33

Means of aphids/trap/week; * significance level at $p<0.05$; ns: not siginificant;

In Embu, no aphid species were specifically affected by the different treatments and the populations were rather low like in the short rains season in Kabete. Table 6.6 represents the situation in the long rains season which was repeated in the dry and short rains seasons with lower population densities (Table 6.6).

Table 6.6 Effect of aphid repellant sources on mean number of individual aphid species populations in Embu during the long rains season (May to September, 2009)

Treatments	A. gosypii	B. brassicae	A. pisum	R. maidis
Yellow trap	6.0	1	2.6	3.4
Reflective mulch	2.2	1	0	1.0
Mineral oil	4.7	1.4	1.0	2.2
Untreated	7.1	1	1.4	3.3
P- value	ns	ns	ns	ns
Lsd (p<0.05)	4.0	1.1	2.6	2.7

Ns: not significant

6.4 Discussion

The results obtained from this study show that aphids were present in passionfruit orchards and were reduced by aphid repellant sources when the population densities were high. The aphid population in Kabete in 2008 and the first two seasons in 2009 reached their peaks offering a challenge to the treatments. High population density was present in the first season 2009 in Embu site. *Aphis gosypii, R. maidis and A. pisum* were the most abundant species in both Kabete and Embu sites during the seasons. These are polyphagous insect pests in addition to being reported as vectors of a number of plant viruses including CABMV and CMV (Bashir *et al.*, 2002; Zanic *et al.*, 2009)

The white reflective mulch was a better treatment achieving the lowest total aphid numbers. It reduced the total aphid population density and that of *A. gosypii, A. pisum, M. euphorbiae*

and *R. maidis* as individual species. This reduction was more vivid in Kabete where the aphid populations were high and offering a challenge to the treatments for evaluation. Reflective mulch achieved significant low aphid mean numbers especially in the long rains season in Embu and Kabete site. The results are consistent with those of Franki and Liburd (2005). The dramatic reduction of aphids and a significant drop in population could be due to weather conditions such as light intensity, temperatures rainfall and wind velocity interacting with the aphid repellants which affected aphid numbers thus contributing to the result in this study. Aphids are sensitive to light in their flight habit and as light intensities decrease (particularly UV wave lengths) aphids become less active. Aphid populations are generally low in areas with low temperature, abundant rainfall and high wind velocity (Machangi, 2004, Nderitu, 1991, Nderitu and Mueke, 1986).

It is suggested that the reflective mulches acted by reflecting the short wave radiation unattractive to the flying aphids. This effect has been reported by Prokopy and Owens (1983). Mulching also changes the background of brown (soil) thereby altering crop appearance. This effect interferes with the search pattern of immigrating aphids thereby affecting the aphids landing on the crop (Prokopy and Owens, 1983). Straw mulch acts in a similar way like the synthetic reflective mulch but with low intensity. It requires further evaluation in efficiency and cost management to offer an alternative since, plastic reflective mulch is initially expensive to purchase while the straw is labour intensive and more biological degradable. The low abundance of *A. gosypii*, *M. euphorbiae* and *A. pisum* over the reflective mulch agrees with similar observation made by Franki and Liburd (2005) and

Zanic *et al.* (2009). According to these authors (2009) mulch colour affects the total number of aphids in a crop and the presence of individual species.

The treated yellow clothing material trap which was expected to attract insects which would be killed by the chemical on the material was the second best after reflective mulch. Again the difference in reduction between the yellow trap and control (no treatment) was not significant except for some individual aphid species such as *A. gosypii* and *A. pisum*. Yellow colour attracts a number of insects, hence its use to attract insects in many monitoring experiments in sticky or water pan traps (Ranamukharachchi and Wickramarachchi, 2007; Demirel and Yildrim, 2008). In this study, the yellow trap results are inconclusive. Its second rank indicates its potential of reducing entry of aphid populations in the orchards.

The mineral oil, DC tron oil®, reduced total aphid populations but the reduction was not different with that of the control for both sites and trials. The true efficacy of the oil may not have been apparent since the aphids were monitored with yellow water pan traps. This is because the smell of the oil and the supposed film layer on leaves is active when the insects are near the plants. Unfortunately, aphids do not colonize passionfruit vines as a host plant except for one record (Iwai *et al.*, 2006). Hence, the aphids were still in the plots and were attracted to the traps although they may not have been landing on the crop to feed or probe. The mineral oil would better be evaluated by monitoring aphids on the plants or testing the plants for virus presence. Monitoring aphids on plants was part of this experiment but no aphids were observed on the plants or reported colonizing the passionfruit vines during the experimental period.

Varied results have been reported regarding the efficiency of mineral oil in managing spread of viral diseases. Difonzo *et al.* (1997) while working with potato viruses reported the use of mineral oil as effective and that it acted by forming a thin layer on the crop leaves thus preventing entry or drawing of the virus by the aphids when probing or feeding. Umesh *et al.* (1995) reported ineffective field spread suppression of WMV-2 and CMV by mineral oil in melon fields. Thomas *et al.* (1984) reported successful control of aphid transmitted viruses in cucurbits on application of mineral oil and according to Lobeinstein and Racah (1980) mineral oil was able to delay and reduce spread of PVY and CMV in pepperfields. Wang and Pirone (1996) demonstrated the ability of mineral oil in interfering with the retention of *Tobacco etch virus* in the stylets of *M. persicae*. Cradock *et al.* (2001) suggested that the potential of a treatment to control/ reduce virus diseases can be indicated by monitoring aphid numbers. If numbers are reduced, disease severity within the crop is expected to reduce (Cradock *et al.*, 2001). Results of this study indicate that mineral oil has a potential to reduce aphid vector populations within the crop. However, when aphid densities were high mineral oil performed worse than the control. This agrees with the findings of Simons (1982) and Umesh *et al.* (1995) who confirmed that mineral oil is ineffective in the presence of heavy inoculum pressure.

Reflective mulch significantly reduced aphid population density. Reflective mulch reduced total aphid population and that of the individual aphid species such as *A. gosypii, A. pisum, R. maidis* and *M. euphorbiae* (Francki and Liburd, 2005) and can be used to delay entry and spread of viruses in the orchards. The aphid species significantly affected are reported vectors of CABMV from cowpea to cowpea (Bashir *et al.* 2002). It has been demonstrated

that they are able to transmit CABMV from passionfruit to passionfruit in this studies. The reflective plastic mulch can decrease population density and can be useful in repelling aphids, thus delaying disease onset and spread within orchards. The aphid repellant sources showed significant reduction when aphid populations were high than when they were low thus indicating their ability to affect aphid species and total population. This emphasizes the need to have in place repellants during the peak activity/ dispersal of aphid species in the orchards in order to reduce entry and possible spread of the woodiness disease.

For all the aphid repellant sources, only a proportion of the population was reduced, ranging from 20-70%. The low densities which are left after the application of aphid repellant sources can not be underestimated. This is because efficient virulent vectors are capable of introducing virus pathogens in the field despite the populations present. The repellants may be used together or complementing other vector control tactics to reduce disease spread in an Integrated Pest Management strategy.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Discussion

Passionfruit is an important economic crop in the world, an income earner and for food and nutrition security. Its production is threatened by the woodiness disease which is widely distributed. In Kenya reports indicate the presence of viruses but there is limited knowledge on causal agents present in the major growing areas. The study identified viruses present in the passionfruit growing areas, the potential vectors and evaluated the possible aphid repellants to help in the development of an integrated pest management strategy for the woodiness disease and the vectors involved.

From the findings obtained this study, viruses are widely distributed in passionfruit growing areas with moderate to high incidence. This varied incidence could be attributed to the initial introduction of the virus contaminated plants as primary innoculum since farmers use grafted seedlings and exchange planting materials among themselves. The viruses detected are CABMV, CMV and an unidentified potyvirus. The most common virus was CABMV (45%). Prevalence recorded could be due to diversity of flora in this ecosystem including the presence of other hosts especially the leguminous crops (beans, cowpeas), maize and wheat grown as food crops (Tzanetakis *et al.*, 2003).

In this study molecular evidence was presented and showed that CABMV CP gene from the passionfruit which infected samples in Kenya was closely related to CABMV. On the basis of the species demarcation reported by Shukla and Ward (1988), Ward *et al.* (1994) and

Adams *et al.* (2005) the virus infecting passionfruit in Kenya is a CABMV strain. The Cowpea aphid borne mosaic virus isolates displayed low diversity as suggested by serological results, RT-PCR amplification and confirmed by phylogenetic analysis. The information can be used to develop a diagnostic tool for passionfruit viruses based on serology and PCR methods.

The study confirmed the possibility of detecting CABMV and other viruses from passionfruit leaf tissues. Double antibody sandwich ELISA and RT-PCR were both able to detect CABMV from passionfruit leaf tissues. The primer set developed in this study successfully detected the target virus and can be useful for the monitoring of virus incidence and the indexing of the virus-free passionfruit plants. Reverse transcription PCR as a rapid assay for detecting viruses in the infected leaf tissue of passionfruit can help to minimize time and labour which is required for the diagnosis of passionfruit viruses (Yoon and Ryu, 2009). The ability to detect passionfruit viruses particularly CABMV from leaf tissues provides a valuable tool for the certification programs and is useful for the early stage screening of planting materials in nurseries. The techniques used for detection and identification, could be used for breeding and quarantine programs as well as for studies in the epidemiology and control of CABMV in passionfruit orchards. The distribution of viruses and vectors reported in this study will be useful for breeders to incorporate both virus and aphid vector tolerance into passionfruit cultivars.

Aphid fauna monitoring revealed that the passionfruit growing areas are rich and abundant in aphid species that are vectors of many viruses including CABMV and CMV (Bashir *et al.,*

2002). The most abundant species were *Aphis gosypii, Ropalosiphum maidis, Acyrothosiphon pisum Macrosiphum euphorbiae and Aphis fabae.* The aphids were present in the orchards throughout the year as transient alates whose main peak activity was in June. The peak coincides with the food crops growing season and the vigorously growing passionfruit vines attractive for probing and feeding. *Aphis gosypii, Ropalosiphum maidis, Brevicoryne brassicae* and *A. fabae* found in this geographical zone have been confirmed as vectors of the virus isolate (CABMV). Many vector species present in large populations have an implication on the virus disease distribution and management in the growing areas.

The aphid repellants evaluated, in form of plastic reflective mulch, treated yellow clothing material and mineral oil had significant influence in reducing the aphid populations in the orchard. The impact was high when the aphid population density was high. Plastic reflective mulch had the greatest reduction effect by repelling the aphids. It has been reported severally that the reflective mulch has a role to play in reducing aphid population and virus disease incidence in various crops (Cradock *et al.*, 2001, Summers *et al.*, 2004; Murphy *et al.*, 2009). The plastic reflective mulch can be used by the farmers to reduce aphid activity in the orchards. The yellow water traps used for sampling are effective and simple and are suggested for use by farmers for monitoring vector activity and population densities. The treated yellow clothing material showed a potential to reduce the aphids present in the orchards.

7.2 Conclusions

This study has revealed wide spread presence of viruses in the passionfruit growing areas with CABMV, CMV and an unidentified potyvirus as the causal agents of the virus symptoms observed in the field. The most abundant virus was a CABMV strain whose presence and identity was confirmed by comparison with the known CABMV isolates deposited in the Genbank. The virus is associated with woodiness symptoms in the Kenyan passionfruit orchards. Sequence analysis of various isolates CABMV CP from different passionfruit growing areas in Kenya reveal low variation among the isolates. This is, despite the perennial nature of the plant and the exchange of infected planting materials between different growing areas.

Many aphid species which are vectors of CABMV are transient alates in the Kenyan passionfruit orchards throughout the year with peak activity in June. The aphid species identified as potential vectors of viruses are *Aphis gosypii, Ropalosiphum maidis, Acyrothosiphon pisum Macrosiphum euphorbiae, Aphis fabae and Brevicoryne brassicae*. They are influenced by the cropping systems within the vicinity of the orchards. Aphid repellant sources which were evaluated, that is plastic reflective mulch, treated yellow clothing material and mineral oil have the potential for reducing aphid activity in the orchards, particularly in periods of high population density. Plastic reflective mulch repelled individual aphid species that is *Aphis gosypii, R. maidis, A. pisum* and *M. euphorbiae* which are potential vectors of viruses causing woodiness symptoms in the orchards. The use of the pest management components tested that is, monitoring and identification and use of cultural methods are affordable to the farmers.

7.3 Recommendations

- Reflective mulch has the potential of reducing aphid activity in the orchards and is recommended for adoption by farmers as a management strategy with others to reduce disease entry and spread.

- Further studies need to be conducted to identify the unidentified potyvirus present in passionfruit and determine the CABMV strains present in Kenya.

- It is important to validate the RT-PCR method used to detect CABMV for sensitivity and specificity as a diagnostic tool for use in certification programs

- Further studies need to be conducted to determine the factors that may influence the efficiency of plastic reflective mulch and mineral oil in reducing aphid vectors and suppressing aphid transmitted viruses in passionfruit orchards. Their success could offer practical means of managing the aphid transmitted viruses in passionfruit and other horticultural cropping systems in Kenya.

- Polyethylene reflective mulch is durable in the field with a longer life than the straw and is effective in reducing aphid activity and weed incidence. However, more studies are necessary to evaluate the reflective mulch effect on a wider interow spacing $> 2 < 3m$ so as to gain the full benefits of its reflective action and determine what to forego in terms of yield because of the reduced population in the stand.

- Straw acts in a similar manner like the reflective plastic mulch but with low intensity of reflection. It needs to be evaluated for recommendation as an

alternative for the small scale farmers with fewer resources as it can offer comparable level of aphid control.

- Further research work will be needed to develop passionfruit varieties with resistance to pathogens (viruses) and aphids, especially CABMV.

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APPENDICES

Appendix I. Description of agroecological zones covered in the passionfruit growing areas during the survey

Agroecozone	Description	Rainy season
Lower Highlands (LH)	Tea -dairy zone and wheat/maize-pyrethrum zone with permanent cropping possibilities dividable in long to very long cropping seasons followed by medium cropping 1980m -2280m asl., Annual mean temperature 15.2-17.0°C	First rains start mid March - 740mm and the second rainy seasons in Mid October, 550mm rainfall received surpassed in 6 out of 10 years. Average rainfall 1100-1500mm. length of growing season 220-340 days
Upper midlands (UM)	The zone covers the coffee-tea sub-zone with a fully long rain cropping season , intermediate and medium rains, main coffee sub-zone and the marginal coffee sub zone 1450-1800m, annual mean temperature 19-22°C	Bimodal rainfall received with the first rains from mid March and the second rains in mid October, Average rainfall 700-1000mm.Lenth of growing season 150-200 days

Source: Farm Management handbook of Kenya, Volumes 1 & II, 2006

Appendix II. Standard procedure for ELISA technique for detecting viruses

Add 200ul purified immunoglobulin in coating buffer to each well of the plate and incubate for 4 hours at 37°C at room temperature

wash | The plates are sequentially washed in PBS-T and carefully dried before the next step

Add 200ul test sample in PBS-T plus 2% polyvinyl pyrolidone (PVP) and incubate overnight at 4°C

wash | The plates are sequentially washed in PBS-T and carefully dried before the next step

Add 200ul enzyme labeled immunoglobulin (conjugate) in PBS-T plus 2% PVP and incubate for 4 hours at 37°C

wash | The plates are sequentially washed in PBS-T and carefully dried before the next step

Add 200ul of the substrate (p- nitrophenyl phosphate) in substrate buffer (diethanolamine) and incubate for 30 minutes to 1 hour at room temperature

Visual assessment of yellow colour as observed in the ELISA plate wells

Photometric measurement of absorbance at 405 nm wavelength (ELISA plate reader)

Appendix III. Location of CABMV isolates from Kenya

District	Isolate code	Altitude(m)	Latitude	Longitude
Nakuru (Kabazi)	NKR 1	2285	S 00' 00787	E 036' 19993
Nakuru (Kabazi	NKR 2	2285	S 00' 00787	E 036' 19993
Nakuru (Kabazi)	NKR 3	2272	S 00' 00815	E 036' 19987
Nakuru (Subukia)	NKR 4	2245	N 00' 00145	E 036' 19633
Nakuru (Subukia	NKR 5	2245	N 00' 00145	E 036' 19633
Uasin Gishu (Kiplombe)	UsG 1	2048	N 00' 02641	E 035' 23592
Uasin Gishu (Soy)	UsG 2	2132	N 00' 59971	E 035' 28086
Uasin Gishu (Soy)	UsG 2	2124	N 00' 60069	E 035' 27932
Embu (Manyatta)	EM 1	1671	S 00' 43937	E 037' 45919
Embu (Manyatta)	EM 2	1671	S 00' 43937	E 037' 45919
Embu (Manyatta)	EM 3	1671	S 00' 43937	E 037' 45919
Embu (Manyatta)	EM 4	1687	S 00' 43512	E 037' 46705
Embu (Manyatta)	EM 5	1687	S 00' 43512	E 037' 46705
Embu (Manyatta)	EM 6	1687	S 00' 43512	E 037' 46705
Embu (Manyatta)	EM 7	1687	S 00' 43512	E 037' 46705
Embu (Carorina)	EM 8	1422	S 00' 51735	E 037' 48018
Embu (Carorina)	EM 9	1422	S 00' 51735	E 037' 48018
Embu (Manyatta)	EM 10	1666	S 00' 43762	E 037' 46649
Embu (Manyatta)	EM 11	1666	S 00' 43762	E 037' 46649
Embu (Manyatta)	EM 12	1666	S 00' 43762	E 037' 46649
Embu (Manyatta)	EM 13	1657	S 00' 45536	E 037' 45766
Embu (Manyatta)	EM 14	1657	S 00' 45536	E 037' 45766
Embu (Manyatta)	EM 15	1657	S 00' 45536	E 037' 45766
Embu (Manyatta)	EM 16	1649	S 00' 45424	E 037' 45794
Embu (Manyatta)	EM 17	1649	S 00' 45424	E 037' 45794
Embu (Manyatta)	EM 18	1649	S 00' 45424	E 037' 45794
Embu (Manyatta)	EM 19	1656	S 00' 45053	E 037' 45691
Embu (Manyatta)	EM 20	1656	S 00' 45053	E 037' 45691
Embu (Manyatta)	EM 21	1656	S 00' 45053	E 037' 45691
Meru(Nkuene)	MRU1	1724	S 00' 05420	E 037' 62507
Meru(Nkuene)	MRU2	1724	S 00' 05420	E 037' 62507
Meru(Nkuene)	MRU3	1724	S 00' 05420	E 037' 62507
Meru(Nkuene)	MRU4	1713	S 00' 05269	E 037' 62686
Meru(Nkuene)	MRU5	1713	S 00' 05269	E 037' 62686
Meru(Nkuene)	MRU6	1713	S 00' 05269	E 037' 62686

District	Isolate code	Altitude(m)	Latitude	Longitude
Meru(Nkuene)	MRU7	1818	S 00' 04725	E 037' 60975
Meru(Nkuene)	MRU8	1818	S 00' 04725	E 037' 60975
Meru(Nkuene)	MRU9	1818	S 00' 04725	E 037' 60975
Meru(Nkuene)	MRU10	1853	S 00' 04514	E 037' 60368
Meru(Nkuene)	MRU11	1853	S 00' 04514	E 037' 60368
Meru(Nkuene)	MRU12	1853	S 00' 04514	E 037' 60368
Meru(Nkuene)	MRU13	1914	S 00' 04183	E 037' 59532
Meru(Nkuene)	MRU15	1914	S 00' 04183	E 037' 59532
Meru(Nkuene)	MRU16	1914	S 00' 04183	E 037' 59532
Meru(Nkuene)	MRU17	1914	S 00' 04183	E 037' 59532