DISTRIBUTION, CHARACTERIZATION AND MANAGEMENT OF PAPAYA RINGSPOT ASSOCIATED VIRUSES IN KENYA

NAOMI NZILANI MUMO

DOCTOR OF PHILOSOPHY

(Horticulture)

JOMO KENYATTA UNIVERSITY

OF

AGRICULTURE AND TECHNOLOGY

2022

Distribution, Characterization and Management of Papaya Ringspot Associated Viruses in Kenya

Naomi Nzilani Mumo

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Horticulture of the Jomo Kenyatta University of Agriculture and Technology

2022

DECLARATION

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

Signature..... Date.....

Naomi Nzilani Mumo

This thesis has been submitted with our approval as university supervisors.

Signature..... Date.....

Prof. Edward G. Mamati, PhD

JKUAT, Kenya

Signature..... Date.....

Prof. Elijah M. Ateka, PhD

JKUAT, Kenya

Signature..... Date.....

Prof. Fredah K. Rimberia, PhD

JKUAT, Kenya

DEDICATION

To parents, Mr. and Mrs. Francis Mumo for showing me the value of education.

ACKNOWLEDGEMENT

I am very grateful to the Deutscher Akademischer Austausch Dienst (DAAD), Germany in collaboration with Jomo Kenyatta University of Agriculture and Technology for giving me In-country /in-region PhD scholarship. I am sincerely grateful to Biosciences eastern and central Africa–International Livestock Research Institute (BecA-ILRI) Hub in Nairobi for financial support and also for capacity building in molecular biology.

I would like to express my deep appreciation and gratitude to Prof. Edward Mamati, Prof. Elijah Ateka, and Prof. Fredah Rimberia for their advice, guidance, and for supervising my PhD work. I sincerely appreciate the supervision and guidance of Dr. Francesca Stomeo and Dr. Roger Pelle, both of the BecA-ILRI hub for the time they took to go through my work, motivations, and support in diverse ways to complete my research. I hope that our paths cross many more times in the future. I am also thankful to Dr. George Asudi, who has played a very big part in the whole journey of my PhD.

Special thanks go to Eunice Machuka of BecA-ILRI hub for her assistance while undertaking laboratory work and to Joyce Njuguna, for assistance and guidance in sequence data analysis.

I am deeply indebted to the farmers who granted permission to collect information from them and obtain samples from their farms. Their time and response to all the questions are highly appreciated. Their responses form an integral part of developing a management approach for the papaya ringspot in Kenya.

Without my family, none of this would have happened. To my husband John for his encouragement and support. To my daughters, Agnes and Kerean who had to bear with my absence many times I was away, and many times, I worked late hours to complete this work.

Finally, I remain eternally grateful to the Lord God Almighty for the gift of life and the privilege to attain this level of academic achievement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABSTRACT

Papaya (*Carica papaya* L.) is an important fruit crop in Kenya, grown by small and large-scale farmers for local and export markets, providing income to many papaya producers. Ripe fruits are rich in vitamins A and C, minerals and dietary fiber. Consumption of these fruits is important in preventing vitamins and mineral deficiency in developing countries including Kenya. Production of papaya, however, is constrained by papaya ringspot. Farmers' knowledge, perceptions and management practices of the disease in the country is not documented. Further, the virus (es) associated with the disease as well as their incidence and distribution are not established. In addition, the host range of the virus associated with the disease in papaya is unknown. These information are important in designing long-term and sustainable papaya ringspot management strategies both at a community level and in the country. In the current investigations, farmers' knowledge, perceptions, and management practices for the disease, were studied using a semi-structured questionnaire that was administered through face-to-face interviews to 103 smallholder farmers in five major papaya growing regions and 22 counties in Kenya. The results showed that 38.8 % identified the disease, with 48.8 % of those who identified the disease not knowing the cause. The disease was regarded as a moderate to serious constraint to papaya production by the majority of the respondents. As a management measure, slightly more than fifty percent (54.8 %) sprayed plants showing the disease symptoms with chemical insecticides, 4.7 % removed the infected plants from the field while 40.5 % did not apply any management measure on the diseased plants. It is therefore concluded that papaya farmers in the sampled regions have limited awareness and knowledge and management of papaya ringspot. It is therefore recommended that capacity building of the papaya farmers on proper identification and management techniques of the disease be undertaken. The viruses associated with disease were studied in 48 plant samples collected from the 22 counties subjected to Next-generation sequencing (NGS) using the Illumina MiSeq platform. Sequence analysis revealed the presence of *Moroccan watermelon mosaic virus* (MWMV), a potyvirus, and three Carlaviruses; Cowpea mild mottle virus (CpMMV), and two putative Carlaviruses, closely related to cucumber vein-clearing virus (CuVCV). In reference to typical symptoms observed in the infected plants and sequence similarities with CuVCV, the two putative Carlaviruses were named papaya mottle-associated virus (PaMV) and papaya mild mottle-associated virus (PaMMV). Disease incidence was determined from twenty plants in every field surveyed for papaya ringspot-like symptoms. The highest disease incidence of 71.4, 51.4 and 52.8 were reported in Kiambu, Murang'a and Nakuru counties respectively. The least incidence were recorded in Kwale (3.8) and Busia (2.8) counties. The papaya ringspot prevalence differed across the regions surveyed. A 100 % disease prevalence was reported in Elgeyo Marakwet, Embu, Homabay, Kiambu, Nakuru, Kitui and Vihiga counties. The disease severity was mild (with a severity index of 2.9) across the surveyed counties. Two hundred and eighty-seven leaf samples collected from farmers' fields tested for MWMV, CpMMV and PaMV viral infections showed that MWMV was the most widespread with 140/287 samples testing positive from 11 counties. The PaMV was the second most prevalent virus detected in 39/287 and 9 of 22 counties. CpMMV was the least prevalent and was detected in 7/287 of samples collected and in three counties. Occurrences of MWMV

and PaMV were detected in five counties; Embu, Kirinyaga, Meru, Machakos and Makueni while that of PaMV and CpMMV was detected in Baringo, Meru and Kitui Counties. The results showed that MWMV was associated with papaya ringspot in Kenya and is also widespread in the country. Other viruses previously not known to infect papaya were detected which could pose a threat to papaya production in the country. Therefore, screening papaya seedlings for the viruses before planting would be an important strategy in preventing disease spread. The host range of the MWMV infecting pumpkin intercropped with MWMV infected papaya plants was identified through NGS and compared. The MWMV isolate from papaya and pumpkin were sap inoculated onto 14 plant species belonging to four families; *Datura metal*, *D. stramonium*, *Nicotiana clevalendii*, *N. tabacum*, *N. glutanosa*, *N. bentamiana*, *Cucurbita pepo* (zucchini), *C. moschata* (pumpkin), *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *Phaseolus vulgaris* (common bean), *Vigna unguiculata* (cowpea), *V. radiate* (mung bean) and *Carica papaya* L. in the greenhouse. From the results, the MWMV infecting pumpkin was a different strain from that infecting papaya; sharing 83.4-83.7 % nucleotides (92.3-95.1 % amino acids) sequence identities in coat protein. Through sap inoculation, MWMV isolated from pumpkin infected watermelon, cucumber and zucchini, but unable to infect papaya and other plant species tested. Similarly, MWMV infecting papaya infected zucchini but did not infect other plant species tested suggesting the existence of independent strains having different molecular and biological characteristics associated with the host specificity. Further research should focus on determining transmission of the viruses using vectors.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Papaya (*Carica papaya* L.) is a dicotyledonous perennial fruit crop belonging to the family *Caricaceae,* and genus *Carica* with several other closely related species including *Carica pentagona, C. cauliflora, C. pubescens* and *C. stipulate.* Among these, papaya remains the most widely cultivated and best-known species in the tropics and subtropics (Araújo et al., 2010; Krishna et al., 2008; Teixeira et al., 2007; Yogiraj et al., 2014). Papaya has a soft and unbranched stem enabling it to be produced in smallholder farming systems as well as large plantations (OCDE, 2005). It grows rapidly with minimal maintenance resulting in quick returns (Gonsalves, 1998; Okon et al., 2017; Tennant et al., 2007). It also adapts well to diverse soil and climatic conditions and its fruits are widely accepted (Edward & Ballen, 2015). These characteristics have popularized the fruit crop from a home garden to a commercial fruit crop in many tropical countries (Tennant et al., 2007). The plant flowers 5-6 months after transplanting and the fruits ripen 5-6 months after flowering depending on cultivar and ambient temperature (Rimberia et al., 2018). The plant is usually short-lived, producing fruits for 2-3 years under normal conditions with the possibility of extending to more than 20 years (Yeh & Gonsalves, 1994) depending on a wide range of ecological factors. The fruits which range from 0.5-9 kg may be round, pyriform or oval and green when immature, yellow or red-orange when ripe, with yellow-orange to deep red flesh (Milind, 2011) with varying intermediates.

The plant exists in three sex types namely male, female and hermaphrodite which are difficult to differentiate at the seedling stage (Orwa et al., 2009; Yu et al., 2008). For effective pollination, both male and female plants are intercropped while hermaphrodites which self-pollinate are produced commercially bearing pear-shaped fruits that are most preferred in the market (Yu et al., 2008).

Papaya is believed to have originated from tropical America, probably in Southern Mexico and Costa Rica and throughout the Andes of South America through hybridization between two Mexican species (Chan, 2009). From there it was widely distributed in different geographical regions because of its many seeds and long viability period. The Spanish carried papaya to Europe and Pacific Island and was subsequently introduced as a plantation crop to Australia, Hawaii, the Philippines, Sri Lanka, South Africa and India (Krishna et al., 2008; Milind, 2011). The fruit was introduced into Kenya during the colonial times probably from Hawaii, the Philippines, India, or Indonesia (Asudi, 2010). Currently, the fruit crop is widely grown in many countries in the tropics and to a limited extent, in subtropics in protected structures because of very low temperatures during winter which negatively affects fruit set, growth and production (Chan, 2009).

The papaya fruit crop is ranked fourth worldwide among tropical fruit production after banana, mango and pineapple. Globally, papaya production has increased significantly over the last few years. The fruit is an important agricultural export for many developing countries, with export revenues providing incomes for thousands of people. Papaya exports also contribute to the growing supply of healthy food products on the international markets (Edward & Ballen, 2015). Ripe fruits are delicious, healthy, reasonably priced and are powerhouse of nutrients including vitamins A and C, dietary fibers and minerals such as calcium, thiamine, iron and potassium (Aravind et al., 2013; Daagema et al., 2020; Krishna et al., 2008). Based on the recommended daily allowance for vitamins A and C and minerals, papaya is ranked first among 38 common fruits (Ming et al., 2008). Therefore, regular consumption of the fruit ensures a good supply of nutrients and minerals, promoting good health and preventing early childhood blindness (Krishna et al., 2008) in developing countries including Kenya (Oyunga et al., 2016). The fruit contains low levels of calories i.e. 32 Kcal/100 g of ripe fruit, making it favourite fruit for obese people. The levels of carotene in papaya exceed those of apple, guava and plantains, hence the fruit can prevents damage caused by free radicals associated with some forms of cancer (Daagema et al., 2020; Krishna et al., 2008;

Yogiraj et al., 2014). Unripe papaya fruits have high latex content that hinders their raw consumption. However, raw papaya fruits are shredded in salads, and a variety of savoury Asian dishes including pickles and chutneys and for canning in sugar syrup (Daagema et al., 2020; OCDE, 2005). Nevertheless, the latex contains the enzymes papain that effectively treats trauma, allergies and injuries in sports (Daagema et al., 2020).

Papaya is widely accepted in Kenya and offers considerable promise as a commercial crop for local and export markets. In the country, papaya fruits are cheaper than most other fruits in the urban marketplace (Imungi & Wabule, 1990) making them accessible to the resource-scarce community. In the rural areas, a few trees in the backyards produce adequate fruits that satisfy the needs for vitamins A and C in the country (Oyunga et al., 2016). The crop is grown in many regions of the country by both small- and large-scale farmers as a source of income (Asudi, 2010; Rimberia & Wamocho, 2014). The majority of growers are, however, small-scale producing fruits majorly for subsistence and selling the surplus. Large quantities of fruits produced are consumed as desserts or used to make jam while the leaves serve as composts (Asudi, 2010). The fruit crop is extensively adapted to a range of agro-ecological zones. Major production areas are concentrated in the Coast, Eastern, Rift valley, Central, Nyanza and Western parts of the country. Large scale farms are located in the Eastern and Coastal areas where commercial production is practised mainly for export (Asudi, 2010; HCDA, 2016; Ombwara et al., 2014; Rimberia & Wamocho, 2014).

The area under papaya production in the country continues to expand with no significant increase in yields (HCDA, 2016). This is attributed to several constraints that affect the production of the crop at different stages. The constraints include shortages of quality or use of inferior seeds, inadequate number of improved cultivars, and the inability to differentiate between different sexes of seedlings at planting time (Rimberia & Wamocho 2014). Pests and diseases coupled with poor management are the main factors hampering papaya productivity in Kenya. Of these, viral diseases in particular papaya ringspot is the most serious production constraint responsible for drastic losses in many

orchards (Asudi, 2010; HCDA, 2016; Ombwara et al., 2014; Rimberia & Wamocho, 2014).

1.2 Statement of the problem and justification

Papaya ringspot is unquestionably the most important biotic factor affecting papaya production globally (Tripathi et al., 2008). The disease spreads fast in many parts of the world rendering many orchards economically unproductive, resulting in substantial yield reduction and significant economic losses to farmers. Due to irregular production of fruits as a result of the disease infection, a significant decrease in fruits for local and international markets has been observed (Tennant et al., 2007). The disease infects papaya plants at all stages of growth and naturally spreads very fast leading to 100 % infection of the whole orchard within a very short period with severe yield losses ranging from 20 to 100 % (Chandrashekar et al., 2015; Sharma & Tripathi, 2014; Singh et al., 2017; Chalak, 2017; Tripathi et al., 2008). Fruits infected with the disease are of poor quality due to the presence of ring spots and contain at least 50 % or lower sugar levels making them fetch low prices both in local and exports markets (Gonsalves, 1998; Sharma & Tripathi, 2014; Tripathi et al., 2008; Zambrana-Echevarría, et al., 2016). The papaya ringspot infected orchards also have a short lifespan of less than a year (Tennant et al., 2007). The disease has not spared the Kenyan papaya industry and continues to cause significant yield and crop losses in the country (Asudi, 2010; Ombwara et al., 2014; Rimberia & Wamocho, 2014).

The disease is believed to be caused by the papaya ringspot virus (PRSV) (Tripathi et al., 2008). Previously, attempts to detect PRSV in papaya plants in Kenya showing ringspot symptoms using double antibody sandwich ELISA was not conclusive (Ombwara et al., 2014). Further attempts to detect the PRSV using pathogen-specific primers documented in the literature (Hema & Prasad, 2004; Jain et al., 2004; Diallo et al., 2008; Omar et al., 2011; Srinivasulu & Gopal, 2011; Mohammed et al., 2012; Martínez et al., 2014) and primers designed from PRSV sequences in the GenBank

failed (personal observations). This led to the notion that there could be a different strain of PRSV in Kenya or a different virus(es) infecting papaya in the country.

For effective management of a disease, a proper understanding of the identity, distribution, and diversity of the disease-causing strains/isolates in regions is inevitable (Romay et al., 2014). Further, knowledge of the molecular characteristics of viruses present in the farmers' fields is very important in the development of rapid and effective detection procedures critical components of disease management (Villamor et al., 2019). Although papaya ringspot was previously reported in Kenya based on symptoms, the viral strains responsible for the symptoms were not characterized, with little or no information available to predict the Kenyan disease epidemiology (Asudi, 2010; Ombwara et al., 2014).

With the development of Next-generation sequencing (NGS) technology, plant virus discovery, identification, and evolutionary studies have increased and improved enormously (Roossinck, 2017). The technology can be used to identify plant viruses in a given sample with or without prior knowledge of the viral types present and can also reveal the presence of novel and unsuspected agents. The approach has also been helpful for viral co-infection detection in many plants (Akinyemi et al., 2016; Candresse et al., 2014). Therefore, the NGS approach can be used to identify and characterize the virus(es) associated with ringspot on papaya in Kenya.

The disease has been reported in several regions of Kenya, with a severe impact on papaya production and industry (Ombwara et al., 2014; Rimberia & Wamocho 2014). However, farmers' knowledge of the disease and the management options applied are not documented. Such information requires proper documentation because the information could guide papaya ringspot management as well as national extension systems, by identifying the type of action required to promote more effective and sustainable management of the disease. Further, disease incidence, severity, and distribution in papaya growing areas in Kenya are scarcely known although it is very important information in understanding disease epidemiology, estimating crop losses associated

with the disease, and helping in decision making (Bock et al., 2020). Assessment of the incidence and severity of plant disease is important to determine the geographic distribution and status of the disease throughout a region to prioritize research (Gashaw et al., 2014).

Papaya in Kenya is grown widely by small-scale farmers in mixed cropping systems (Asudi, 2010; Rimberia & Wamocho 2014). Although this cropping system has several advantages including efficient utilization of land resources, enhanced returns per unit area, and insurance against crop failure (Malézieux et al., 2009), the system may facilitate disease spread as intercrops can serve as alternate hosts or reservoirs of pathogens, a crucial role in the perpetuation of several diseases in different crop species (Ara et al., 2012; Martins et al., 2016; Ocimati et al., 2018). Determination of the potential alternative hosts for the virus causing papaya ringspot is important in improving the understanding of the disease epidemiology and supporting the improvement of the management approaches.

1.3 Objectives

1.3.1 Overall objective

To characterize and determine the distribution of papaya ringspot associated viruses for the development of better management options

1.3.2 Specific objectives

1. To assess farmers' knowledge, perceptions and management practices of papaya ringspot in Kenya
2. To determine and characterize viruses associated with papaya ringspot in Kenya
3. To determine the occurrence and distribution of viruses associated with papaya ringspot in Kenya
4. To molecular and biological characterize Moroccan watermelon mosaic virus isolates

1.3.3 Null hypotheses

H1: Farmers' knowledge, perceptions and management practices of papaya ringspot in Kenya are not limited

H2: Papaya ringspot in Kenya is not associated with by papaya ringspot virus (PRSV)

H3: There are no alternative hosts' plants of MWMV isolates

CHAPTER TWO

LITERATURE REVIEW

2.1 Papaya ringspot

Papaya ringspot named because of the presence of 'ring spots' on the fruits of infected plants, is the greatest constraint to papaya production globally (Gonsalves, 1998; Tripathi et al., 2008; Yeh & Gonsalves, 1984). In Kenya, the dark green circular or concentric ring spots of different sizes are common on mature green and ripe papaya fruits and leaves in commercial orchards, small-holder farms, nurseries, and markets (Asudi, 2010; Ombwara et al., 2014) signifying the widespread nature of the disease in the country. The disease has also been reported in Tanzania and Uganda with an incidence ranging from 4 to 100 % (Ndunguru, & Rajabu, 2002).

Early symptoms appear first on young leaves in the crown of the plant and include yellowing followed by mosaic patterns, mottling, vein clearing, distortion, puckering, blistering, and shoe stringing of the leaves. The upper parts of the stems and petioles have irregular water-soaked or oily streak marks. Streaks on the petioles are normally lighter and mix into the normal color of the petiole. Infected plants are characterized by reduced growth and canopy, production of small flowers, and deformed fruits or death (Jain et al., 2004; Sharma & Tripathi, 2014; Ventura et al., 2004). These symptoms, however, differ in intensity depending on the age of infection, viral strain or isolate, and the response of the cultivar (Alviar, 2012).

The disease naturally spreads very fast infecting the whole papaya orchard within a short period. This negatively impacts the cultivation of the crop, forcing many farmers to abandon farming in harshly infested regions leading to a 50 % or more decline in production. The disease infects plants at all stages of growth, from seedling to maturity with symptoms appearing between 3 and 4 weeks after infection (Kumar et al., 2010; Singh et al., 2010). If infection occurs early at the seedling stage or within two months

after planting, the tree fails to produce mature fruits normally (Mowlick & Akther, 2008). The yield losses range from 20 to 100 % depending on the incidence level and the stage of infection (Martins et al., 2016; Gonsalves, 1998; Sharma & Tripathi, 2014; Chalak, 2017; Tripathi et al., 2008).

2.2 Epidemiology and transmission of papaya ringspot

The disease is caused by papaya ringspot virus (PRSV), belonging to the family *Potyviridae* and genus *Potyvirus* (Gonsalves, 1998). The PRSV virions are filamentous, non-enveloped, and flexuous measuring 760–800 nm x 12 nm, and consist of positive-sense single-stranded (ss) RNA with 9000–10,336 nucleotides (nt) long excluding the poly (A) tract. Virus particles (virions) contain 94.5% protein and 5.5% nucleic acid. The genome is monocistronic and is expressed via a large polypeptide of 381 kDa that is subsequently cleaved by the virus-encoded proteinases to yield functional proteins (Sharma et al., 2010).

The PRSV is classified into two serologically indistinguishable biotypes, namely biotype P (PRSV-P) which infects and causes damages in cucurbits and papaya, and biotype W (PRSV-W), which infects cucurbits only (Yeh & Gonsalves, 1984; Gonsalves, 1998; Tripathi et al., 2008). PRSV also produces local lesions on *Chenopodium quinoa and C. amaranticolor*, plants used as indicators of the viruses (Gonsalves, 1998). *Cucurbita pepo* var. cylindrica (zucchini) and *C. moschata* (pumpkin) are hosts of PRSV-P (Noa-Carrazana et al., 2006). Further, PRSV-P can infect zucchini squash, watermelon and other cucumber species through mechanical inoculation with severe symptoms and impact (Mansilla et al., 2013). Other weed species *Cnidoscolus chayamansa* belonging to the Euphorbiaceae family and Momordica charantia L (*Momordica charantia* L.) in cucurbitaceae, also harbour PRSV (Noa-Carrazana et al., 2006; Chin et al., 2007).

PRSV is transmitted by several species of aphids including *Myzus persicae, Aphis coreopsidis, A. craccivora, A. fabae, A. gossypii* and *Toxoptera citricidus* in a non-persistent manner and mechanically through grafting and sap inoculation (Yeh &

Gonsalves 1984; Ventura et al., 2004). Transmission of the virus via seed is possible but rare (Villegas et al., 1990).

Moroccan watermelon mosaic virus (MWMV) belonging to the family *Potyviridae* and genus *Potyvirus* (McKern et al., 1993; Lecoq et al., 2001) also causes ringspot in papaya (Arocha et al., 2008; Mumo et al., 2020; Read et al., 2020). The MWMV has a positive sense ssRNA (~9.7 kb) genome with a single open reading frame that is translated into a large polyprotein cleaved by the virus-encoded proteases P1, HC-Pro and NIa into ten functional proteins (Ibaba et al., 2016; Yakoubi et al., 2008). The MWMV has a narrow host range restricted mainly to cucurbits and papaya, with wide distribution in Africa and the Mediterranean region (Lecoq et al., 1997; Lecoq & Justafré, 2007; Arocha et al., 2008; Yakoubi et al., 2008; Ibaba et al., 2016; Mumo et al., 2020; Read et al., 2020). MWMV is transmitted to cucurbits in a non-persistent manner by *M. persicae nicotianae, Aphis gossypii, A. spiraecola, A. fabae,* and *A. nerii* (Owolabi & Ekpiken, 2014; Chatzivassiliou et al., 2016).

2.3 Management of papaya ringspot

Several strategies for controlling and managing papaya ringspot have been investigated (Mansilla et al., 2013; Ventura et al., 2004). Presently, disease management mainly focuses on the integration of cultural practices, conventional breeding for disease-resistant cultivars, cross-protection and genetic engineering approaches (Yeh & Gonsalves 1994; Gonsalves 1998; Ventura et al., 2004).

2.3.1 Cultural practices

The use of chemical sprays to reduce the levels of the disease damage by spraying against aphids is not very effective because of the rapid transmission of the virus to healthy plants by vectors and the late manifestation of disease symptoms (Ventura et al., 2004). Aphids also have a rapid life cycle with a high reproductive and dispersal rate, making virus dissemination rapid. However, regular and frequent preventative sprays

using systemic insecticides can be used to control aphids starting at the nursery stage (Chalak et al., 2017) and during the peak activity period to reduce the viruliferous aphids (Kumar et al., 2010).

Rouging (Chalak et al., 2017; Ventura et al., 2004) prevents the vectors from spreading the viruses to the nearby trees through periodic removal of infected plants manifesting symptoms. Once removed, the plants dry and die and become less attractive to aphids reducing disease spread. However, it is difficult to fully control the spread of the disease through rouging because of the quick and effective transmission of the viruses by aphids. Further, to avoid financial losses, some growers delay rouging of the infected trees to enable them to harvest more fruits. This increases the chances of the disease spreading leading to greater financial losses to the grower and also to the nearby farms (Ventura et al., 2004). Hence the practice is not an everlasting solution for an area without geographic isolation, because it is expensive and difficult to get rid of the virus sources where the disease has become endemic (Yeh & Gonsalves, 1994). Nevertheless, systematic roguing has been used to effectively manage the disease in Espírito Santo and Bahia states which are the major papaya-producing states in Brazil over the last 25 years (Mansilla et al., 2013; Ventura et al., 2004). In Hawaii, regular surveying and rouging of infected trees in the Hilo and Keaau areas kept the disease from spreading (Gonsalves, 1998).

Pumpkins, watermelon, cucumber and squash play a major role in the epidemiology of papaya ringspot by harbouring the disease-causing virus in papaya and aphid species (*A. gossypii)*, vector for the disease (Bateson et al., 2002; Chin et al., 2007; Gonsalves, 1998; Mansilla et al., 2013). Hence, growers are advised to avoid planting cucurbits near or within papaya plantations because they are potential sources of inoculums and also rogue diseased papaya plants and cucurbits susceptible to natural infection of the virus (Chin et al., 2007; Mansilla et al., 2013). Growing the main crop amid non-host crops (of virus) decreases the spread of viruses. For example, banana and maize used as border crops in papaya orchard reduces the transmission efficacy of the virus by aphids and lower the disease incidence (Chandrashekar et al., 2015; Sharma et al., 2010). Border

crops reduce papaya ringspot incidence because aphids lose the virus inoculum when probing for suitable hosts (Kumar et al., 2010).

Isolation involves stern quarantine measures to restrain the disease in areas with an outbreak. For instance in Australia, the disease was strictly confined to South Queensland. In Brazil, Mexico, Philippines, India and Hawaii papaya growers moved to new areas once the growing regions were infested (Fitch, 2010). Unfortunately, the disease followed the papaya industry and it was more difficult to establish new farms to temporarily escape the virus (Gonsalves, 1998). The challenge with isolation is increasing distance from the market and the need to set up facilities in the new areas (Fitch, 2010).

Several farms in Taiwan have resorted to growing papaya under protective netting to eliminate aphids until the trees have produced a good canopy of fruit. The nets are then removed to allow more sunlight to the trees and thus increase the sugar concentrations of fruit. Trees subsequently become infected, but fruit production is assured for several months. Raising papaya under large net houses is extremely costly but is economically viable because the returns on papaya are very high (Gonsalves, 1998; Ventura et al., 2004).

2.3.2. Host resistance

2.3.2.1. Conventional resistance

Natural resistance of *C. papaya* cultivars to papaya ringspot does not exist (Bateson et al., 1994; Martínez et al., 2014; Zambrana-Echevarría et al., 2016) there are, however, several cultivars developed including ‘Cariflora’, ‘Harichaap’ and Sinta that are tolerant to the disease (Alviar et al., 2012; Crane et al., 1995; Roff & Lumpur, 2007; Singh et al., 2005). These cultivars tend to be symptomless or express only mild symptoms depending on virus strain and produce economically useful yields (Zambrana-Echevarría et al., 2016). Further, levels of resistance have been reported in some wild species of

'highland papaya' from the related genus *Vasconcellea* (Dillon et al., 2006; Sharma & Tripathi, 2014). Some of these species of *Vasconcellea,* include *V. cauliflora, V. cundinamarcensis, V. quercifolia*, and *V. stipulata* (Drew et al., 1998). Conventional breeding for the disease resistance using these wild relatives of *Vasconcellea* has shown only limited success due to interspecific reproductive barriers, leading to the production of infertile progeny with reduced resistance, making it difficult to incorporate resistance genes into *C. papaya* (Drew et al., 1998; Gonsalves, 1998; Srinivasulu & Sai Gopal, 2011). The process is also very slow hindering its application (Zambrana-Echevarría et al., 2016).

2.2.3.2. Cross protection

Cross protection is a phenomenon in which plants systemically infected with one strain of a virus are protected from superinfection by a second related strain of the same virus (Fletcher, 1978; Gal-On & Shiboleth, 2005; Jun et al., 1988). This phenomenon has been attempted to control papaya ringspot in different countries with varying degrees of success (Mowlick et al., 2007; Wang et al., 1987). However, the technique has not been effective in controlling the disease (Azad et al., 2014) due to the adverse effects of the mild strain on papaya plants, the need for extra agricultural practice and care, strain specificity, technical difficulties associated with the development of pure strains of the mild virus, the unavailability of such strains, the breakdown of the protection with time and under heavy disease pressure and also the reluctance of farmers to infect their trees with a virus. Other challenges of this approach include the additional cost of inoculating and indexing the seedlings; difficulties in propagation and preservation of the inoculum and lack of consistent economic returns to farmers (Gonsalves, 1998; Sharma et al., 2014; Yeh & Gonsalves, 1994; Tripathi et al., 2008).

2.2.3.3 Transgenic resistance

The development of the concept of pathogen-derived resistance to combat plant viruses effectively has offered a new approach to controlling papaya ringspot and a lot of

research has been diverted toward developing resistant papaya using coat protein gene (Kumari et al., 2015; Zambrana-Echevarría et al., 2016). Transgenic papaya has been one of the most successful and safe genetically modified (GM) products among horticultural crops (Kumari et al., 2015). Several countries have developed transgenic papaya for viral resistance such as Hawaii (Tennant et al., 2005; Tripathi et al., 2007), Brazil (Júnior et al., 2005), China (Wei et al., 2006), Jamaica (Fermin et al., 2004), Taiwan (Bau et al., 2004), India (Chandra et al., 2010), and Venezuela (Fermin et al., 2004). However, transgenic development of papaya cultivars resistant to specific strains of the virus causing the disease is a challenge due to limiting bio-safety as well as legal frameworks involved in supporting the release of transgenic cultivars and breakdown of resistance by more virulent strains with time (Kertbundit & Juĝíþek, 2010; Kung et al., 2015; Zhao et al., 2016). Further, transgenic plants are virus isolate specific (Martínez et al., 2014; Tripathi et al., 2008).

2.4 Role of alternative hosts in the epidemiology of papaya ringspot

The host range reflects the diversity of species that viruses can naturally infect (Fermin, 2018). Plant viruses have a host range that includes several species from one or more different plant families (Lefeuvre et al., 2019), which provide a reservoir of the pathogens as alternative hosts, from which economically important crop plants may become infected. These alternative hosts are epidemiological bridges for the inoculum between crops of the main hosts (Ara et al., 2012; Dinoor, 1974). Since the alternate hosts contribute to the infection of crop plants in the field by supplying inoculum and acting as important initial sources of infection from which the viruses spread into or within a crop, identification of these alternate hosts is important for studying epidemiological aspects of plant viruses and also in formulating control measures as well as management practices against the virus diseases (Ara et al., 2012; Thresh, 1982).

Cucurbita pepo var. cylindrica (zucchini) and *C. moschata* (pumpkin) host PRSV-P which systemically infects *C. papaya* (Noa-Carrazana et al., 2006). Further, PRSV-P can infect zucchini squash, watermelon and other cucumber species through mechanical

inoculation with severe symptoms and impact (Mansilla et al., 2013). Other weed species belonging to the Euphorbiaceae family also harbour both PRSV and Papaya mosaic virus (PapMV) (Noa-Carrazana et al., 2006). A common weed, *Momordica charantia* L., found growing on fences or the ground along the periphery in papaya orchards, was reported to harbour PRSV with prominent vein clearing symptoms in western Jamaica (Chin et al., 2007). The transmission of the virus using the insect vector revealed high rates of transmission from *Momordica charantia* L. to papaya, papaya to *Momordica charantia* L. and *Momordica charantia* L. to *Momordica charantia* L indicating its potential as a major reservoir of PRSV and its role in the epidemiology of the pathogen (Chin et al. 2007).

2.5. Knowledge, perceptions, and their implications on management practices of plant viral disease

The concept of knowledge, perception, and practices articulates what people know about a problem, how they feel it, what they perceive to be the severity and the cause of the problem, and the type of actions they undertake to deal with it. It assumes that changes in farmers' practices are a collective result of changes in farmers' knowledge, attitudes, and perceptions (Schreinemachers et al., 2015). Because, viral diseases are difficult for farmers and non-experts to identify due to similarity in symptoms to those caused by abiotic stresses (soil nutrients or mineral deficiency), insects or pests damage; knowledge about transmission and infection cycles are important in their control (Islam, 2017; Schreinemachers et al., 2015). Several studies evaluating farmers' knowledge about plant viral diseases have shown a lot of confusion among farmers on proper disease identification and management strategies. This confusion has resulted in to increase in economic losses due to improper methods of disease management with most of the farmers applying unsuitable chemicals as a management strategy leading to environmental pollution (Khan et al., 2014; Lwin et al., 2012; Nagaraju et al., 2002; Schreinemachers et al., 2015).

Farmers in developing countries such as Kenya, India, and Tanzania have long been using indigenous knowledge such as pruning, and roguing to manage crop diseases caused by different pathogens (Asudi et al., 2015; Hubert et al., 2016). Modern scientific knowledge on the identification and management of papaya ringspot has also grown and disseminated through agricultural extension systems (Ventura et al., 2004). Thus, for the successful management of the disease, researchers can integrate the existing indigenous knowledge of the papaya farming community with scientific knowledge in the management of the disease. More importantly, the use of the two folds of knowledge (indigenous and conventional science) can effectively guide papaya ringspot management action, as well as in the national agricultural extension systems. However, the understanding of the level of farmers' knowledge, perceptions and management practices for the disease in Kenya is limited.

2.6 Disease assessment

Plant diseases account for major yield losses of modern agricultural production and continue to cause damage to nearly all crops, where crop production is practiced (Mumford, et al., 2016). Detecting and assessing plant disease are therefore useful in forecasting yield losses associated with disease, monitoring and predicting epidemics, assessing host resistance/susceptibility in plant breeding, making cost-effective disease control/ management decisions, and also in studying essential biological host-pathogen processes such as co-evolution and disease epidemiology (Bock et al., 2010; Bock et al., 2021; Merga, 2018; Mumford et al., 2016). "Remote sensing" is a term that is used in quantifying disease on plants by measuring symptoms (Bock et al., 2010) and visual assessment is probably the most widespread method used for quantifying plant disease (Bock et al., 2011). Various terms are used in estimating and measuring plant disease (see review by Bock et al., 2010) including i. Disease intensity a term that describes the amount of disease present in a population. ii. Disease prevalence is the proportion (or percent) of fields, counties, states, etc. where the disease is detected. iii. Disease incidence is the proportion (or percent) of plants (or plant units, leaves, branches, etc.)

diseased out of a total number assessed and iv. Disease severity is the area (relative or absolute) of the sampling unit (leaf, fruit, etc.) showing symptoms of the disease.

2.7 Plant virus diagnostics and detection

Viral diseases cause losses of several billion dollars every year threatening sustainable and productive agriculture worldwide hence accelerating the current food supply deficiency in which at least 800 million people are inadequately fed (Mumford et al., 2016; Rubio et al., 2020; Strange & Scott, 2005). To combat the losses caused by disease, it is indispensable to define the problem and seek solutions (Strange & Scott, 2005). Correct identification and detection of disease-causing pathogens remain a key focus, particularly in the field of plant virology (Roy et al., 2013; Strange & Scott, 2005). This is because, due to the lack of host immune systems and post-infection therapy options for virus infections, early and correct pathogen detection is the best tool for the prevention of disease epidemics (Roy et al., 2013).

Virus identification and characterization allow the development of reliable detection methods which are key components in disease management (Villamor et al., 2019). Further, using the developed detection methods, virus population structure can be studied allowing further improvement of the method to accommodate different isolates and strains. The reliable detection methods can be applied in plant breeding to study virus resistance, testing of planting materials and in the implementation of effective management strategy targeting the virus vector at the beginning of the infection more so in high-value perennial crops (Villamor et al., 2019).

2.7.1 Symptomatology

Symptoms portrayed on diseased plants are generally used to identify a viral disease of known etiology and also assist in removing diseased plants as a way to control the disease spread. This is because visual scrutiny is quite easy when symptoms depicted have clear characteristics of a specific disease (Naidu & Hughes, 2003). However,

symptoms expressed can be deceptive (Candresse et al., 2014), and may not be a precise indication of virus identity and its interpretation should be treated with caution (Hamilton et al., 1981). Diagnosis of viral diseases based on symptoms is more difficult than in other pathogens (Jeong et al., 2014). This is because sometimes, the type of symptom may be indicative of a distinct virus or virus group, or due to the influence of prevailing environmental conditions (Hamilton et al., 1981; Bock, 1982; Tripathi et al., 2008; Schreinemachers et al., 2015). As a result, visual inspections for symptoms in the field should be used in conjunction with other confirmatory tests to ensure accurate viral disease diagnosis (Bock, 1982).

2.7.2 Biological indexing

Biological indexing (assay) is a method of virus identification and diagnosis based on the ability of certain susceptible plants, known as indicator plants, to produce symptoms when the virus is mechanical, graft, and vector transmitted onto them (Legrand, 2015). Biological indexing ensures that plants for planting are free from regulated diseases. Some indicator plants are susceptible to many viruses and virus-like diseases and can therefore be used to detect a wide range of pathogens. However, several factors can influence the results of biological indexing, affecting the analytical specificity, sensitivity, and reliability of the tests. Hence biological assays should only be considered for use in conjunction with serological or molecular tests to detect and identify pathogens (Legrand, 2015).

2.7.3 Physical properties

Physical properties of a virus such as thermal inactivation point (TIP), dilution endpoint (DEP), and longevity in vitro (LIV) as a measure of virus infectivity in sap extracts are used to identify plant viruses. These methods are still used in some laboratories for diagnostic and taxonomic purposes, although they have largely outlived their usefulness (Hamilton et al., 1981). This is probably because physical properties are not good taxonomic indicators more so for viruses that are neither particularly stable nor unstable.

2.7.4 Microscopy

Microscopy provides important information on the morphology of the virus particles and is normally used for virus detection when electron microscopy (EM) amenities are readily available. Viruses that are rod-shaped and filamentous such as tobamoviruses, potyviruses and potexviruses can more readily be differentiated in negatively stained leaf-dip preparations than isometric viruses and other viruses (Naidu & Hughes, 2003). Different plant viruses induce distinguishing intracellular inclusions or develop large crystalline accumulations of virus particles making their detection by EM simple, rapid, and less expensive methods to confirm viral infection. Due to the uniqueness of inclusions produced as a result of infection by some viruses, unknown viruses can occasionally be identified based on inclusion bodies observed using selective stains (Naidu et al., 1998). However, EM for viral disease identification requires a lot of experience. Further, it is labour intensive and cannot be used for the rapid processing of multiple samples.

2.7.5 Serological methods

Rapid and specific serological techniques such as enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses have been developed in the last decades (Rubio et al., 2020). ELISA is based on the ability of specific viral proteins to bind with antibodies (Clark & Adams, 1977). To achieve a precise and reproducible result, the timing and development conditions of the enzyme-substrate reaction need to be optimized. ELISA has been used as a very common assay to identify plant viruses within plant material, insect vectors, and seeds (Clark & Adams, 1977; Naidu & Hughes, 2003; Webster et al., 2004). Advantages of ELISA include sensitivity, a high number of samples can be processed at a given time, the amount of antibodies required for disease detection is little, and the process can be semi-automated (Naidu & Hughes, 2003). However, ELISA has limitations including the requirement for a relatively large amount of samples to capture an antigen of interest in wells coated with the capture antibody.

The large surface area of the wells and the hydrophobic binding of capture antibodies can result in a non-specific binding and increased background (Baker et al., 2012)

2.7.6 Molecular techniques

Molecular hybridization relies on binding viral nucleic acids with sequence-specific DNA or RNA probes, due to their sequence complementarity. The polymerase chain reaction (PCR) technique is one of the methods of virus detection using the DNA amplification approach. The technique is used to create millions of copies of a particular DNA sequence within a small reaction tube (Jeong et al., 2014). Before amplification, the DNA is denatured by heating at 90°C to 95°C to separate the double-stranded DNA (dsDNA) template into single strands. Two sets of primers (oligonucleotides) are allowed to bind the start and end of the target DNA by cooling at 40°C to 60°C (annealing), leading to DNA synthesis by the DNA polymerase. By extending heating from 70°C to 75°C, the thermostable DNA polymerase synthesizes new DNA strands starting from the primer (Jeong et al., 2014; Rubio et al., 2020).

PCR approach has many applications in molecular biology including cloning, gene manipulation, gene expression analysis, genotyping, sequencing, and mutagenesis. Currently, PCR is common too for the diagnostic of plant viruses in the laboratory and is normally used in molecular experiments (Jeong et al., 2014; Webster et al., 2004).

2.7.7 Next-generation sequencing

Next-generation sequencing (NGS) also known as high-throughput sequencing (HTS) technologies has impacted hugely on the life sciences with wider utility (Kulski, 2016). The approach sequences the total nucleic acid content in a sample for the subsequent identification of pathogens by bioinformatics tools, an approach referred to as metagenomics (Riesenfeld et al., 2004; Wu et al., 2015). Early in the development of NGS, complete new viral genomes were determined using 454 sequencing of nucleic acids extracted from diseased plants. In 2005, the former Solexa (today Illumina)

developed a technology based on sequencing by synthesis using reversible dye-terminator chemistry, which is presently widely used by many researchers (Blawid et al., 2017).

The viral metagenomics employing the NGS has aided the identification of recognized and unidentified viral pathogens and those that occur at extremely low titers. These viruses include the detection of two quarantined mastreviruses in sugarcane that had gotten away from routine quarantine viral detection (Candresse et al., 2014), Apricot vein clearing associated virus (AVCaV) in apricot (Marais et al., 2015), cytoplasmic Citrus leprosis virus (CiLV) infecting citrus (Roy et al., 2013), *Grapevine rupestris vein feathering virus* and *Grapevine yellow speckle viroid 1* in grapevine (Kreuze et al., 2009; Roy et al., 2013; Wu et al., 2015), pumpkin polerovirus characterized in pumpkin (Kidanemariam et al., 2019), and the common mosaic necrosis virus cucumber mosaic virus and *Phaseolus* vulgaris alphaendornaviruses 1 and 2 (Mutuku et al., 2018) infecting beans. Other viruses identified through NGS approach include maize lethal necrosis, maize chlorotic mottle virus, sugarcane mosaic virus (SCMV), maize streak virus and the maize yellow dwarf virus infecting maize and sorghum in Kenya (Wamaitha et al., 2018). Three dicistroviruses including aphid lethal paralysis with intra-species recombination, *rhopalosiphum padi*, and big Sioux river viruses characterized in aphids and maize using NGS shotgun metagenomics in Kenya (Wamonje et al. 2017). The NGS viral metagenomics approach has shown the diversity of viruses and strains and their genetic differences from isolates and has also proven to be a crucial tool for understanding viral community structure and discovering novel genes. Further, the approach provide insight into virus-host interactions, an important step in understanding the co-evolution of host and viral genomes as well as uncovering the presence of viral types not previously described in certain environments (Rosario & Breitbart, 2011).

CHAPTER THREE

FARMERS' KNOWLEDGE, PERCEPTIONS AND MANAGEMENT PRACTICES OF PAPAYA RINGSPOT IN KENYA

Abstract

The production of papaya, an important fruit crop in Kenya is severely constrained by papaya ringspot. Understanding farmers' knowledge, perceptions and practices is a prerequisite to establishing an effective disease management strategy at the community and national levels. Field surveys were conducted in five major papaya growing regions in Kenya namely Coast, Western, Rift valley, Central and Eastern to determine farmers' knowledge, perceptions and management practices of the disease. A total of 103 papaya farmers were identified and interviewed during the field surveys using a semi-structured questionnaire. About thirty-nine percent (38.8 %) of the respondents were aware of the disease, reported its occurrence on their farms, perceived the rate of spread as fast, and described the disease as a moderate to a serious problem in papaya production. Of the respondents of the disease, 48.8 % did not know the cause. As a management practice, 54.8 % sprayed insecticides on plants showing ringspot disease symptoms while 40.5 % did not apply any measures. These findings indicate knowledge, as well as management of papaya ringspot, are limited for most papaya farmers in the surveyed regions. As such, there is a need for capacity building of the Kenya papaya farmers on proper identification and management techniques of the disease. These may include equipping the farmers with identification of the disease symptoms as well as educating them on the integrated management techniques for the disease.

3.1 Introduction

Papaya is an important fruit crop in Kenya grown for domestic consumption, and local and export markets (Asudi, 2010; Rimberia & Wamocho, 2014; HCDA, 2016). It is one of those fruit crops that produce fruits throughout the year under optimal management.

The fruits are rich in vitamin A and C. Vitamin A deficiency is a major nutritional and public health problem among children under 5 years in Kenya (Oyunga et al., 2016). The ripe fruits are also a major source of income for farmers, especially the resource-scarce farmers in rural areas. Its production is however severely constrained by biotic and abiotic stressors, with viral disease infections playing an important role (Asudi, 2010; Rimberia & Wamocho 2014; HCDA, 2016).

Papaya ringspot is the most destructive viral disease affecting papaya production in Kenya (Ombwara et al., 2014; Rimberia & Wamocho, 2014). The disease affects papaya plants at all stages of growth, eventually causing yield losses of up to 100 % (Tripathi et al., 2008; Sharma & Tripathi, 2014; Chalak et al., 2017). Papaya ringspot infected plants are easily recognized by symptoms including the presence of ring spots on the fruits and mosaic, mottling, vein clearing, puckering, shoe stringing, downward leaf curling and distortion. Additionally, the infected papaya plants have stems and petioles with irregular oily or water-soaked marks (Arocha et al., 2008; Tripathi et al., 2008). Some of these symptoms closely resemble those caused by other plant stressors, such as insect or pest damage, and soil nutrient toxicity and/or deficiency (Tripathi et al., 2008; Schreinemachers et al., 2015). The infected papaya fruits have low sugar content, which together with the ring spots lower their quality and hence make the fruits to fetch low prices in the markets (Tripathi et al., 2008; Sharma & Tripathi, 2014).

A fundamental part of designing integrated disease management approaches in agriculture is the knowledge and perceptions of farmers on the disease as well as its implications on management practices. Knowledge, for instance, informs how farmers know and appreciate a problem, and perceptions inform how farmers feel about the problem, and its cause and this information certainly influences the farm cultural practices they carry out (Lwin et al., 2012; Schreinemachers et al., 2015). Farmers in developing countries have for a long time been using local knowledge to manage crop diseases caused by different pathogens (Asudi et al., 2015; Hubert et al., 2016). For successful management of the disease, researchers can integrate the existing indigenous knowledge of the papaya farming community with scientific knowledge in the

management of the disease. More importantly, the use of indigenous and conventional science can effectively guide papaya ringspot management actions. However, the level of farmers' knowledge, perceptions and management practices for papaya ringspot in Kenya is limited. Therefore, the objective of this study was to determine farmers' knowledge, perceptions and management practices of papaya ringspot in major papaya growing regions of Kenya.

3.2 Materials and Methods

3.2.1. Study areas and data collection

The study was conducted in 22 counties in five major papaya growing regions in Kenya namely Coast (Taita Taveta, Kwale, and Kilifi), Western (Siaya, Kisumu, Homabay, Migori, Bungoma, Busia and Vihiga), Rift valley (Nakuru, Baringo and Elgeyo Marakwet), Central (Kiambu, Murang'a, Kirinyaga) and Eastern (Makueni, Embu, Tharaka Nithi Meru Machakos and Kitui) (Figure. 3.1). These regions were selected because of the relatively high number of farmers growing papaya (Asudi, 2010; Ombwara et al., 2014).

Surveyed sites were mapped using a global positioning system (GPS; Magellan GPS 315, San Dimas, CA). The altitudes of the surveyed sites ranged from; 11 to 1116 m above sea level (a.s.l.) at the Coast, 784 to 1568 m a.s.l. in the Eastern, 1020 to 1914 m a.s.l. in the Rift Valley, 1160 to 1523 m a.s.l. in the Western, and 877 to 1576 m a.s.l. in the Central region.

Papaya farmers in the counties within these regions were randomly selected for the study. When farmers resided within the same county, only those spaced at a minimum distance of 5 km were interviewed. Data for the study were collected using a semi-structured questionnaire administered through face-to-face interviews (Appendix 1). The questionnaire was pre-tested on five farmers before conducting the study. The data collected included socioeconomic characteristics of the farmers, papaya production,

farmers' knowledge, perceptions as well as management practices of the disease. Farmers' knowledge was assessed by asking if they were aware of papaya ringspot and its occurrences on their farms. The responses to the knowledge questions were recorded in a series of binary responses (1 for yes and 0 for no) as described previously (Asudi et al., 2015); Khan et al., 2014). A4-sized photographs of a papaya plant with ringspot infected leaves, stems, petioles and fruits were used to assess farmers' knowledge of the disease (Plate 3.1). The photos had no text to ensure the identification was based on visual cues by correlating the symptoms in fields with those in the pictures.

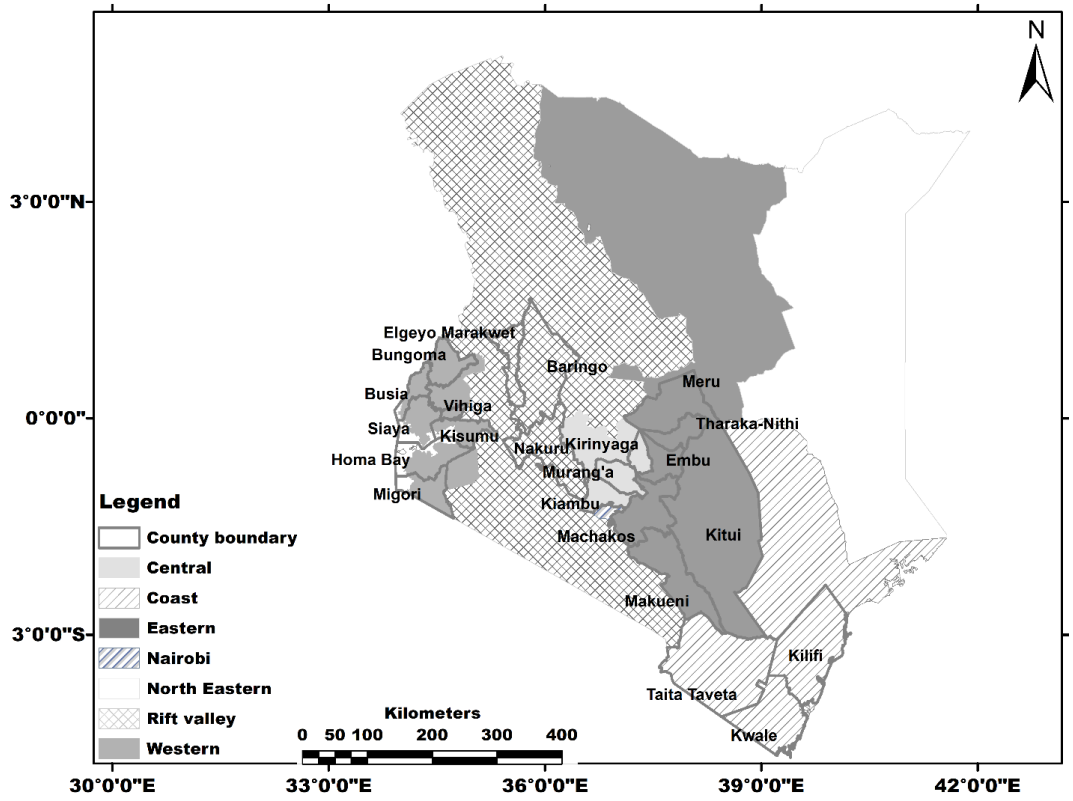

Figure 3.1: A map of Kenya showing the study regions for farmers' knowledge, perceptions and management practices of papaya ringspot.

When needed, the disease symptoms were described to the farmers. The perception of the disease problem and its rate of spread was captured as a categorical variable using a 4-point Likert scale rating (Khan et al., 2014; Asudi et al., 2015). For this, farmers were

asked to rate the disease problem on a scale of 0 to 3, where 0 = no problem, 1 = moderate problem, 2 = severe problem and 3 = very severe problem. The rate of disease spread in the past year was scored on a scale of 0 to 2 where 0 = no spread, 1 = slow spread and 2 = fast spread. Interviewed farmers were also asked to name the papaya cultivars they grew, the purposes for which papaya were cultivated, the source of planting materials, the cropping system, the seasonal prevalence of the disease on their papaya crops and the control measures they practised (Appendix 1).

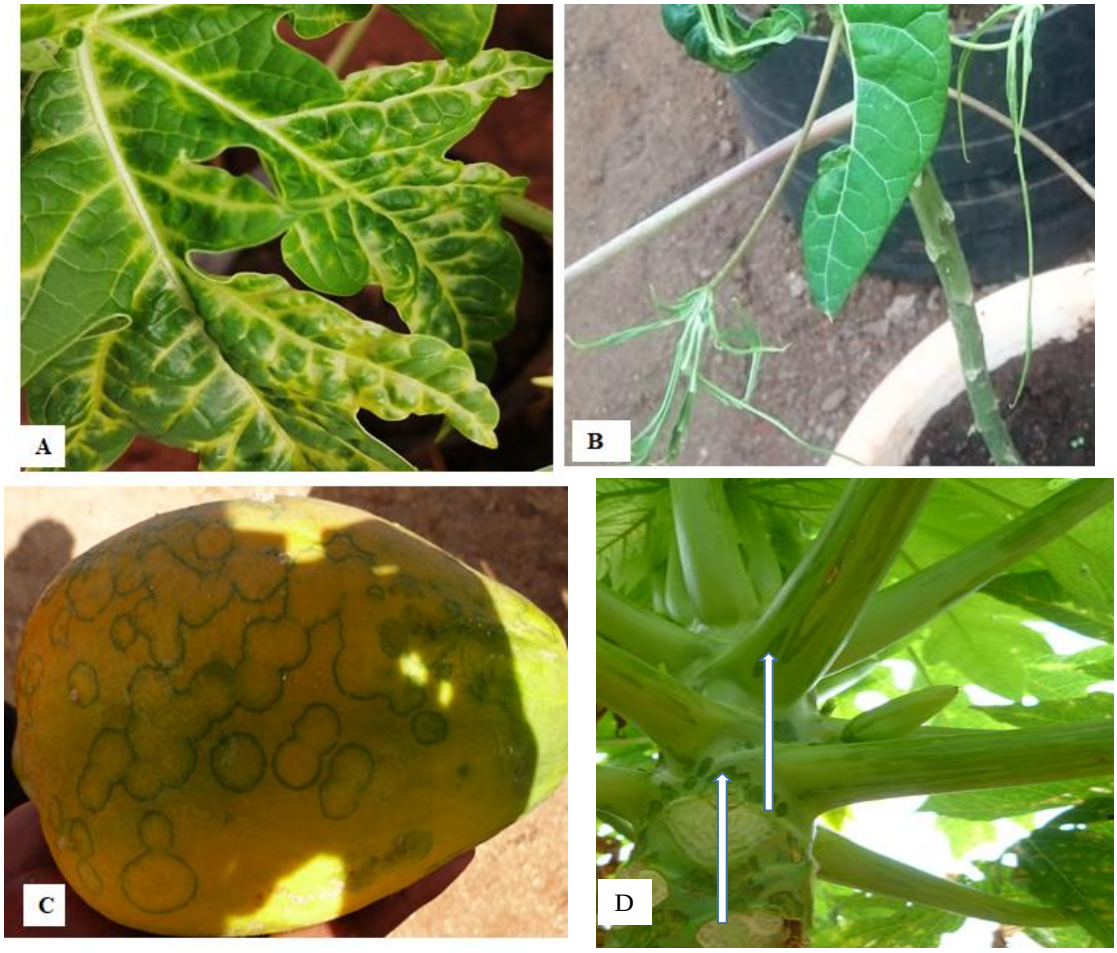

Plate 3.1: Pictures of papaya ringspot symptoms used in evaluating farmer's knowledge. (A): Leaf distortion, puckering, mosaic and vein clearing; (B): Shoe stringing; (C): Ringspots on fruit and (D): Water or oil-soaked marks on stem and petioles (shown with arrows).

3.2.2 Data analysis

Data collected were cleaned and analysed descriptively by frequencies and percentages in the Statistical Package for Social Sciences (SPSS, ver. 20; SPSS Inc. Chicago, USA).

3.3 Results

3.3.1 Socio-economic profile of respondents

A total of 103 farmers (28, 14, 37, 8 and 16 from Central, Coast, Eastern, Rift Valley and Western, respectively) were interviewed (Table 3.1). Slightly over half (58.3 %) of the respondents were male. The respondents' age was between 20 and above 60 years, and majority (41.7 %) of them were between 41 to 50 years (Table 3.1). Half (50 %) of the respondents had attained secondary school education (12 years of basic education). Most (56.3 %) of the farmers interviewed produced papaya for use at home and sold the surplus. Central, Coast and Eastern regions had 70.3 %, 50 % and 57.1 % of the households producing papaya for subsistence and market, respectively, while in the Rift Valley region, 50 % produced papaya fruits for the market. In the Western region, 56.2 % of the respondents cultivated papaya for subsistence (Table 3.1). About 30 % of the farmers sold between 26 and 50 % of their produce. However, the overall proportion of farms under papaya cultivation was very low with 51.5 % of the respondents allocating less than 2 % of their total land to papaya cultivation.

3.3.2. Papaya cultivars and cropping system

Majority (87.3 %) of the respondents practiced an intercropping system with papaya being grown mixed with other crops (Table 3.2). In Central, Coast and Eastern regions, at least 9 in 10 farmers (91.9 %, 92.9 %, and 92.9 % respectively) intercropped papaya with other crops. The proportion of farmers who intercropped papaya with other crops in Western was much lower (80 %) and lowest in Rift valley (50 %). Maize, banana, mango, cowpea, sweet potato, coffee, pigeon pea, cassava, citrus, passion fruit, vegetable crops and cucurbits, were some of the crops intercropped with papaya. A

majority (74 %) of the respondents did not know the names of the papaya cultivars in their fields (Table 3.2). 'Solo sunrise' was the most common cultivar grown by 16.3 % of the papaya farmers followed by 'SP' (7.7 %) and 'Mountain' (6.7 %). The respondents from central region reported the highest number of these cultivars (Table 3.2). The survey also recorded 'Malaysian 5', 'Red royale', Vega F1 and 'Sinta F1', in the Central region. These cultivars were recently imported to Kenya.

Table 3.1: Socio-economic characteristics of the respondents interviewed in five regions of Kenya

Variable	Region					Mean
	Central	Coast	Eastern	Rift Valley	Western	
Number of farms surveyed	28	14	37	8	16	103
Gender of the respondents (%)						
Male	62.2	42.9	60.7	62.5	56.2	58.3
Female	37.8	57.1	39.3	37.5	43.8	41.7
Age (years) (%)						
20-30	-	-	5.4	25.0	25.0	7.8
31-40	32.1	42.8	29.7	37.5	31.2	33.0
41-50	57.2	35.7	45.9	37.5	12.5	41.7
51-60	10.7	4.2	16.2	-	12.5	12.6
>60	-	7.1	2.7	-	18.7	4.8
Education levels (%)						
Below primary	–	–	3.6	14.3	18.8	4.9
Primary	27.0	28.6	28.6	14.3	50.0	30.4
Secondary	54.1	64.3	53.6	42.9	25.0	50.0
Tertiary	18.9	7.1	14.3	28.6	6.2	14.7
Utilization of papaya (%)						
Subsistence	5.4	28.6	28.6	12.5	56.2	23.3
Subsistence and market	70.3	50.0	57.1	20.0	37.5	56.3
Market	21.6	21.4	14.3	50.0	6.2	19.4
Proportions of papaya fruits sold						
0 –25 %	20.6	33.3	40.0	14.3	14.3	26.2
26 –50 %	29.4	8.3	25.0	28.6	85.7	30.0
51 –75 %	35.3	25.0	10.0	–	–	21.2
>75 %	14.7	33.3	25.0	57.1	–	22.5
Proportion of farm allocated to papaya cultivation						
0 –2 %	35.1	57.1	57.1	37.5	81.2	51.5
3 –4 %	43.2	14.3	14.3	12.5	18.8	25.2
5 –8 %	13.5	7.1	10.7	–	–	8.7
>8 %	8.1	21.4	17.9	50.0	–	14.6

All the Western region respondents (100 %) did not know the cultivars they planted (Table 3.2). About 76.9 % of the respondents saved seeds from healthy-looking ripened fruits from their farms, while 9.6 % source seeds from the nearest neighbouring farms or National Research Institutes such as the Kenya Agricultural Livestock and Research Organizations (KALRO). Nine farms (3.8 %) in the Central region bought seeds from commercial seed companies (Table 3.2).

Table 3.2: Cropping system, source of planting materials and papaya cultivars grown by farmers in the selected regions

Variable	Region					Mean
	Central N = 28	Coast N = 14	Eastern N = 37	Rift Valley N = 8	Western N = 16	
Papaya cropping system						
Intercrop	91.9	92.9	92.9	50	80	87.3
Monocrop	8.1	7.1	7.1	50	20	12.7
Papaya cultivars grown by farmers (%)						
Not sure of the name	51.4	85.7	92.9	50.0	100	74.0
Solo sunrise	27.0	7.1	7.1	50	–	16.3
Mountain	8.1	14.3	3.6	–	–	6.7
SP	18.9	7.1	–	–	–	7.7
Malaysia	2.7	–	–	–	–	1.0
Sinta F1	2.7	–	–	–	–	1.0
Red Royale	5.4	–	–	–	–	1.9
Vega F1	2.7	–	–	–	–	1.0
Source of planting materials (%)						
Farmer's own seed	59.5	92.9	82.1	75.0	93.8	76.9
Neighbours	10.8	7.1	10.7	1.5	6.2	9.6
Market	2.7	–	–	–	–	1.0
Imported	10.8	–	–	–	–	3.8
KALRO	16.2	–	7.1	25.0	–	9.6

'N' is the number of farmers surveyed, (-): no reported case.
KALRO: Kenya Agricultural and Livestock Research Organizations.

3.3.3 Awareness of papaya ringspot

Knowledge of papaya ringspot among respondents was minimal with 38.8 % of the respondents able to correctly recognize infected plants based on the symptoms exhibited indicating awareness of the disease. In Central, Eastern, Coast and Rift valley regions, 59.7 %, 39.3 %, 35.7 %, and 25 % of the farmers interviewed, respectively were aware of the disease. The respondents from the western region, surprisingly, did not recognize the disease symptoms (Table 3.3). Of those knowledgeable, 95.5 % were aware of the disease's presence on their farms. The spread of the disease was rated as fast, with 100 %, 60 %, 57.1 % and 50 % of the respondents from Central, Coast, Eastern and Rift valley regions, in the respective order, indicating that papaya ringspot was spreading fast on their farms. Half (50 %) of the respondents regarded papaya ringspot as a moderate constraint to papaya production, with 40.5 % regarding it as a serious problem. Fifty-seven percent of the respondents perceived symptoms of the disease on papaya crops to be more prevalent during the dry season, while 26.2 % of the respondents were not aware of when the symptoms were prevalent. In Central, Rift valley and Eastern regions, 71.4 %, 100 %, and 42.9 % of the respondents, respectively perceived the disease to be prevalent during the dry season, while 60 % of the respondents in Coast region were not aware when the symptoms were most prevalent (Table 3.3).

Table 3.3: Awareness of papaya ringspot among farmers in selected regions of Kenya

Variable	Region					Mean
	Central N = 28	Coast N = 14	Eastern N = 37	Rift Valley N = 8	Western N = 16	
Farmers' awareness (%) of ringspot						
Yes (%)	59.5	35.7	39.3	25.0	–	38.8
Farmer's awareness of the presence of papaya ringspot in their farms						
Yes (%)	95.5	83.3	100	100	–	95.5
Is the disease spreading on the farm? (%)						
Yes	100	100	92.9	100		97.6
Rate of spread of ringspot disease (%)						
Slow	–	40.0	42.9	50.0	–	22
Fast	100	60.0	57.1	50.0	–	78
Magnitude of papaya ringspot problem (%)						
No problem	–	–	–	–	–	–
Low	4.8	20.0	14.3	–	–	9.5
Moderate	57.1	40.0	42.9	50.0	–	50.0
High	38.1	40.0	42.9	50.0	–	40.5
Season when is the disease more prevalent (%)						
Dry season	71.4	20.0	42.9	100.0	–	57.1
Cold season	–	–	–	–	–	–
Long rains	9.5	–	–	–	–	4.8
Short rains	–	20.0	7.1	–	–	4.8
Always	9.5	–	7.1	–	–	7.1
Not aware	9.5	60.0	42.9	–	–	26.2

'N' is the number of farmers surveyed, (-): no reported case.

3.3.4 Farmers knowledge on the cause and management of ringspot

Of the respondents, 48.8 % did not know the cause of papaya ringspot in papaya plants (Table 3.4). Other respondents thought ringspot is caused by insect attack (18.6 %), bacteria (2.4 %), fungi (4.8 %), virus (11.9 %) and changes in weather (13.5 %). About 73.2 % of the respondents were not aware of when newly grown papaya plants get infected, with 22.5 % observing the disease symptoms on newly planted papaya plants

after three months, while 2.4 % observed the symptoms two months and 2.4 % observed the disease symptoms more than three months after planting. A majority (54.8 %) of the respondents sprayed symptomatic plants with chemicals, and 40.5 % applied no management measures, while 4.8 % practiced rouging of infected plants to manage the disease (Table 3.4). In Central, Coast, Eastern and Rift valley, 54.5 %, 20 %, 61.5 % and 100 % of the respondents respectively, sprayed chemical insecticides as a disease management measure. On the other hand, 40.9 % of the respondents in Central, 80 % in Coast and 30.8 % in Eastern did not apply any control measures, while 4.5 % in Central and 7.7 % in Eastern regions removed and destroyed papaya plants showing symptoms (Table 3.4).

Table 3.4: Farmer's knowledge on the cause, time of symptom expression and management practices of papaya ringspot

Variable	Region					Mean
	Central N = 28	Coast N = 14	Eastern N = 37	Rift Valley N = 8	Western N = 16	
Cause of ringspot (%)						
Virus (yes %)	10	–	13.3	50.0	–	11.9
Fungus (Yes %)	10	–	–	–	–	4.8
Insects (Yes %)	19.0	20.0	20.0	–	–	18.6
Bacteria (Yes %)	–	20	–	–	–	2.4
Weather (yes %)	20	–	13.3	–	–	13.5
Don’t know	42.9	60	53.3	50.0	–	48.8
Time growing plants get affected (%)						
One month	–	–	–	–	–	–
Two months	5.0	–	–	–	–	2.4
Three months	35.0	–	7.1	50.0	–	22.5
More than 3 months	–	–	7.1	–	–	2.4
Not aware	60.0	100	85.7	50.0	–	73.2
Control measures for the disease						
Rouging	4.5	–	7.7	–	–	4.8
Spraying with chemicals	54.5	20.0	61.5	100	–	54.8
Do not control	40.9	80.0	30.8	–	–	40.5

‘N’ is the number of farmers surveyed, (-): no reported case.

3.4 Discussion

To implement a successful integrated papaya ringspot management program, adequate knowledge of how farmers perceive the problem, and their attitudes and practices to papaya crop protection are required. A survey was conducted in the five major papaya-producing regions of Kenya to unravel farmers' knowledge, perceptions and management practices of papaya ringspot in the country. The study showed that farmers' papaya ringspot knowledge is limited with only a few of the respondents being able to identify the disease. This implies that knowledge is a likely obstacle to ringspot management in papaya farming in Kenya. Other studies with a focus on farmers' crop disease knowledge have found a correlation between farmers' knowledge and perceptions of crop pests and disease management. Lwin et al. (2012), for example, reported farmers' lack of knowledge on pests and diseases affecting tomato farming in the Inlay Lake region of South East Asia. Similarly, Khan and Damalas (2015), cited farmers' low/poor knowledge as negatively affecting farming of cotton by small acreage holding farmers in Pakistan.

The results showed that majority of respondents who were relatively knowledgeable of the disease especially those from Central, Eastern, Coast and Rift valley regions had attained higher education (secondary school education level). Furthermore, the respondents in addition to producing papaya for subsistence also farmed the crop for sale. These results further suggest that it is likely that there is a link between farmers' level of education and the general knowledge of the disease in papaya farming in Kenya. It was noted during the survey that farmers from these regions tended to have a greater interest in the quality and quantity of the papaya they produced, suggesting, in addition to their higher attained education, the need to produce quality papaya fruits to compete for market motivated them to notice obvious changes on their papaya plants that are likely to lower quality and quantity, and subsequently their potential income. The motivation is likely to push the farmers to seek information on the problem, including identification of the symptoms of the disease. Knowledge is directly related to education level (Adam et al., 2015). The Western region where the majority of the respondents had

only attained primary education and produced papaya for subsistence purposes, possibly contributed to their ignorance on the disease despite being present on their farms. There are several recorded respondents from Central and Rift valley regions who obtained their planting materials from research institutes. This further suggests the presence of the institutes in those regions most likely provided the farmers with information on crop production, challenges, and disease management which further boosted their knowledge of crops pests and diseases.

The study showed the majority (95.5 %) of the respondents knowledgeable and aware of papaya ringspot acknowledged the presence of the disease on their farms. These respondents could narrate the disease's general causative agents, its spread rate and severity seasons on their farms, and its effects on papaya production. These results demonstrate how important farmers' crop disease knowledge is and could motivate the potential disease management practice to be adopted. Indeed, farmers' crop disease experience has been shown to positively impact the practised management of Napier grass stunt disease in western Kenya (Khan et al., 2014). The result of a few respondents (26.2 %) not being aware when the disease symptoms are more prevalent is however of concern. Technically, this result implies that the lack of knowledge of the disease is likely a major hindrance to the management of papaya ringspot by Kenyan farmers. Lack of knowledge of the causative agents of agricultural diseases has been reported to hinder the production of legumes and vegetable crops in Asia (Schreinemachers et al., 2015) and also on Napier grass in East Africa (Asudi et al., 2015).

This study's results show that though the majority of the respondents (54.8 %) managed the disease by spraying chemical insecticides, a good proportion (40.5 %) did not apply any measure. Spraying of chemical insecticides is one of the integrated management approaches to managing papaya ringspot because it decreases the aphids population and distribution, reducing the level of damage they cause (Ventura et al., 2004; Kalleshwaraswamy & Kumar, 2008). However, the use of chemical insecticides as management practice could be less successful in the absence of an understanding of the role of aphids in the spread of the virus causing papaya ringspot (Kalleshwaraswamy &

Kumar, 2008). Furthermore, insect control can be effective if practised before symptoms of the disease appear, which requires farmers' knowledge about the epidemiology of the disease (Schreinemachers et al., 2015). Hence there is an urgent need for a robust sensitization on the use of chemical insecticides to control vectors transmitting the disease-causing virus and other cultural practices such as roguing to manage papaya ringspot in Kenya. Roguing of infected plants, which is also another effective integrated management practice of papaya ringspot (Ventura et al., 2004), was only reported in Central and Eastern regions, suggesting the practice is minimally used by Kenya papaya farmers.

The observation that the majority of the farmers have insufficient knowledge on the identification and cause of the ringspot disease on papaya, calls for urgent farm-level training to increase farmers’ awareness and knowledge about papaya ringspot.

CHAPTER FOUR

METAGENOMIC ANALYSIS OF VIRUSES ASSOCIATED WITH PAPAYA RINGSPOT IN KENYA

Abstract

Carica papaya L. is an important fruit crop grown by small and large-scale farmers in Kenya for local and export markets. However, its production is constrained by papaya ringspot. The disease is believed to be caused by the papaya ringspot virus (PRSV). Preliminary attempts to detect PRSV in the papaya plants showing disease symptoms using enzyme-linked immunosorbent assay (ELISA) and Reverse transcriptase (RT)-PCR procedures with primers specific to PRSV, did not yield conclusive results. Therefore, the nature of the virus(es) responsible for the ringspot was elucidated in papaya leaves collected from 22 counties through Illumina MiSeq next-generation sequencing (NGS) and validated by RT-PCR and Sanger sequencing. Viruses were detected in 38 out of the 48 leaf samples sequenced. Sequence analysis revealed the presence of four viruses: a Potyvirus named *Moroccan watermelon mosaic virus* (MWMV), and three viruses belonging to the genus Carlavirus. The Carlaviruses included *Cowpea mild mottle virus* (CpMMV), and two putative Carlaviruses, closely related but distinct from *cucumber vein-clearing virus* (CuVCV) with amino acid and nucleotide sequence identities of 75.7-78.1 % and 63.6-67.6 %, respectively, in the coat protein gene. In reference to typical symptoms observed in the field in the infected plants, the two putative Carlaviruses were named papaya mottle-associated virus (PaMV) and papaya mild mottle-associated virus (PaMMV). Surprisingly and in contrast to previous studies in other parts of the world, PRSV was not detected. The majority of the viruses were detected as a single viral infection, while a few were found co-infecting with another virus for example MWMV and PaMV. Furthermore, the NGS and RT-PCR analysis identified MWMV to be strongly associated with ringspot symptoms on infected papaya fruits. This study has provided the first complete genome sequences of these viruses isolated from papaya in Kenya, together with primers for their detection, an

important step towards the design of long-term, sustainable disease management strategies.

4.1 Introduction

Papaya is an important fruit crop both in the tropics and subtropical regions (Mishra et al., 2007), ranked fourth worldwide among tropical fruits (Evans & Ballen, 2015). Ripe fruits are very rich in vitamins A and C and minerals (Ming et al., 2008). The fruit is reasonably priced and is rich in nutrients making it a common man's fruit. Papaya is also a source of papain, a proteolytic enzyme obtained by collecting and drying the latex exuded from scratches on the surfaces of slightly immature papaya fruits. The enzyme is purified and used in foods, beverages, pharmaceuticals and manufacturing industries (Yogiraj et al., 2014).

Despite its importance, the national economies of many papaya-growing nations are jeopardized by the papaya ringspot. The disease affects papaya plants at all stages of growth and naturally spreads very fast leading to infection of the whole orchard within 3-7 months with severe yield losses of up to 100 % (Ventura et al., 2004; Tripathi et al., 2008; Sharma & Tripathi, 2014). A typical characteristic symptom of the disease on infected plants is the production of ringed spots on the fruits (Gonsalves, 1998; Sharma & Tripathi, 2014). Other symptoms of the disease include vein clearing, mottling, mosaic, chlorotic spots, leaf curling, green blisters and distortion of leaves termed shoe stringing. Reduction in size of the leaf canopies, as the disease advances, results in stunted growth of the plant. Irregular oily or water-soaked streaks/marks are seen on stems and leaf petioles. These symptoms can occur together or separately. Fruits affected by this disease are of poor quality with low sugar levels attracting low prices both at local and exports markets (Tripathi et al., 2008; Sharma & Tripathi, 2014). In addition, if plants are infected with the disease either at the seedling stage or within two months after planting, they fail to produce mature fruits and the affected papaya orchards have a short lifespan of less than a year (Gonsalves, 1998; Tennant et al., 2007). The impact of the disease on rural farming communities has been extreme

because papaya trees can no longer be grown without a high possibility of being damaged (Sakuanrungsirikul et al., 2014). The disease is known to be caused by Papaya ringspot virus (PRSV), a *Potyvirus* in the family *Potyviridae* (Sharma &Tripathi, 2014; Tripathi et al., 2007; 2008).

A study conducted in Kenya to document papaya cultivation between 2008 and 2009 reported papaya ringspot as the main constraint to papaya production in several regions of the country including the Coast, Central, Rift Valley, Western and Eastern regions (Asudi, 2010; Ombwara et al., 2014). Several attempts to detect PRSV in papaya plants showing the disease symptoms using double antibody sandwich ELISA were not conclusive (Ombwara et al., 2014). Reverse transcriptase-polymerase chain reaction (RT-PCR) procedures using published PRSV primers (Hema and Prasad, 2004; Jain et al., 2004; Diallo et al., 2008; Omar et al., 2011; Srinivasulu and Gopal, 2011; Mohammed et al., 2012; Martínez et al., 2014) also failed to detect the virus. The same failure occurred using primers designed based on PRSV sequences available in GenBank to amplify the virus in symptomatic plants. This led to the notion that there could be a different strain of PRSV in Kenya or a different virus(es) infecting papaya in the country.

With the development of Next-generation sequencing (NGS) technology, plant virus discovery, diagnostics, and evolutionary studies have increased and improved enormously (Roossinck, 2017). The technology can be used to identify plant viruses in a given sample with or without prior knowledge of the viral types present and can also reveal the presence of novel and unsuspected agents. The approach has also been helpful for viral co-infection detection in many plants (Candresse et al., 2014; Roossinck, 2015; Akinyemi et al., 2016; Blawid et al., 2017). Therefore, the NGS approach coupled with RT-PCR and Sanger Sequencing was used to identify and characterize the virus(es) causing symptoms associated with ringspot disease on papaya in Kenya. This is the first application of NGS technology in assessing viruses associated with ringspot infecting this important fruit crop in Kenya and is likely to help in the design of long-term and sustainable disease management strategies in the country.

4.2 Materials and Methods

4.2.1. Sample collection

Field surveys and sampling were carried out during the months of February to April 2017 in 22 administrative regions (counties) in Kenya namely Taita Taveta, Kwale, Kilifi, Bungoma, Busia, Siaya, Vihiga, Kisumu, Homabay, Migori, Nakuru, Baringo, Elgeyo Marakwet, Kiambu, Murang'a, Kirinyaga, Embu, Tharaka Nithi, Meru, Makueni, Machakos and Kitui (Figure 3.1). These Counties were selected based on reported papaya production and the presence of papaya ringspot-related symptoms (Asudi, 2010; Ombwara et al., 2014). A total of 287 leaf samples (200 with the disease symptoms and 87 symptomless) were collected from randomly selected plants using sterile forceps and immediately immersed in RNA*later*® (Invitrogen™) solution to prevent the degradation of RNA. The samples were then transported to the Biosciences eastern and central Africa–International Livestock Research Institute (BecA-ILRI) Hub in Nairobi, Kenya and stored at 4°C until RNA extraction. Forty-eight samples; 34 with and 14 without ringspot disease symptoms, were randomly selected for NGS analysis based on the region and the symptoms observed. In every county, a representative sample with or without symptoms was selected and in counties where more than one sample was chosen, differences in symptoms exhibited by the plants were considered.

4.2.2. RNA extraction, library preparation, and Illumina MiSeq sequencing

Leaf samples selected for NGS analysis were dried using a clean absorbent paper towel to remove the RNA*later*® and then powdered in liquid nitrogen with sterile mortars and pestles. Total RNA was extracted from 100 mg of leaf samples using the RNeasy® plant mini Kit (Qiagen Inc.) following the manufacturer's instructions. The integrity of extracted RNA was checked using agarose gel electrophoresis where 0.8 % of agarose was dissolved in 100 ml of 0.5 X TAE (Tris-acetate-EDTA) buffer, stained with 3 µl of GelRed® Nucleic Acid Gel Stain (Biotium) and run at 100 V for 30 minutes in a gel tank. The gel was visualized in a gel imaging system with a UV transilluminator. The

quantity of extracted RNA was measured using the ssRNA assay on the Qubit® 2.0 fluorometer (Invitrogen™) system. The extracted RNA was then stored at -80 °C. The libraries were prepared from 1 μg of the total RNA using the Illumina TruSeq® RNA sample preparation protocol according to the manufacturer's instructions (Illumina, San Diego, California). Briefly, poly-A containing mRNA molecules were purified using oligo-dT and fragmented into small pieces using the Illumina "Elute, Prime, Fragment Mix". The fragmented RNA was copied into the first-strand using reverse transcriptase and random primers and second-strand complementary DNA (cDNA) was synthesized using DNA polymerase I and RNase H. The double-stranded cDNA (ds cDNA) was purified using Agencourt AMPure ®XP magnetic beads (Beckman Coulter, Inc. Indianapolis, IN). The end-repair of synthesized cDNA was performed using End Repair mix. Thereafter, 3′ ends were adenylated and unique adaptors for each library ligated to the 5′ and 3′ ends ds cDNA. The ds cDNA was enriched through PCR to create the final cDNA library under the following cycling conditions; one cycle of 98°C for 30 seconds; 15 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 30 seconds; with a final extension of 72°C for 5 minutes.

The final size and concentration of the cDNA libraries were estimated with the Agilent Tape Station 2200 system (Agilent Technologies, Santa Clara, CA) and Qubit® 2.0 fluorometer (Invitrogen™), respectively. The cDNA libraries, each with a unique adaptor, were normalized to 4nm and pooled for multiplex sequencing. A pooled library consisted of 24 biological samples, each at equal molar concentration hence two flow cells were used. The libraries were sequenced using a 2×300 cycle PE V3 Illumina kit (Illumina, San Diego, California). Paired-end reads were generated using the Illumina MiSeq System at the BecA-ILRI Hub in Nairobi, Kenya.

4.2.3 RNA sequence processing and de novo assembly

Paired-end reads generated in the Illumina MiSeq System were checked for quality using FastQC. The low-quality reads and sequencing adapters were removed using Trimmomatic V 0.33 (Bolger et al., 2014). The host genome was removed by mapping

all the reads to the papaya plant genome (GenBank accession number ABIM01000000) (Ming et al., 2008) using Bowtie2 V 2.2.8 (Langmead and Steven, 2013). The remaining reads (unmapped) were then assembled *de novo* to obtain contigs using metaSPAdes V 3.9.0 (Nurk et al., 2017) with default settings.

4.2.4 Virus identification and reference mapping of the assembled de novo contigs

The resulting *de novo* contigs were compared with other sequences in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/) (Benson et al., 2012) and Plant Virus Genome Database (Camacho et al., 2009) using BLASTn search, and the top hit accession used for virus identification. For each viral species identified, the most frequent annotated accessions in the NCBI were used as a reference for the alignment and in the estimation of sequence similarity. Krona web-based tool (Ondov et al., 2011) was used to visualize BLAST results.

Reference assemblies were performed for complete virus genome sequences by mapping the *de novo* sequences against the most similar existing viral genomes using the read mapping module of CLC genomics workbench version 5.5.1 (www.clcbio.com). The sequences were assigned as complete genomes based on comparison with the reference sequences used in the mapping process obtained from BLASTn search results. The *de novo* consensus sequences and consensus sequences from reference mapping were then compared through visual inspection of individual mappings to ensure no artefacts were incorporated as a result of sequencing errors or errors during genome assembly. *De novo* sequences were however chosen over the consensus of reference assembly as a precautionary measure in case the viruses identified had considerably diverged from similar viral genome sequences in the GenBank database.

4.2.5 Validation of assembled virus sequences through RT-PCR and Sanger sequencing

The assembled viral sequences were validated through reverse transcription (RT) followed by polymerase chain reaction (PCR) and sent to Macrogen (Europe) for Sanger sequencing. Briefly, viral sequences generated from the Illumina MiSeq were aligned using CLC genomics and consensus sequences were used for designing primers using Primer 3 (Untergasser et al., 2012). Designed primers were evaluated for specificity using Primer-BLAST (Ye et al., 2012) and tested on the samples in which the viruses had earlier been detected (by NGS). To test the virus, RNA was extracted from the samples using RNeasy® plant mini Kit (Qiagen Inc.) following the manufacturer's instructions and used for cDNA synthesis. Synthesized cDNA was stored at -20°C before use as a template for the PCR process.

Before the amplification of viruses, the PCR process was optimized to evaluate the optimal annealing temperature and cycling condition for the primers. Briefly, a 10 µl PCR reaction mixture comprising 5 µl of AccuPower® Taq PCR 2X Master Mix (Bioneer, Korea), 3.6 µl of nuclease-free water, 0.2 µl of 10 µM each of forward and reverse primers (Macrogen) and 1 µl of cDNA (50 ng/µl) was prepared. Positive and negative controls were included in the reaction. The positive control constituted a sample infected with the virus from NGS results while a negative control comprised nuclease-free water used in place of nucleic acid. The PCR reactions were carried out on a thermal cycler (Eppendorf Mastercycler Nexus Gradient) under the following cycling conditions; 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55-66 °C, and 1 min at 72 °C; and a final extension at 72 °C for 5 min for the respective sets of primers. Amplified PCR products alongside O'GeneRuler ™ 1-Kb plus DNA ladder (Invitrogen™, USA) were separated on 2 % (wt/vol) agarose gels, and the bands were visualized on a UV trans-illuminator before documentation by digital photography.

After optimizing the annealing temperature and cycling conditions for each primer designed, the viruses were amplified through PCR. The viral amplicons were purified

using the QIAquick PCR Purification Kit (Qiagen Inc.) according to manufacturer instructions and quantified using the NanoDrop Spectrophotometer (Thermo Scientific™). The purified products were checked for quality and quantity using gel electrophoresis and Bioanalyzer respectively before shipping to Macrogen Europe for Sanger sequencing.

4.2.6 Analysis of virus sequences associated with papaya ringspot

The obtained viral sequences from the Illumina MiSeq system were used to determine percentage sequence identity, open reading frames (ORFs), conserved motifs, and phylogenetic analysis. Sequence identities were computed using the Sequence Identity and Similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html) search for ORFs was done using the ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/), conserved protein domains were identified using NCBI conserved domain search program (Marchler-Bauer et al., 2011) whereas the conserved motifs were identified through comparisons with known viral sequences. The sequences from this study and other previously identified viral sequences retrieved from the GenBank database were used to determine phylogenetic relationships among members of the same genus. Briefly, sequences were imported into the CLC Genomics Workbench, aligned and exported in FASTA format, converted to Molecular Evolutionary Genetics Analysis (MEGA) format and used for distance and phylogenetic analysis using MEGA 6 software (Tamura et al., 2013). The phylogenetic trees were constructed using the maximum likelihood method based on the JTT matrix (Jones et al., 1992) as determined in the program Modeltest (Posada & Crandall 1998), using 1000 replicates for bootstrap analysis. The recombination detection program (RDP)4 package (Martin et al., 2015) was used to detect recombination in the nucleotide sequences of the identified viruses using RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), Bootscan (Martin et al., 2005), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), 3Seq (Boni et al., 2007) and SiScan (Gibbs et al., 2000) programs implemented in the package with default parameters. Sanger sequences were quality checked, trimmed and assembled using the CLC Genomics Workbench version 8.03 with the default settings.

The consensus sequences were used for BLASTn search in the NCBI and for comparison with the sequences generated on the Illumina MiSeq System.

4.3 Results

4.3.1 Illumina MiSeq Sequencing statistics

To provide an insight into viruses associated with ringspot symptoms in papaya in Kenya, extracts from 48 leaf samples were sequenced using the Illumina MiSeq platform. A total of 50,247,269 reads of length between 35 and 151 bp were generated from 2 runs. The raw reads were filtered to remove those of low quality, leaving a total of 47,800,743 reads with read-length ranging from 60 to 151 bp. The number of reads/sample ranged from 465,116 in S1 to 1,809,690 in S43. The GC content ranged from 42 to 48 %. Viruses were detected in 38 out of the 48 samples sequenced (Table 4.1).

4.3.2 Viruses detected in symptomatic and asymptomatic papaya leaf samples

The reads were assembled into 49 contigs ranging from 469 bases in S11 collected from Kiambu County to 10,292 bases in S4 collected from Nakuru County (Table 4.2). The BLASTn search of the *de novo* assembled sequences against the NCBI non-redundant database indicated the presence of *Moroccan watermelon mosaic-like virus* (MWMV), cowpea mild mottle-like virus (CpMMV) and cucumber vein-clearing like virus (CuVCV). The BLASTn results of 35 de novo assembled sequences from 31 samples shared between 80 to 90 % sequence similarities with MWMV genome sequences previously reported in Tunisia, South Africa, Democratic Republic of Congo and Morocco (GenBank accession numbers LN810061, EF579955, KU315176 EF211959 and AF305545 respectively). The CuVCV genome sequences were recorded in 11 samples with nucleotide sequence similarities of 72 - 77 % to CuVCV previously reported in Tanzania (GenBank accession number JN591720). Three samples showed sequences closely related to CpMMV and had between 76 and 86 % nucleotide sequence

similarities to CpMMV sequences reported previously in North America, Brazil and Ghana (GenBank accessions KC774020, KF554101 and HQ184471 respectively) (Table 4.2).

Cases of single and co-infections of the viruses were observed in the samples. Single virus infections of MWMV were detected in 26 samples collected from Makueni, Nakuru, Homabay, Taita Taveta, Kiambu, Busia, Kilifi, Murang'a, Kirinyaga, Embu, Meru and Machakos counties whereas single CuVCV infection was found in two samples collected from Makueni and Kwale (Table 4.2). The co-infections of MWMV and CuVCV were the majority and were detected in seven samples collected from Kitui, Embu, Machakos, Tharaka Nithi, Meru and Makueni counties. The existence of mixed infections of MWMV and CpMMV, CuVCV and CpMMV, and, MWMV, CpMMV and CuVCV were reported in one sample each from Baringo, Kitui and Meru counties respectively. Due to the misleading interpretation of partial sequences for virus identity (Jo et al., 2017), complete genome sequences of the viruses identified through BLASTn search were used in subsequent analysis. In the GenBank database, the complete genome of MWMV is about 9.7 kbp and CpMMV is 8.1 kbp while the CuVCV genome sequence is partial (5218 bp) (JN591720). However, it was possible to designate sequences of CuVCV as complete or near-complete genomes based on the information of genome features in the species *Carlavirus* in the database. Out of the 22 counties surveyed, 22 complete virus genome sequences detected in 11 counties namely Homabay, Kisumu, Busia, Kiambu, Meru, Embu, Kirinyaga, Murang'a, Machakos, Makueni and Nakuru were closely related to MWMV, the most prevalent virus in the samples analysed (Table 4.2). The CuVCV was the second most prevalent and eight complete viral genome sequences were detected in samples collected from Makueni, Kwale, Tharaka Nithi, Meru, Kitui and Machakos counties. Only one complete genome of CpMMV with 8196 bases was detected in a sample collected from Kitui County (Table 4.2).

Table 4.1: Illumina MiSeq sequencing statistics obtained from papaya samples with and without ringspot symptoms from 22 counties in Kenya

Sample No	County of collection	Raw reads	Length (bp) before QC	Trimmed reads	Length (bp) after QC	% GC content	Virus*
S1	Makueni	465116	35-151	436423	60-151	44	+
S2	Makueni	878230	35-151	824664	60-151	46	+
S3	Kiambu	484883	35-151	450236	60-151	45	–
S4	Nakuru	992553	35-151	927987	60-151	46	+
S5	Nakuru	840783	35-151	788212	60-151	45	+
S6	Baringo	1045411	35-151	981992	60-151	45	+
S7	Taita Taveta	678903	35-151	638032	60-151	44	–
S8	Homabay	608410	35-151	569076	60-151	44	+
S9	Kwale	818104	35-151	770565	60-151	44	–
S10	TaitaTaveta	882906	35-151	832445	60-151	42	+
S11	Kiambu	1145487	35-151	1073453	60-151	45	+
S12	Kitui	1107196	35-151	1042103	60-151	42	+
S13	Busia	781327	35-151	733690	60-151	44	+
S14	Kisumu	1240288	35-151	1167504	60-151	44	+
S15	Taita Taveta	587524	35-151	551297	60-151	44	+
S16	Kilifi	895032	35-151	842086	60-151	46	+
S17	Makueni	1080677	35-151	1018184	60-151	44	+
S18	Kitui	1075787	35-151	1004375	60-151	44	–
S19	Murang'a	1288194	35-151	1212378	60-151	45	+
S20	Murang'a	845593	35-151	791310	60-151	46	+
S21	Kirinyaga	1082909	35-151	1016693	60-151	45	+
S22	Kirinyaga	1118095	35-151	1053334	60-151	44	+
S23	Embu	1309867	35-151	1237337	60-151	42	+
S24	Embu	831762	35-151	782762	60-151	44	+
S25	Kwale	1207561	35-151	1153706	60-151	44	+
S26	Machakos	933695	35-151	900984	60-151	45	+
S27	Homabay	1124740	35-151	1080401	60-151	46	+
S28	Embu	1236829	35-151	1183709	60-151	45	–
S29	Tharaka Nithi	1289557	35-151	1243865	60-151	42	+
S30	Tharaka Nithi	600317	35-151	578568	60-151	48	–
S31	Meru	1110859	35-151	1068658	60-151	45	+
S32	Meru	729236	35-151	702649	60-151	45	–
S33	Meru	1387548	35-151	1329158	60-151	44	+
S34	Meru	1373080	35-151	1320794	60-151	44	+
S35	Kiambu	698466	35-151	668678	60-151	45	+
S36	Kiambu	1060572	35-151	1021341	60-151	45	+
S37	Kilifi	1142123	35-151	1098926	60-151	46	–
S38	Baringo	1635383	35-151	1560252	60-151	45	–
S39	Kitui	1401904	35-151	1352125	60-151	44	+
S40	Kisumu	955874	35-151	918727	60-151	45	+
S41	Makueni	802399	35-151	766667	60-151	46	+
S42	Kirinyaga	1174298	35-151	1132021	60-151	44	+
S43	Machakos	1809690	35-151	1746884	60-151	43	+
S44	Machakos	1151750	35-151	1089401	60-151	45	+
S45	Makueni	1727221	35-151	1656070	60-151	44	+
S46	Murang'a	1413519	35-151	1362841	60-151	44	+
S47	Kirinyaga	1395345	35-151	1347231	60-151	45	+
S48	E.Marakwet	800266	35-151	770949	60-151	45	–
		50,247,269		**47,800,743**			

Key: *Presence or absence of virus is indicated by + or – respectively.

Table 4.2: Viruses identified in papaya and their sequence similarity (%) with closest homologues in the databases

Sample No.	Symptomatic/Asymptomatic	Symptoms expressed[a]	Virus identified[b]	Accessions in NCBI[#]	Length	De novo coverage	Similarity (%)	Identities	E-value
S1	Symptomatic	WS	MWMV	EF211959	930	2.0	82	766/929	0
S2	Symptomatic	ML, LD, RS	MWMV	KU315176	9769	305.4	80	7726/9645	0
S3	Asymptomatic	-	nd	-	-	-	-	-	-
S4	Symptomatic	ML, RS	MWMV	KU315176	10292	442.6	80	7820/9767	0
S5	Symptomatic	ML, LC	MWMV	EF211959	1010	1.9	87	877/1003	0
S6*	Asymptomatic	-	CpMMV	KF554101	787	2.6	78	331/424	8.00E-65
			MWMV	EF211959	587	1.0	83	356/431	1.00E-101
S7	Asymptomatic	-	nd	-	-	-	-	-	-
S8	Asymptomatic	-	MWMV	KU315176	3229	5.4	83	2668/3223	0
S9	Symptomatic	MO	nd	-	-	-	-	-	-
S10	Symptomatic	MO, LC	MWMV	EF211959	842	4.9	88	741/842	0
S11	Symptomatic	WS	MWMV	AF305545	469	1.2	90	414/462	2.00E-163
S12*	Asymptomatic	-	CuVCV	JN591720	2376	8.5	76	528/697	3.00E-89
			MWMV	LN810061	6337	7.3	81	5164/6348	0
S13	Symptomatic	WS	MWMV	LN810061	9808	400	80	7717/9621	0
S14	Symptomatic	MO	MWMV	LN810061	9772	616	80	7722/9635	0
S15	Asymptomatic	-	MWMV	EF211959	769	2.9	87	627/720	0
S16	Symptomatic	ML, LC	MWMV	EF579955	491	1.6	83	408/489	2.00E-123
S17	Symptomatic	MO	CuVCV	JN591720	9023	324.7	76	1270/1664	0
S18	Symptomatic	MO	nd	-	-	-	-	-	-
S19	Symptomatic	MO, SS, PU	MWMV	LN810061	9804	2239.7	80	7738/9643	0
S20	Symptomatic	ML, WS	MWMV	LN810061	9844	1703.8	80	7728/9636	0
S21	Symptomatic	MO, SS, PU	MWMV	LN810061	9723	3027.0	80	7730/9636	0
S22	Symptomatic	WS, RS	MWMV	KU315176	9706	262.4	80	7727/9624	0
S23*	Symptomatic	MO, PU, VC, LC	MWMV	KU315176	9722	1002.2	80	8197/9763	0
			CuVCV	JN591720	2229	5.0	77	729/949	8.00E-144
S24	Symptomatic	ML, WS	MWMV	LN810061	9861	1723.4	80	7719/9636	0
S25	Symptomatic	ML	CuVCV	JN591720	9081	3155.4	75	1220/1637	0
S26*	Asymptomatic	-	MWMV	LN810061	2004	1.4	84	1688/2000	0
			CuVCV	JN591720	5596	63.9	73	1077/1468	9.00E-142
S27	Symptomatic	ML, PU	MWMV	LN810061	9772	767.0	80	7728/9637	0
S28	Asymptomatic	-	nd	-	-	-	-	-	-

Sample No.	Symptomatic/Asymptomatic	Symptoms expressed[a]	Virus identified[b]	Accessions in NCBI[#]	Length	De novo coverage	Similarity (%)	Identities	E-value
S29*	Asymptomatic	-	CuVCV	JN591720	9079	567.4	72	1124/1554	1.00E-122
			MWMV	EF579955	2011	1.4	81	1629/2006	0
S30	Asymptomatic	-	nd	-	-	-	-	-	-
S31	Symptomatic	PU, LC	MWMV	LN810061	9792	2454.9	80	7724/9636	0
S32	Asymptomatic	-	nd	-	-	-	-	-	-
S33*	Symptomatic	MO, SS, VC	MWMV	LN810061	9717	612.7	80	7727/9634	0
			CuVCV	JN591720	9080	2192.4	73	1072/1466	2.00E-136
S34*	Symptomatic	MO	CuVCV	JN591720	9069	891.9	73	1074/1466	7.00E-140
			CpMMV	HQ184471	3204	5830	76	507/668	3.00E-85
			MWMV	EF211959	1433	5.0	85	1222/1432	0
S35	Symptomatic	RS	MWMV	KU315176	9675	84.7	80	7708/9610	0
S36	Symptomatic	RS	MWMV	KU315176	9726	1252.6	80	7726/9639	0
S37	Asymptomatic	-	nd	-	-	-	-	-	-
S38	Asymptomatic	-	nd	-	-	-	-	-	-
S39*	Symptomatic	ML	CpMMV	KC774019	8196	532.5	86	7046/8217	0
			CuVCV	JN591720	9028	74.2	75	611/813	1.00E-98
S40	Symptomatic	ML, LC	MWMV	LN810061	9722	921	80	7700/9637	0
S41	Symptomatic	MO, SS, RS	MWMV	LN810061	9677	27.5	80	7705/9598	0
S42	Symptomatic	MO, VC	MWMV	LN810061	9734	2454.4	80	7732/9635	0
S43*	Symptomatic	VC, ML	MWMV	KU315176	10221	1129.8	80	7805/9747	0
			CuVCV	JN591720	9072	3740.8	73	1065/1462	2.00E-131
S44	Symptomatic	MO, SS, PU	MWMV	KU315176	9747	742.0	80	7818/9643	0
S45*	Symptomatic	ML, PU	MWMV	KU315176	9723	1106.2	80	7732/9653	0
			CuVCV	JN591720	9080	1001	73	1073/1469	7.00E-135
S46	Symptomatic	MO, SS, PU	MWMV	LN810061	9786	5911	80	7735/9643	0
S47	Symptomatic	MO, SS, PU	MWMV	LN810061	1556	3.0	82	1254/1528	0
S48	Asymptomatic	-	nd	-	-	-	-	-	-

Key: *Indicates co-infections, nd-not detected [a]Symptom description; WS: water-soaked marks on the stem/petioles; ML: Mottling; MO: Mosaic; LD: leaf distortion; LC: leaf curl; SS: shoe stringing of leaves; PU: Puckering; VC: Vein clearing and RS: Ringspots on the fruits; [b]virus identified; MWMV: Moroccan watermelon mosaic virus, CuVCV: Cucumber vein-clearing virus and CpMMV: Cowpea mild mottle virus; #: Blastn search showing only topmost accession hit

4.3.3. Relationship between viruses and disease

The symptoms observed on infected leaves varied significantly and ranged from mottling, mosaic, shoe stringing, curling, and puckering. Similarly, on fruits, symptoms such as concentric water-soaked lesions, circular ring spots and necrotic rings were observed. On the upper part of the stem and leaf petioles, numerous water-soaked lesions were seen (Table 4.2; Plate 4.1). There was an association between the virus present and symptoms in papaya plants. Hence, the majority of the symptomatic plants (32/34) tested positive for virus(es) infection. There were, however, cases in which plants were asymptomatic but viruses were detected (6/14) (Table 4.2). More interesting, in cases where fruits exhibited concentric water-soaked lesions, circular ring spots and necrotic rings, a single infection with MWMV was detected while in a single infection of CuVCV, mottling was observed. Co-infections of MWMV and CuVCV were found in plants that were either asymptomatic or those exhibiting mottling, mosaic, vein clearing, or puckering symptoms. Co-infection of CuVCV with CpMMV was detected in plants exhibiting mottling symptoms. To exclude the presence of PRSV and to confirm that MWMV was associated with ringspots, the spots were excised from the infected fruits (Plate 4.2), and the viruses nucleic materials were concentrated using the protocol described by Blomström et al. (2010). RNA was extracted using TRIzol LS Reagent (Invitrogen) and further purified using RNeasy Mini Kit columns (Qiagen). The RNA was sequenced using the Illumina Miseq system and a *de novo* assembly obtained a 9,700 bp sequence that shared 80 % sequence similarity with MWMV sequences in GenBank (accession numbers LN810061 and KU315176). This strongly suggested that MWMV is associated with the ringspot symptoms and is the putative cause of the ringspots on papaya fruits in Kenya.

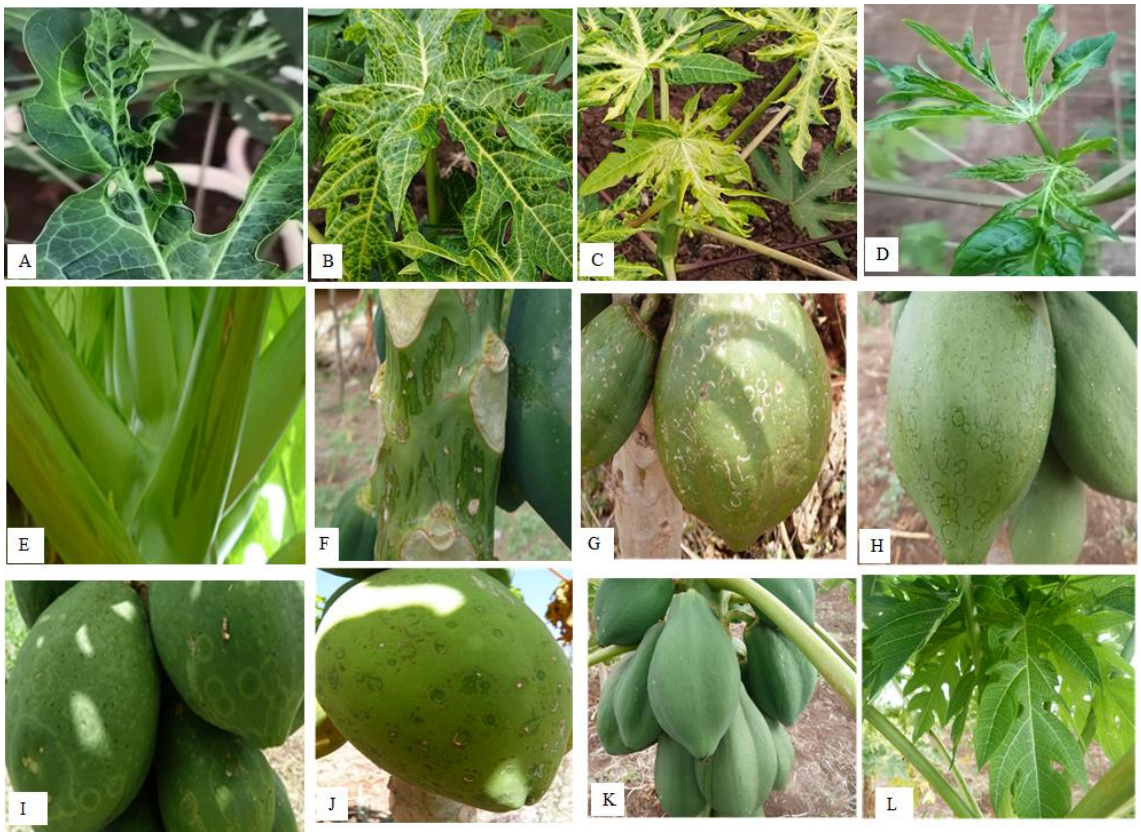

Plate 4.1: Diversity in papaya ringspot symptoms observed during the field survey (A): Puckering; (B): Mottling; (C): Mosaic; (D): Leaf deformation; (E) Oily streaked petioles (F): Oily streaked stem; (G): Necrotic rings; (H) and (I): Circular rings: (J): Concentric water-soaked lesions; (K): asymptomatic fruits and (L): asymptomatic leaves

4.3.4 RT-PCR and Sanger sequencing validation of viruses

Sanger sequencing of the viral amplicons obtained from RT- PCR yielded sequences that were 100 % identical to those generated *de novo* from assembled Illumina sequences, confirming that the *de novo* assembly gave accurate sequences of the viruses' genomes. The primers designed from de novo sequences, the target region of the genome, and the expected size of the virus amplicon are presented in Table 4.3.

Table 4.3: Marker primers for papaya ringspot-associated viruses with a target region of the genome and the expected size of PCR fragments

Virus	Gene	Primer Name	Primer size	Sequence (5'>3')	Amplicon size (bp)
MWMV	Coat Protein	CP F1_p	20	ATCATCGCAGAACCAAGGCA	697
		CP R1_p	20	ATCAACAGTGTGCCTCTCCG	
	Cylindrical inclusion	CI F1_p	20	TCTCAGCTAGCACGCAACAA	315
		CI R1_p	20	CGGTGTTGAGCCAAACGAAG	
Cucumber vein clearing like virus	Coat protein	4FCVCV	20	AGACCAAAGAGTGCTTCGGG	304
		4RCVCV	20	TAGGAACTCCCAGTCCCTCG	
	RdRp	8FCVCV	20	AGTGGTTGCGAGTTGTTCCA	420
		8RCVCV	20	CAACCAAAGTCCCCATCCGA	
CpMMV	Coat protein	39F2CpMMV	20	AACATGGCGACAGCTGAAGA	694
		39R2CpMMV	20	GAAGAGCGACCAGTTCCCAA	
	RdRp	39F4CpMMV	20	CTGACCAGGCTCTTTGGGAG	971
		39R4CpMMV	20	TTCAAAAGCCAGCATTCGCC	

To further authenticate the association of MWMV with ringspot symptoms on fruits, extracts from ringed spots (Plate 4.2A) were tested using RT-PCR approach in a few samples among the 48 sequenced indicating a strong association of the virus with ringspots on the fruits (Plate 4.2B).

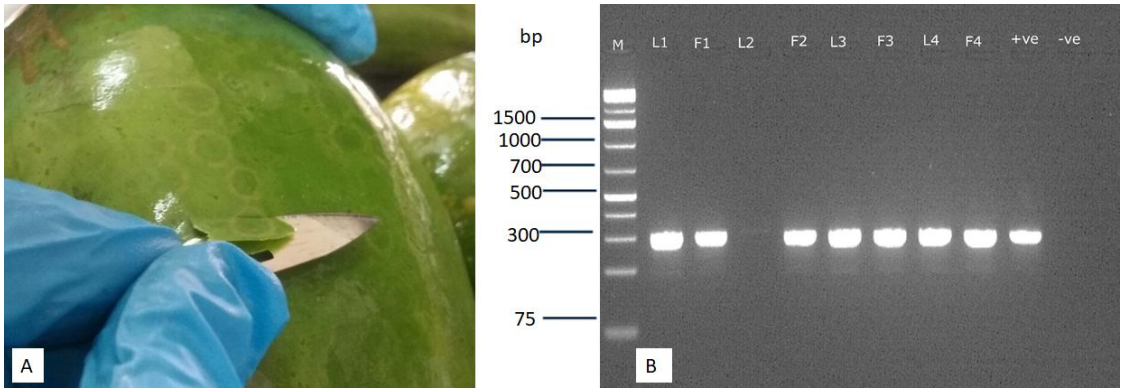

Plate 4.2: Extraction of ringspots from infected fruit (A) and the use of RT-PCR to confirm the presence of MWMV detected by Illumina sequencing in sampled papaya leaf tissues and in ringspots extracted from fruits. A band at 315bp in A and B represented presence of MWMV M= O'GeneRuler ™ 1-Kb plus DNA ladder, +ve = positive control, -ve=negative control. Numbers F1, F2, F3 and F4 represents virus extracted from the ringed spots on fruits while L1, L2, L3 and L4, represent virus extracted from leaves.

4.3.5. Genome organization of MWMV in this study and determination of its phylogenetic affinities

The obtained 11 viral genome sequences of MWMV were deposited in GenBank under accession numbers MH595736-MH595746. The genomes are 9,712-9,725 nucleotides (nt) long organized into 142-155 noncoding nt at their 5´ terminus followed by 9,375 nt encoding the polyprotein, from which all the proteins of the virus are derived, and 194-197 noncoding nt at 3´ terminus. The polyprotein codes for 3,124 amino acids (aa) with a molecular weight of between 353.9 and 354.5 kDa. The base composition includes 31.4-31.6 % adenine, 18.8-19.0 % cytosine, 23.3-23.4% uracil and 26.1-26.4% guanine.

The genomes are single-stranded positive-sense RNA viruses with a single ORF that is translated into a single large polyprotein. The polyprotein has nine putative cleavage sites, yielding 10 functional proteins. The length (nt and aa) and organization of the10 proteins are as follows: P1 (1035/345), helper component protease (Hc-Pro) (1371/457), P3 (1041/347), 6K1 (156/52), cylindrical inclusion

(CI) (1905/635), 6K2 (171/57), VPg (570/190), nuclear inclusion a (NIa) (717/239), nuclear inclusion b (NIb) (1550/517) and coat protein (CP) (855/285).

Several conserved motifs found in potyviruses were identified in the MWMV genomes detected in this study. In the HC-Pro gene, there are highly conserved "RITC" "CSC" and "PTR" motifs, which are associated with virus transmission (Blanc et al., 1998; Huet et al., 1994) and "FRNK$(X)_{12}$CDN" that is involved in symptom development (Gal-on, 2000). The RNA helicase function motifs ''GAVGSGKST'' and ''PTR'' were found to be conserved in the N-terminal region of the CI. Three RdRp motifs ''YCDADGS'', ''GNNSGQPSTVVDNTLMV'' and ''NGDDL'' responsible for potyviral genome replication (Hong & Hunt, 1996) were present in NIb. The well-characterized DAG motif, highly conserved among all aphid transmissible *potyviruses* (Atreya et al., 1995) was found in the N-terminus of the CP. A stretch of glutamic acid and lysine repeats (KE repeats) was found after the "DAG" motif in the N-terminus of the CP in all MWMV signatures in this study.

Potyviruses are usually classified based on the percentage of sequence identity in the polyprotein or CP. Viruses sharing more than 75 % nt and 80 % aa sequence identity in the CP or polyprotein are considered the same species (Adams et al., 2005a). The MWMV viruses from this study share 98.1-98.6 % aa (97.5-98.5 % nt) sequence identity amongst themselves in the polyprotein region. They also share 89.8-90.0 % aa (79.2-79.6 % nt) with isolate from Tunisia (GenBank accession EF579955) and 90.3-90.5 % aa (79.6-79.7 % nt) with isolate from South Africa (GenBank accession KU315176) in the polyprotein. Furthermore, sequence identity of 93.7-95.1% aa (84.3-84.9 % nt) and 94.1-95.1% aa (83.9-85.3 % nt) with Tunisian and South African MWMV isolates in the CP was observed, respectively (Table 4.4).

Table 4.4: Nucleotide and amino acid sequence identities (%) between Kenyan MWMV isolates and those from Tunisia and South Africa

Genome features[a]	**Among Kenyan isolates (MH595736-46)**		**Between Kenyan and a Tunisian isolate (EF579955)**		**Between Kenyan and a South African isolate (KU315176)**	
	aa	**nt**	**aa**	**nt**	**aa**	**nt**

Polyprotein	98.1-98.6	97.5-98.5	89.8-90.0	79.2-79.6	90.3-90.5	79.6-79.7
P1	94.2-96.5	96.4-97.8	65.1-68.1	69.4-70.5	67.8-69.0	70.3-71.3
HC-Pro	97.4-98.9	97.0-98.8	93.7-94.7	79.7-80.3	93.7-94.7	79.5-80.4
P3	97.9-99.3	97.5-98.8	87.4-88.5	79.3-79.9	89.2-90.1	79.9-80.7
6K1	100	96.8-99.4	94.2	80.8-81.4	94.2	82.1-83.3
CI	98.9-99.7	97.8-98.6	95.3-95.7	80.9-81.7	95.7-96.4	80.9-81.3
6K2	98.2-100	96.5-99.4	86.0-87.7	74.9-76.6	89.4-91.2	75.4-77.2
VPg	97.9-100	97.4-99.3	83.6-85.2	78.1-79.1	83.7-84.7	75.1-76.1
NIa	98.7-99.6	97.4-98.7	92.1-92.5	78.9-79.5	92.5-93.7	79.9-80.6
NIb	98.6-99.4	97.5-98.6	94.4-95.0	80.5-81.1	94.2-94.8	81.2-82.1
CP	97.6-99.7	97.6-99.2	93.7-95.1	84.3-84.9	94.1-95.1	83.9-85.3

[a] HC-Pro = helper component-protease; CI=cylindrical inclusion; Vpg= viral genome-linked protein; NIa =nuclear inclusion A; NIb= nuclear inclusion B; CP=coat protein.

Phylogenetic analysis built using the complete polyprotein aa sequences showed that all the MWMV isolates detected in this study formed a single cluster separate from South Africa, Tunisia and Greece sequences. A clear geographical clustering was also observed. Isolates from South Africa clustered separately from the Mediterranean ones. The PRSV that was previously believed to be responsible for the ringspot symptoms showed a distinct evolutionary pathway from MWMV as depicted in the phylogenetic tree (Figure 4.1).

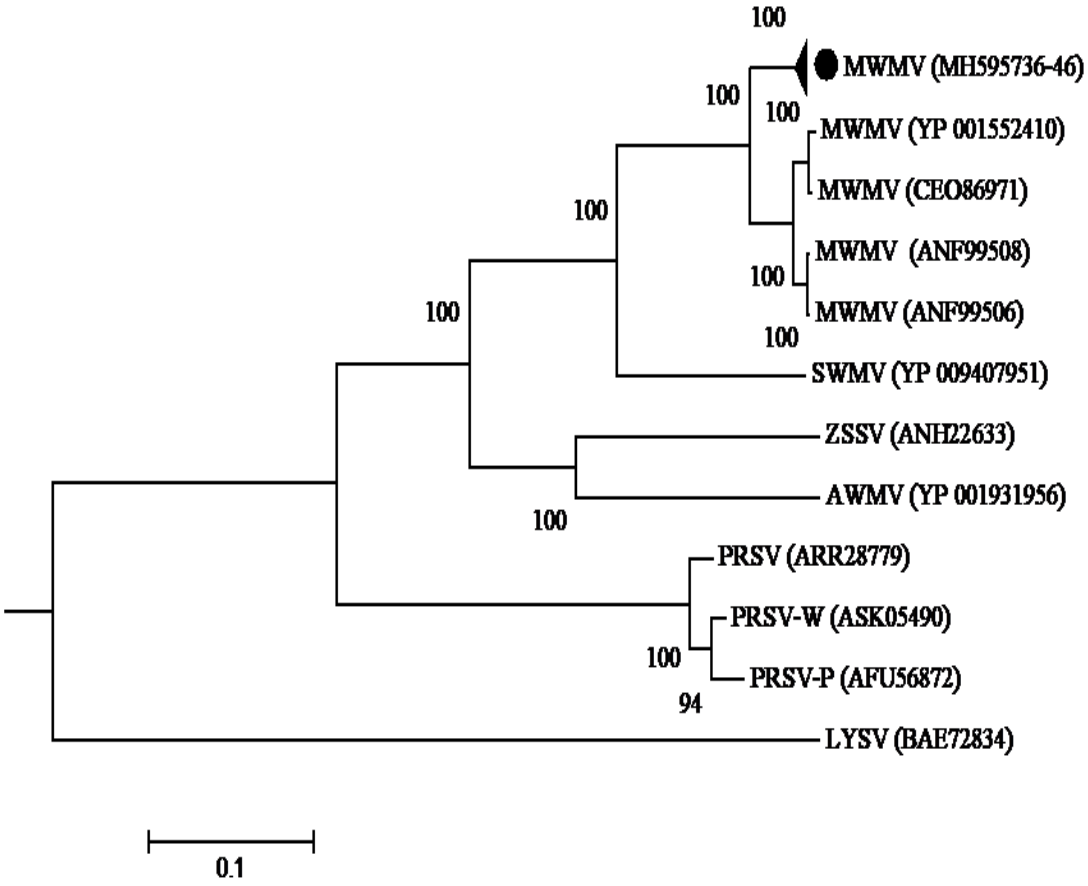

Figure 4.1: Phylogenetic relationships among MWMV isolates and closely related potyviruses. The phylogenetic tree was generated using the maximum likelihood method based on JTT matrix-based model. The sequence generated in this study is shown by black circle. MWMV-Moroccan watermelon mosaic virus; SWMV: Sudan watermelon mosaic virus; ZSSV: Zucchini shoe stringing virus; AWMV: Algerian watermelon mosaic virus; PRSV-W: Papaya ringspot virus biotype w; PRSV-P: Papaya ringspot virus biotype p and LYSV: Leek yellow stripe virus.◄ indicates 11 isolates of MWMV from papaya from Kenya.

4.3.6 Identification and phylogeny of Cowpea mild mottle virus

The complete genome sequence of the CpMMV identified in this study is 8151 nt long, excluding the poly-A tail (GenBank accession number MK984605). The genome is single-stranded, positive-sense with six ORFs. The ORF1 encodes a RdRp

gene consisting of 1859 aa with an estimated molecular weight of 211.3 kDA with four conserved motifs including viral methyltransferase (RdRp) (Koonin, 1991), *Carlavirus* endopeptidase (Peptidase C23) (Lawrence et al., 1995) and viral (superfamily 1) RNA helicase (Viral_helicase1) (Gorbalenya & Koonin, 1989). The ORFs 2, 3 and 4 encode the triple gene block proteins (TGB1-3p, with a molecular weight of 25.8, 11.6 and 7.6 kDa respectively), that are essential for virus movement. The ORF 5 encodes the CP comprising of 288 aa with a molecular weight of 32 kDa and contains a strong conserved motif "His-X_8Asp-X_{15}Thr-Gly-Gly" at aa position 246-273, in the C-terminal region of the CP (Naidu et al., 1998). The ORF 6 encodes a cysteine-rich protein (CRP) with nucleic acid-binding protein (NaBP) consisting of 109 aa with a molecular mass of 12.3 kDa.

The CP sequence comparison of CpMMV isolate from this study with sequences in the database, indicated that the Kenyan CpMMV shares 84.7 %, 84 % and 82.6 % aa sequence identities with Brazilian (GenBank accession AGS13100), Ghanaian (GenBank accession YP-004035878) and Indian (GenBank accession ATV94962) isolates, respectively. However, in the RdRp gene, the Kenyan CpMMV isolate shares 90.7 %, 88.6 % and 72 % aa sequence identities with the Indian, Brazilian and Ghanaian isolates, respectively. CpMMV sequences in this study clustered together with those of several other CpMMV isolates from the GenBank with strong bootstrap support of 100 % based on either CP or RdRp gene (Figures 4.2 and 4.3).

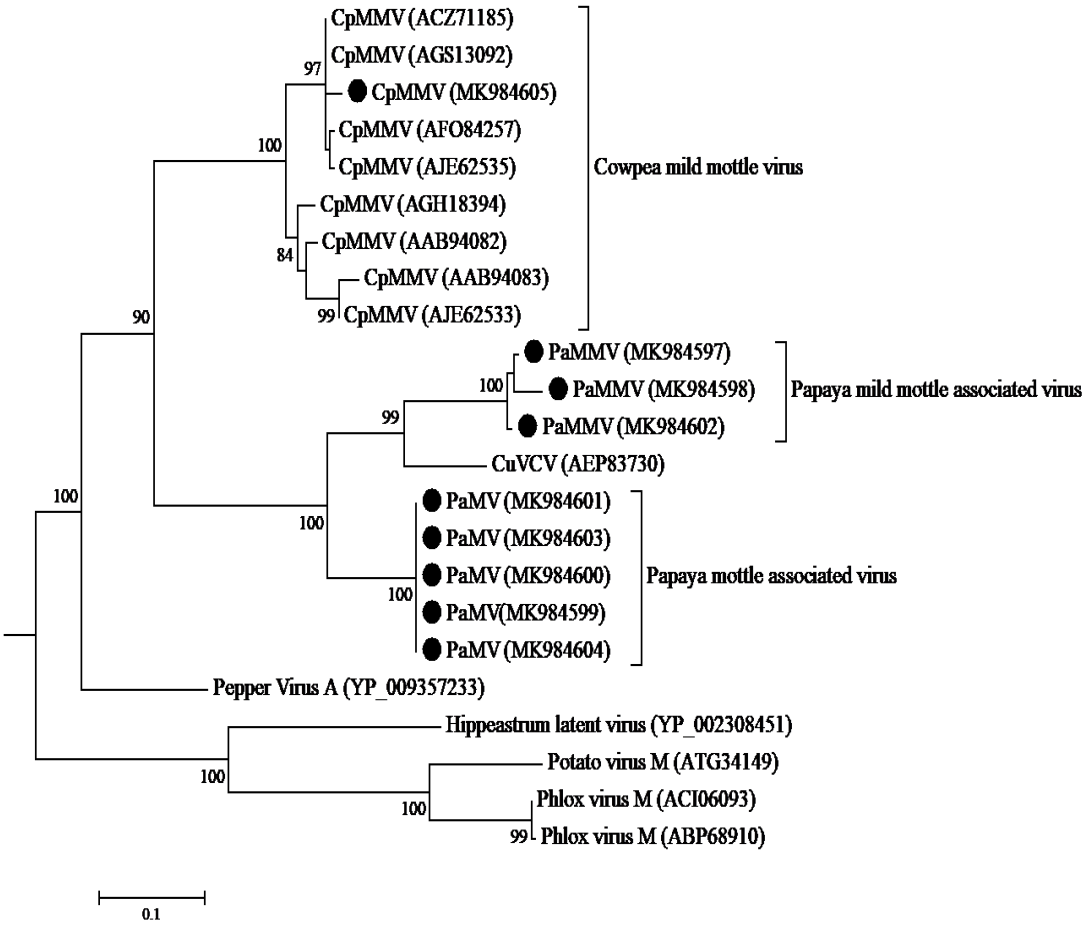

Figure 4.2: Phylogenetic analysis of coat protein amino acid sequences among CpMMV, PaMMV, PaMV and closely related Carlavirus generated using maximum likelihood method based on JTT matrix-based model. The sequences generated in this study are shown by black circles.

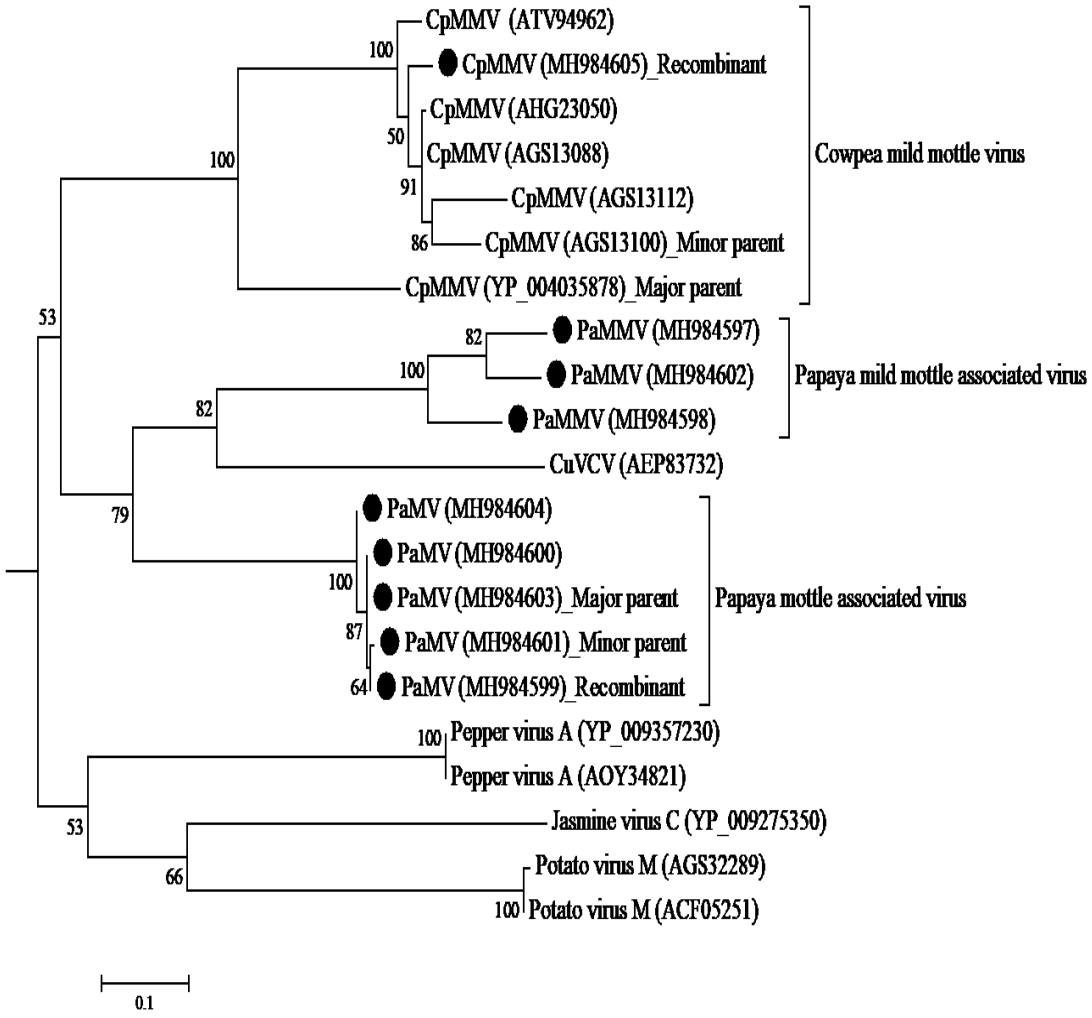

Figure 4.3: Phylogenetic analysis of RdRp amino acid sequences among CpMMV, PaMMV, PaMV and closely related *Carlavirus* generated using maximum likelihood method based on JTT matrix-based model. The sequences in this study are shown with black circles.

Analysis of the ORF1 nucleotide sequences using seven different algorithms showed evidence of recombination. Recombination was detected with the major parent being a Ghanaian isolate (YP-004035878) at positions 1-34 and 5330-5650 and the minor parent being a Brazilian isolate (AGS13100) at positions 35-5329. The recombination was detected by four programs; MaxChi Chimaera, Siscan and 3Seq with P value of 1.893 E-06. No recombination was detected in the other genes.

4.3.7 Molecular characterization of putative Carlaviruses detected in papaya

The genome sequences of the putative *Carlavirus* (PaMV and PaMMV) are linear, single-stranded positive-sense RNA viruses with a poly-A tail and consist of six ORFs encoding the following proteins; RdRp, movement proteins i.e., triple gene block, CP and CRP with NaBP with arrangement typical of the genus *Carlavirus* (Adams et al., 2012b; Martelli et al., 2007). A BLAST search using individual ORF sequences revealed that these sequences belong to the genus *Carlavirus* in the family *Betaflexiviridae*. Functional protein domains known to be conserved in *Carlaviruses* were also detected in isolates from this study. In the ORF1, there are viral RNA methyltransferase (Vmethyltransf), RdRp_2 Superfamily, *Carlavirus* endopeptidase (Peptidase_C23) and Viral (Superfamily 1) RNA helicase (Viral_helicase1). The RdRp domain also contains the characteristic core motif $SGX_3TX_2NT_{22}GDD$ found in *Carlaviruses* (Adams et al., 2012b; Martelli et al., 2007) while the CP has the conserved CP domain of carlviruses Flexi_CP_N and Flexi_CP.

These viruses were found in a mixed infection with MWMV or CpMMV. The association of symptoms to them hence becomes problematic. However, in cases where these viruses were identified as a single infection through NGS, severe and mild mottling symptoms were observed. Accordingly, we have proposed naming these viruses papaya mottle-associated virus (PaMV) and papaya mild-mottle associated virus (PaMMV).

The PaMV detected in this study is 9,061-9,071 nt long, excluding the poly-A tail (GenBank accessions numbers MK984599, MK984600, MK984601, MK984603 and MK984604) and were obtained from samples collected from Machakos, Meru, Tharaka Nithi and Makueni counties. The size of the ORFs is as follows; ORF1 1,558 aa (partial) for MK984600 and 2,162 aa (complete) (175.1-248 kDa), ORF2 228 aa (25.3 kDa), ORF3 108 aa (11.6 kDa), ORF4 63 aa (6.9 kDa) ORF 5 276 aa (30.8 kDa) and ORF 6 102 aa (11.6 kDa). The PaMMV, on the other hand, was identified in three samples collected from Makueni, Kwale and Kitui counties (GenBank accession numbers MK984597, MK984598 and MK984602). The genomes are 9,028, 9,023 and 9,070 nt long respectively excluding the poly-A tail.

The ORF1 comprises of 2,154 aa (245 kDa), ORF2 228 aa (25.3 kDa), ORF3 108 aa (11.6 kDa), ORF4 75 aa (8.1 kDa), ORF 5 288 aa (32.1 kDa) and ORF 6 103 aa (11.9 kDa).

Although BLASTn search of PaMV and PaMMV top hit the CuVCV in the GenBank database (Table 4.2), differences in the size of ORF1, ORF4, ORF5 and ORF 6 was observed between the two viruses. The ORF1 in PaMV comprises 2162 aa whereas in PaMMV has 2154 aa. The ORF4 consists of 63 and 75 aa, ORF5 276 and 288 aa, and ORF6 102 and 103 aa for PaMV and PaMMV, respectively. There were insertions/ deletions (indels) of aa sequences observed on these ORFs when sequence alignment was performed contributing to differences in the sizes of these ORFs. These indels are unlikely to be due to sequencing error because they were also found in other published *Carlaviruses*. Furthermore, these variations mapped to a common area in the samples analyzed (as was the case on the 5' end of the ORFs).

Based on the species demarcation criterion of 72 % nt and 80 % aa similarity in the CP or RdRp among *Carlviruses* (Adams et al., 2012b), it is clear that the two viruses could be considered distinct species within the genus *Carlavirus*. The percentage of sequence identity indicated that the isolates in this study shared 75.7-78.1 % aa and 63.6-67.6 % nt sequence identities in the CP gene with CuVCV isolate from Tanzania (GenBank accession number AEP83730) (Table 4.5), values below the threshold for species discrimination in *Carlviruses*. Additionally, PaMV and PaMMV shared less than 80% aa and 75 % nt sequence identities in the CP thereby qualifying them to be different species of the same *Carlavirus* genus.

Table 4.5: Sequence identities (%) in the coat protein of PaMV and PaMMV *Carlaviruses* with the closest homologue; CuVCV

		PaMMV			**PaMV**					**CuVCV**
	GenBank Acc. No.	1	2	3	4	5	6	7	8	9
PaMMV	1. MK984597	-	92.0	93.4	75	75	75	75	75	77.4
	2. MK984598	**73.2**	-	91.7	72.9	72.9	72.9	72.9	72.9	76.7
	3. MK984602	**71.2**	**74.1**	-	74.7	74.7	74.9	74.7	74.6	78.1
PaMV	4. MK984599	**64.6**	**62.9**	**63.5**	-	100	100	100	100	75.7
	5. MK984600	**65.7**	**64.5**	**64.4**	**77.3**	-	100	100	100	75.7
	6. MH984601	**66.7**	**63.4**	**64.2**	**75.4**	**74.6**	-	100	100	75.7
	7. MK984603	**66.3**	**63.2**	**65.4**	**72.3**	**76.9**	**75.7**	-	100	75.7
	8. MK984604	**66.8**	**63.7**	**64.9**	**73.6**	**75.5**	**75**	**76.7**	-	75.7
CuVCV	9. AEP83730	**66.4**	**65.3**	**66**	**65.8**	**65.9**	**63.6**	**67**	**67.6**	-

Percentage nucleotide sequence identity in coat protein (Bold numbers) and amino acids (regular text)

The phylogenetic trees generated using the CP and the RdRp gene (Figures 4.2 and 4.3 respectively) support the proposed species classification within the genus *Carlavirus*. The PaMV isolates formed a monophyletic group whereas PaMMV isolates clustered together, closer to the CuVCV isolate from Tanzanian (AEP83730) (Figures 4.2 and 4.3).

A recombination event was detected in PaMV isolate MK984599, collected from Tharaka Nithi County with the major parent being MK984603 from Machakos County (at positions 1-1169 and 2280-6806) and the minor parent being MK984601 from Meru County (at positions 1170-2279) (Figure 4.3). The recombination was supported by four programs; MaxChi, Chimaera, Siscan and 3Seq with P value of 4.718 E-06. No recombination was detected in the coat protein gene.

4.4. Discussion

Through Illumina MiSeq sequencing, complete genome sequences of MWMV, a *Potyvirus*; CpMMV, a *Carlavirus* and two novel yet divergent *Carlaviruses* namely PaMV and PAMMV in symptomatic and asymptomatic papaya leaves collected from Kenyan fields were identified. This study provides the first report of these viruses in papaya in Kenya and also reports for the first time the infection of papaya with CpMMV, PaMMV and PaMV. The presence of MWMV in papaya crops in Kenya would suggest that either the virus is increasing its geographical distribution or it has been present in papaya and/or in other host plants but went undetected before. The study also suggests the emergence of new viruses (CpMMV, PaMMV and PaMV) or that the viruses have been present but have recently moved to papaya from other hosts, and are now posing a serious threat to papaya production in the country.

The characterization of a plant virus disease of known etiology usually relies on the symptoms expressed in the host plants because they are easy to recognize especially if disease-specific. Symptoms also aid in roguing diseased plants as a strategy for preventing virus spread (Naidu & Hughes, 2003). Potyviruses have limited host ranges and can be identified based on the characteristic symptoms they produce in certain host plants (Shukla & Ward, 1989). In this study, the symptoms observed on papaya plants included those that are attributed to PRSV infection (Tripathi et al., 2008; Zhao et al., 2016), although PRSV was not detected in the samples. This explains why earlier attempts to detect PRSV in diseased plants through ELISA and RT-PCR procedures using primers specific to PRSV (Ombwara et al., 2014) were unsuccessful. Failure to detect PRSV in papaya plants exhibiting the above symptoms shows the limitations of using symptoms for disease diagnosis (Candresse et al., 2014).

Establishing associations between a specific viral infection and symptoms expressed in host plants can be further complicated by mixed viral infections (Marais et al., 2015). For instance, in the co-infection of papaya with MWMV and PaMV, it was not possible to associate specific symptoms with either virus. However, Illumina sequencing of RNA extracted from the ringed spots of fruits strongly suggested

MWMV be associated with the ringspots on papaya in Kenya. This virus has also been reported in papaya plants exhibiting ringspots in Congo (Arocha et al., 2008). However, papaya plants showing ringspots symptoms could also be infected with other viruses like PaMV, PaMMV, or CpMMV as observed in this study. The occurrence of mixed viral infections in papaya has also been reported in Mexico (Noa-Carrazana et al., 2006). In many samples sequenced in this study, MWMV was found with other viruses suggesting that co-infection of these viruses in papaya plants is not an uncommon phenomenon. The co-infection of MWMV with CuVCV has also been reported in watermelon in Tanzania (Menzel et al., 2011). Plant viruses co-infecting the same host may generally interact in either a synergistic or an antagonistic way (Syller, 2012). Whether this is the case for viruses in papaya in Kenya remains to be determined.

The association between virus presence and symptoms expression in papaya plants was observed with the majority of the symptomatic plants testing positive for virus(es) infection. Some of the samples, despite not having clear visual viral symptoms showed the presence of virus(es) when sequenced on the Illumina MiSeq platform. Most of these viral sequences from asymptomatic leaves were partial except in one instance in Tharaka Nithi County where the complete viral genome of PaMV was recovered. Several factors may have contributed to the absence of symptoms in these infected samples, including papaya variety or cultivar, plant age and the number of days post-infection at the time samples were collected (Singh & Shukla, 2011) and virus titres (Ghoshal & Sanfacon, 2015). Further, masking of symptoms occurs in virus-infected papaya plants depending on the environmental conditions during the season (Kabir et al., 2017). Thorough screening of asymptomatic plants is therefore paramount for better disease management.

Since the discovery of MWMV in papaya in Congo more than a decade ago (Arocha et al., 2008), the virus has not been reported again in papaya. This work represents the second report of MWMV in papaya worldwide. MWMV has been reported in Africa and the Mediterranean region mostly in cucurbits such as *Cucurbita pepo* (Yakoubi et al., 2008; Ibaba et al., 2016; Kidanemariam et al., 2019), *Cucumis melo* (Lecoq et al., 2001), *Lagenaria bleviflora* and *Adenopus breviflorus* (Owolabi et al.,

2012; Mofunanya & Edu 2015). These findings suggest that papaya is an additional natural host for MWMV and that there could be more wild or cultivated hosts that need to be determined.

Phylogenetic analysis of MWMV revealed a clear geographical clustering pattern showing the Kenyan isolates on one clade and the South African and Mediterranean ones in separate groups. A similar geographical grouping of MWMV isolates was also reported in Tunisia (Yakoubi et al., 2008). If this clustering is based on host or geographic origin remains to be determined. Nevertheless, all MWMV isolates in this study show high sequence identity values despite their different Counties of collection suggesting a recent introduction in the country.

Recombination and mutation events are major forces attributed to evolution in plant viruses and are associated with adaptation to new hosts, often leading to the emergence of new variants and resistance breaking strains (Ohshima et al., 2002; García-Arenal et al., 2003; Nagy, 2008; Kwak et al., 2015; Xie et al., 2016). BLASTn search of PaMV and PaMMV sequences showed CuVCV to be their closest species genetically. Similarly, these viruses seem to have a common ancestor (from the phylogenetic analysis). Moreover, recombination events were detected in PaMV sequences within the RdRp genes although these recombination events did not change the phylogenetic groupings of the isolates. Further comparison between PaMV and PaMMV ORFs showed several indels. From the results, these two viruses likely evolved from a common ancestor. However, a detailed analysis of these viruses from different hosts and locations will be critical in elucidating their evolutionary paths and for determining if these events have any biological significance such as host range and virulence. The CpMMV under this study is a likely recombinant between Ghanaian (YP_004035878) and Brazilian (AGS13100) isolates. In the phylogenetic tree, it clustered away from both parents. This result point to the likelihood that CpMMV from Kenya is a separate strain from its parents which could have arisen out of a recombination event.

The detection of single or co-infections of *potyviruses* and *carlaviruses* associated with papaya ringspot in papaya fruit crops in Kenya in both symptomatic and asymptomatic samples is a cause for concern. These viruses cause symptoms resembling other viral diseases and could escape routine detection resulting in a considerable reduction in fruit yield and quality. The inability to recognize a symptomless plant harboring a virus could also result in inadvertent exposure of other crops in the country to a potential inoculum source. Although the insect vectors transmitting these viruses and the mode of transmission in papaya are yet to be established, the MWMV in papaya is probably transmitted by aphids while CpMMV could be vectored by whiteflies (Naidu et al., 1998). Because of the close relationship between PaMMV and PaMV with the white-fly transmitted cucumber vein-clearing virus, there is a likelihood that they are also transmitted by whiteflies (Menzel et al., 2011). Papaya in Kenya is propagated by seeds and the possibility that any of these viruses is seed transmitted cannot be ruled out. However, further studies are needed to identify specific insect vectors and examine the likelihood of virus transmission to papaya in Kenya and their likely wild hosts of these viruses.

As the identified viruses continue to impact negatively on the livelihoods of many farming householders, there is, an urgent need to develop an integrated management strategy for the different virus diseases. Current management practices include the use of chemicals to control the insect vectors and reduce their populations and limit the spread of viruses, and also by rouging diseased plants which act as sources of viral inoculum. However, these measures are mostly not effective. A larger percentage of farmers in Kenya also do not employ any control measures. Papaya cultivars resistant to pathogens or less attractive to insect vectors are also not available to poor farmers. Therefore, promoting and implementing quarantine measures could help prevent the spread of these viruses to areas that are currently virus-free.

In conclusion, MWMV is the causal virus of papaya ringspot in Kenya. Other viruses CpMMV and two newly discovered viruses infecting papaya, tentatively named PaMV and PaMMV, were detected in papaya ringspot infected plants. Further, virus-specific primers developed in the current study will help to regularly monitor both

symptomatic and asymptomatic plants where necessary and discover new infections. These will help prevent the future spread of the viruses as well as develop ways of combating and reducing their effects on papaya crops. In the future, management and control options such as the identification of tolerant germplasm as well as alternative hosts need to be explored. Further studies are needed for the complete classification of PaMV and PaMMV and to understand the risk they pose to papaya and other crops. The sequencing strategy used in this study targeted viruses with poly-A tail as per the TruSeq RNA Illumina protocol used. The possibility of more and new viruses falling outside this detection approach infecting papaya crops cannot be ruled out. Additional viral metagenomics studies could help in elucidating the complete diversity of viruses infecting papaya in Kenya.

CHAPTER FIVE

OCCURRENCE AND DISTRIBUTION OF VIRUSES ASSOCIATED WITH PAPAYA RINGSPOT IN KENYA

Abstract

Papaya ringspot is a serious threat to papaya production in Kenya. For effective management, it is important to determine the occurrence and distribution of the viruses associated with the disease. A survey was conducted in 2017 covering a total of 103 papaya fields in major papaya production areas in the country. To determine the disease incidence, 20 plants per field were inspected for symptoms associated with the disease including mosaic, mottling, vein clearing, puckering, shoe stringing, and distortion on leaves; water-soaked marks on stems and petioles; ringspots on fruits and general stunted growth of the plant. Disease severity was evaluated on a scale of 1 to 5 while disease prevalence was determined as a proportion of fields showing the disease symptoms per county expressed as a percentage. A total of 287 leaf samples were collected from surveyed fields and tested for Moroccan watermelon mosaic virus (MWMV), Cowpea mild mottle virus (CpMMV), and Papaya mottle-associated virus (PaMV) using polymerase chain reaction (PCR)-based techniques. The highest (71.4%) disease incidence was recorded in Kiambu county while the lowest of 2.8 was recorded in Siaya and Bungoma counties (0.0 %). The mean disease incidence among counties surveyed was 21.1 %. Disease prevalence ranged from 0 to 100 % with Elgeyo Marakwet, Embu, Kiambu, Kitui, Murang'a, Nakuru and Vihiga counties recording 100 % prevalence. Mean disease prevalence among counties surveyed was 65.6 % while the lowest prevalence of 0.0% was reported in Bungoma and Siaya counties. The highest disease severity of 4.0 was reported in Baringo county while the lowest (2.0) was reported in Kwale, Kilifi and Taita Taveta counties. Overall, mean disease severity of 2.9 was recorded among the counties surveyed. MWMV was the most prevalent with 140/287 samples testing positive and also widespread having been detected in 11 of 22 counties surveyed. The PaMV was the second most prevalent and widespread detected in 39/287 and in 9 of 22 counties. CpMMV was the least prevalent, detected 7/287

samples and in three counties. The occurrence of both MWMV and PaMV was detected in five counties; Embu, Kirinyaga, Meru, Machakos and Makueni while that of PaMV and CpMMV was detected in Baringo, Meru and Kitui counties. The presence of MWMV, PaMV and CpMMV was detected in Meru county. The results of this study show the viruses associated with papaya ringspot are widespread in papaya growing regions, with some counties reporting 100 % disease prevalence. These findings are important for the development of control strategies for the disease. Further, this information calls for the implementation of papaya ringspot control measures. Important drivers influencing disease spread in the country are critical for effective papaya ringspot control.

5.1 Introduction

Carica papaya L., is an important fruit crop in Kenya grown by small and large-scale farmers for subsistence, local and export markets. However, statistics regarding its production in the country are not satisfactory. For instance, there has been a steady increase in the area under papaya production over recent years with no substantial increase in yields (HCDA, 2016). The low papaya yields in the country is mostly attributed to poor agronomic practices, lack of improved varieties, and damage caused by pests and diseases (HCDA, 2016; Kansiime et al., 2020; Rimberia & Wamocho, 2014).

Viral diseases threaten cultivated plants by impairing their growth and vigor, leading to a decrease in gross yields. The diseases also spoil the quality of produce decreasing marketable yield (Woolhouse et al., 2005). Among the disease infecting papaya, papaya ringspot is the most important biotic constraint worldwide. The disease is very destructive, threatening both small- and large-scale growers of papaya in several parts of the country (Ombwara et al., 2014; Rimberia & Wamocho 2014; Mumo et al., 2020). The impact of the disease in the country is being felt with farmers in some regions abandoning the growing of papaya in favour of other crops (Mumo et al., 2021) calling for an urgent need to develop disease management measures.

In Kenya, the disease was established to be associated with a potyvirus MWMV (Mumo et al., 2020). Other viruses such as cowpea mild mottle virus (CpMMV) virus, and papaya mottle-associated viruses (PaMV and PaMMV) have also been reported in papaya plants. However, occurrences and distribution of these viruses in the country are scarcely known, although this is important in disease management. This is also important for the extension services in terms of disease incidence, prevalence and severity to facilitate the coining of the appropriate extension packages to address the farmers' needs. The objective of this study, therefore, was to establish disease incidence, prevalence and severity and distribution of the viruses associated with the disease in the country.

5.2 Materials and Methods

5.2.1 Sampling sites and sampling procedure

Surveys of papaya fields and sampling of papaya plants were carried out between the months of January and April 2017 in 22 counties. The counties included Taita Taveta, Kwale, Kilifi, Kisumu, Homabay, Migori, Siaya, Bungoma, Busia, Vihiga, Nakuru, Baringo, Elgeyo Marakwet, Kiambu, Murang'a, Kirinyaga, Embu, Tharaka Nithi, Meru, Makueni, Machakos and Kitui. Fields with papaya crops established as a pure stand or intercropped were purposefully surveyed along selected routes. In each county, a particular representative route that captured the area of interest was discussed and agreed upon by the survey team and adopted. Amongst issues considered included the sample area and availability of suitable papaya fields. When farmers resided within the same county and papaya fields were close to each other, sampling was done on those spaced at a minimum distance of 5 km, otherwise, a distance interval of 10 km between fields was adopted. A transect was drawn diagonally in the field from both directions ending up with two transects (Sseruwagi et al., 2004). During sampling, representative plants were randomly selected along X-shaped transects in each field to reduce biases. In total 103 papaya fields were surveyed.

5.2.2 Incidence, severity and prevalence of papaya ringspot in selected counties in Kenya

Twenty plants per field were visually inspected for papaya ringspot symptoms on leaves, stems, petioles, and fruits. The general vigour of the inspected plants was also recorded. The disease severity scale was based on the area or the proportion of symptomatic plant tissue. The scale 1-5 (Ombwara, et al., 2014) was adopted where; 1=No visible symptoms, 2 = 1-25 % of plant tissues portraying symptoms such as mild mottling and mild mosaic patterns on the leaves, little distortion of leaves, mild oily streaked petioles/stems, apparent but negligible stunting, 3 = 26-50 % of plant tissues portraying symptoms: moderate yellow and mosaic patterns on the leaves, moderate distortion of leaf shape, moderate oily streaked petioles/stems, moderate stunting, moderate ringspot symptoms on fruits, 4 = 51-75 % of plant tissues portraying symptoms: severe yellow and mosaic patterns on leaf, severe leaf distortion with reduced size, severe oily streaked petioles/stems severe ringspot on fruits, plant partially stunted and 5 = more than 75 % of plant tissues portraying symptoms: very severe yellow and mosaic patterns symptoms on leaf, very severe leaf distortion and reduced size, very severe oily streaked petioles/stems, plant severely stunted and very severe ringspot symptoms on fruits. Scores of '1' (no visible symptoms) were excluded in calculating the mean severity per field to allow for a true evaluation of the degree of damage caused to the diseased plants. Disease incidence was determined as the proportion of the plants showing symptoms out of 20 examined expressed as a percentage. The prevalence of papaya ringspot was determined in every county as the proportion of fields with at least one diseased plant of the total number of fields observed in that county expressed as a percentage.

5.2.3 Sample collection and virus detection

Two hundred (200) symptomatic and 87 asymptomatic leaf samples were collected randomly from 2 to 5 plants per field. This involved harvesting the second youngest fully developed leaf from the shoot apex of symptomatic and asymptomatic plants using sterile forceps. The number of papaya leaf samples collected per field depended on the disease severity across the field and the plant population. The

collected leaf samples were preserved in RNAlater™ (Invitrogen™) stabilization solution to prevent RNA degradation and transported to the Biosciences eastern and central Africa–International Livestock Research Institute (BecA-ILRI) Hub, Nairobi laboratory and stored at 4°C before RNA extraction.

5.2.4 RNA extraction and PCR process

Leaf samples were removed from the RNA*later*™ solution using sterile forceps and the solution was blotted away using a sterile absorbent paper towel. Total RNA was extracted from the samples using RNeasy® plant mini Kit (Qiagen, Inc.) following the manufacturer's instructions. The integrity of extracted RNA was checked using agarose gel electrophoresis where 0.8 % of agarose was dissolved in 100 ml of 0.5 X TAE (Tris-acetate-EDTA) buffer, stained with 3 µl of GelRed® Nucleic Acid Gel Stain (Biotium) and run at 100 V for 30 minutes in a gel tank. The gel was visualized in a gel imaging system with a UV transilluminator. The quantity of RNA was checked using Qubit™ 2.0 Fluorometer system (Invitrogen™) following the manufacturer's instructions and normalized to 5 µg before cDNA synthesis. The cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Invitrogen™) and stored at -20°C before use as a template for the PCR process.

Samples were screened for viruses in PCR using a set of primers specific to the respective viruses; 5' TCTCAGCTAGCACGCAACAA 3' and 5' CGGTGTTGAGCCAAACGAAG 3' for MWMV, 5' AGACCAAAGAGTGCTTCGGG 3' and 5' TAGGAACTCCCAGTCCCTCG 3' for PaMV and 5' AACATGGCGACAGCTGAAGA 3' and 5' GAAGAGCGACCAGTTCCCAA 3' for CpMMV (Table 4.3, Mumo et al., 2020). The primers were designed to amplify a 315 bp fragment for MWMV, 304 bp for PaMV and 694 bp for CpMMV. Briefly, a 10 µl PCR reaction mixture comprising 5 µl of AccuPower® Taq PCR 2X Master Mix (Bioneer, Korea), 3.6 µl of nuclease-free water, 0.2 µl of 10 µM each of forward and reverse primers (Macrogen) and 1 µl of cDNA (50 ng/µl) was prepared. A positive control comprised a sample infected with the virus while a negative control comprised nuclease-free water used in place of nucleic acid. The PCR reactions were carried on a thermal cycler (Eppendorf

Mastercycler Nexus Gradient) under the following cycling conditions; 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C; and a final extension at 72 °C for 5 min for all sets of primers. Amplified PCR products alongside O'GeneRuler ™ 1-Kb plus DNA ladder (Invitrogen™, USA) were separated on 2 % (wt/vol) agarose gels and the bands were visualized on a UV trans-illuminator before documentation by digital photography.

5.3 Data analysis

Data on disease incidence, prevalence and severity were analysed by computing means among the counties surveyed. The presence of viruses was scored based on the presence or absence of the right size of the amplified fragment in the gel electrophoresis.

5.4 Results

5.4.1 Incidence, prevalence and severity of papaya ringspot in Kenya

Papaya ringspot was observed in the majority of the counties surveyed (Table 5.1). It was evident that the disease is widely distributed countrywide with an average incidence of 21.1 %. The highest (71.4 %) disease incidence was reported in Kiambu county, followed by Murang'a and Nakuru counties with means of 51.4 % and 52.8 %, respectively. The least incidence was recorded in Busia county with a mean of 2.8 %. However, Bungoma and Siaya counties had zero incidence (Table 5.1).

The disease prevalence differed within the counties' surveyed regions. An average of 65.5 % disease prevalence among the counties surveyed was observed. Elgeyo Marakwet, Embu, Homabay, Kiambu, Nakuru, Kitui and Vihiga, counties recorded the highest (100 %) disease prevalence (Table 5.1). No disease prevalence was observed in Bungoma and Siaya counties. Generally, mild disease severity (2.9) across the counties was recorded. The highest disease severities 4.0 were recorded in Baringo county followed by Kirinyaga and Murang'a counties with a mean of 3.8. The least disease severities were recorded in Kwale, Kilifi and Taita Taveta counties

with a mean of 2.0 (Table 5.1). No disease severity was recorded in Bungoma and Siaya counties (Table 5.1).

5.4.2 Viruses associated with papaya ringspot in Kenya

The viruses detected in the collected samples are shown in Table 5.2. The viruses were detected based on the detection of the respective viruses in the samples by PCR (Plate 5.1) and confirmed through Sanger sequencing. A sharp band of 315 bp, 304 bp and 694 bp in gel electrophoresis indicated the presence of the MWMV, PaMV and CpMMV, respectively. When only one virus was detected in a sample, it is reported as a single viral detection. In cases where more than one virus was detected in the same field surveyed and sampled, mixed infections are reported (Plate 5.1; Table 5.1). Dual infections occurred when more than one virus was amplified in a sample.

Table 5.1: Incidence, prevalence and severity of papaya ringspot in major papaya producing counties of Kenya

County	Disease incidence (%)	Disease prevalence (%)	Disease severity
Baringo	7.7	75	4.0
Bungoma	0.0	0	1.0
Busia	2.8	50	3.0
Elgeyo Marakwet	7.2	100	2.7
Embu	35.4	100	3.7
Homabay	20.3	100	3.2
Kiambu	71.4	100	2.7
Kilifi	6.7	33.3	2.0
Kirinyaga	36.0	77.7	3.8
Kisumu	13.3	50.0	3.1
Kitui	19.4	100.0	2.9
Kwale	3.8	25.0	2.0
Machakos	33.8	90.9	3.2
Makueni	12.9	57.1	2.5
Meru	36.9	75.0	3.1
Migori	38.1	50.0	2.8
Murang'a	51.4	100	3.8
Nakuru	52.8	100	3.1
Siaya	0.0	0.00	1.0
Taita Taveta	12.4	33.3	2.0
Tharaka Nithi	11.9	45.5	2.4
Vihiga	14.3	100.0	3.3
Mean	21.1	65.5	2.9
LSD (P=0.05)	2.6		3.0

Severity was visually assessed using a scale of 1-5 (Ombwara et al., 2014)

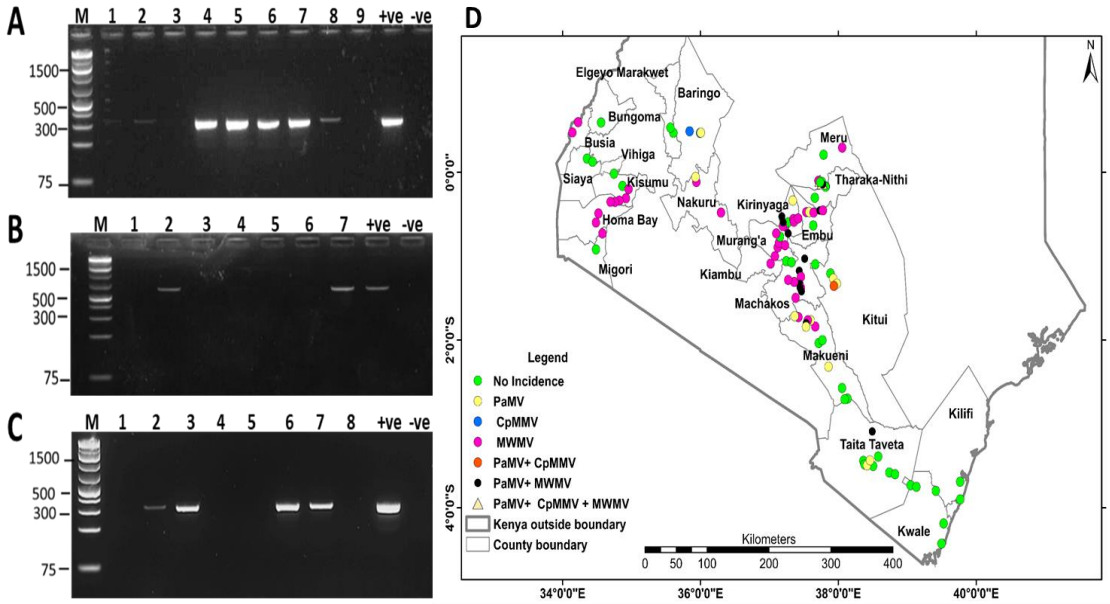

Plate 5.1: Gel electrophoresis for diagnostic studies of MWMV PaMV and CpMMV in Kenyan papaya. A band at 315 bp in (A), 694 bp in (B) and 304 bp in (C) show the presence of MWMV, CpMMV and PaMV, respectively. M indicates the O'GeneRuler™ 1 kb plus DNA ladder. +ve is a positive control, -ve is negative control. Numbers 1-9 = papaya samples. (D) A section of the map of Kenya showing combinations of viruses associated with the disease as determined through RT-PCR approach in selected counties in Kenya. The map was developed using QGIS software (QGIS Development Team, 2019)**.**

From the PCR-based detection, 180 of 287 samples collected tested positive for at least one virus infection. MWMV was the most widespread virus detected alone, in mixed infections and dual infections. The virus was reported in 11 of 22 counties surveyed namely Nakuru, Busia, Homabay, Kisumu, Migori, Embu, Kiambu, Kirinyaga, Meru, Makueni, Murang'a, and Machakos and in 140 of 287 samples collected (Table 5.2). PaMV was the second most widespread virus and was detected in 9 of 22 counties surveyed namely Baringo, Embu, Kirinyaga, Meru, Tharaka Nithi, Kitui Machakos and Taita Taveta and in 39 of 287 samples collected (Table 5.2). CpMMV was the least prevalent virus and was detected in only three counties including Baringo, Meru and Kitui (Table 5.2).

Mixed infections of MWMV and PaMV were detected in samples collected from 5 of 22 counties namely, Embu, Kirinyaga, Meru, Machakos and Makueni while that of PaMV and CpMMV were detected in Baringo, Meru and Kitui counties. The presence of all the three viruses (MWMV, CpMMV and PaMV) was obtained in Meru county (Table 5.2; Plate 5.1D). Detections of more than one virus in fields sampled were encountered in some counties. Fifteen samples from 4 of 22 counties namely, Embu, Kirinyaga, Machakos and Makueni had dual infections of MWMV and PaMV while 2 of 8 samples from Kitui county were co-infected with CpMMV and PaMV (Table 5.2).

Detection of the disease symptoms signified the presence of viral infection in some counties. In other instances, the presence of PRS-like symptoms was not an indicator of viral presence or absence. In Vihiga county, for instance, plants displayed symptoms and none of the three viruses was detected in them. In Baringo, Migori, Embu, Kiambu, Tharaka Nithi and Taita Taveta counties, 8/13, 10/10, 28/28, 10/10, 3/12, 4/12 samples respectively displayed symptoms, however, the viruses were detected in 3/13 (1 PaMV and 2 CpMMV), 4/10 (MWMV), 17/28 (15 MWMV and 2 PaMV), 9/10 (MWMV), 2/12 (PaMV) and 3/12 (PaMV) in the respective order in each county. In some instances, the number of plants infected with viruses was higher compared to the number of symptomatic plants. For instance, in Kirinyaga, Makueni and Kisumu counties, 28/42, 13/25 and 6/12 plants respectively displayed symptoms whereas viruses were detected in 40/42 (40 MWMV and 4 MWMV+PaMV), 19/25 (8 MWMV and 11 PaMV) and 10/12 (MWMV) plants, respectively (Table 5.2). The most prevalent symptoms in plants included vein clearing, mosaic patterns, mottling, leaf distortion, puckering, shoe-stringing on leaves, water-soaked marks on the petioles and stems, ringspot on fruits, and stunted growth.

Papaya plants singly infected with MWMV displayed puckering, vein clearing, leaf distortion, shoe stringing, mottling water-soaked marks on stems and petioles, ringspot on fruits and stunted growth. On the other hand, papaya plants infected with PaMV displayed mottling, puckering and leaf distortion symptoms (Table 5.2). The symptoms of plants dually infected fields with MWMV and PaMV were severer,

including leaf distortion, mosaic, mottling, vein clearing, ringspots, water-soaked marks, shoe stringing, puckering and stunted growth (Table 5.2). Papaya plants infected with PaMV and CpMMV showed mild symptoms such as mottling and stunted growth.

Table 5.2: Incidence (%) of viruses associated with papaya ringspot in 22 counties of Kenya

County	No. of samples collected[a]	No. of symptomatic samples[b]	Symptoms [c]	MWMV	PaMV	CpMMV	MWMV+PaMV	CpMMV+PaMV
Baringo	13	8	Mo	-	1	2	-	-
Elgeyo marakwet	8	2	SG, M	-	-	-	-	-
Nakuru	7	7	Mo, LD, RS, SG	7	-	-	-	-
Bungoma	3	0	None	-	-	-	-	-
Busia	4	2	WS	2	-	-	-	-
Homabay	14	14	PU, VC, WS, LD, SG	14	-	-	-	-
Kisumu	12	6	Mo, PU, WS, LD	10	-	-	-	-
Migori	10	10	Mo, M, VC	4	-	-	-	-
Siaya	2	0	None	-	-	-	-	-
Vihiga	2	2	Mo, VC	-	-	-	-	-
Kiambu	10	10	Mo, RS, WS, VC, LD, SG	9	-	-	-	-
Kirinyaga	42	28	LD, Mo, VC, RS, WS, SS, PU, SG	40	4	-	4	-
Meru	13	10	VC, M, Mo, PU, LD, SS, SG, WS	4	4	2	2	-
Murang'a	16	12	LD, M, RS, Mo, VC, SS, WS, PU	12	-	-	-	-
Tharaka Nithi	12	3	Mo	-	2	-	-	-
Embu	28	28	LD, VC, PU, M, Mo, WS, LC	15	2	-	2	-
Kitui	8	8	Mo, SG	-	5	3	-	2
Machakos	26	22	Mo, PU, RS, SS, WS, SG	15	7	-	5	-
Makueni	25	13	Mo, LD, WS, M, PU, RS, SG	8	11	-	2	-
Kwale	12	6	Mo, M	-	-	-	-	-
Kilifi	8	2	Mo, LD	-	-	-	-	-
Taita taveta	12	4	Mo, PU, LD	1	2	-	-	-
Total	**287**	**200**		**140**	**39**	**7**	**15**	**2**

[a] Number of samples collected per county for virus detection using PCR approach.

[b] Number of samples collected from plants exhibiting papaya ringspot symptoms

[c] symptoms exhibited by plants M: Mosaic patterns on the leaves; Mo: Mottling symptoms on the leaves; VC: vein clearing; PU: Puckering; SS: shoe stringing; LD: leaf distortion: WS: water-soaked marks on stems and petioles; RS: Ringspots on fruits and; SG: Stunted growth of the plant.

(-), not detected; MWMV, Moroccan watermelon mosaic virus; PaMV, Papaya mottle virus; CpMMV, Cowpea mild mottle virus

5.5 Discussion

Papaya ringspot disease is a major threat to papaya production in Kenya. The impact of the disease in the country is becoming serious that many growers have abandoned the fruit crop in favour of other crops. This study provides information on the incidence, severity and prevalence of papaya ringspot and maps out its distribution which are important aspects for the development of an effective management approach.

Papaya plants showing symptoms associated with the disease were observed in 20 out of 22 counties surveyed, causing minimal to severe levels of damage. Prevalence levels of up to 100 % were also reported in some counties signifying the widespread and the threat of the disease to papaya production in the country. The highest disease severities were reported in Kirinyaga, Murang'a Makueni, Machakos and Kiambu counties. The situation could partly be attributed to a lack of management measures as observed during the survey due to minimal knowledge of the disease and its causal agents (Mumo et al., 2021). In these counties, some farmers also cultivated papaya as a monocrop on large fields for commercial purposes, which could have encouraged fast disease spread because of the high host density and large size of cropped area (Kumar et al., 2010; Piper et al., 1996). Furthermore, monoculture facilitates easy movement of vectors from plant to plant during their transitional flights as they probe for a suitable host (Kumar et al., 2010), a situation that could contribute to the high disease incidences in these counties.

Three viruses MWMV, PaMV and CpMMV were detected in both symptomatic and asymptomatic papaya samples collected during the survey in farmers' fields in the major growing counties in Kenya. MWMV was the commonest and was widely distributed. The virus is one of the most common cucurbit viruses in Africa (Ibaba, Laing, & Gubba, 2016; Kidanemariam et al., 2019; Lecoq et al., 2001; Yakoubi et al., 2008). Although the virus was reported for the first time in papaya more than a decade ago in Congo (Arocha et al., 2008), its wide distribution in the country indicates that the virus is well established in papaya and there is an urgent need to develop management strategies is of paramount importance. The PaMV was recently

discovered and described as a ‘new’ virus infecting papaya in Kenya (Mumo et al., 2020). However, little is known about its impact on papaya crops, its vectors and mode of transmission as well as alternate hosts. Nevertheless, the virus poses a serious production challenge to papaya because of its wide distribution and occurrences of dual infections with other viruses. The CpMMV infecting papaya is recombinant (Mumo et al., 2020) and its incidences in papaya production counties are very low. The detection and low incidences of the CpMMV in papaya could be attributed to the recent host jump from cowpea to papaya after recombination and mutation leading to an increase in the host range (Legg & Thresh, 2000; Monci et al., 2002; Woolhouse et al., 2005). The CpMMV has been reported in leguminous and solanaceous crops in Africa (Jeyanandarajah & Brunt, 1993). During the survey, it was observed that cowpea plants were intercropped with papaya. Therefore, there is a chance that the whitefly transmitted the virus from cowpea to papaya, but this needs to be confirmed empirically.

Some plants displayed papaya ringspot symptoms, although no viruses were detected. For example, in Baringo, Migori, Embu, Kiambu, Tharaka Nithi and Taita Taveta counties, the number of plants infected was lower compared to the number of symptomatic plants. The absence of viruses in symptomatic plants could be attributed to other viral or non-viral diseases, nutrient disorders, insect damage (Schreinemachers et al., 2015) and viral load/titer and or existence of variants that may not be detected by the primers used (Ghoshal & Sanfacon, 2015). In other instances, the number of plants infected with the viruses was higher compared to the number of symptomatic plants (e.g. in Kirinyaga, Makueni and Kisumu counties). The absence of symptoms on virus-infected plants could probably be because the plants had just been infected and had not developed symptoms at the time the survey was carried out, the time of the year/season when the plant was infected, antagonisms due to co-infection with another virus or tolerance of the plant to the viruses (Kumar et al., 2010; Mowlick & Akther, 2008; Singh & Shukla, 2011).

The distribution of individual virus infections in Kenya is not region-specific. For instance, single PaMV infections occurred in Tharaka Nithi (Eastern) and Taita Taveta (Coast) while MWMV single infections were recorded in Kiambu, Murang’a,

Nakuru, Kisumu, Homabay, Migori and Busia counties which are either located in Central, Rift Valley or Western. The difference may be a result of the different frequency of distribution of individual viruses. The two viruses, PaMV and MWMV were found in Kirinyaga, Embu, Makueni and Machakos Counties which are located in the central and eastern regions. The PaMV and CpMMV were found in Baringo (Rift valley), Meru (Central) and Kitui (Eastern) Counties. No dual infection with MWMV and CpMMV was detected.

In conclusion, the incidence, severity, prevalence and distribution of papaya ringspot-associated viruses in Kenya have been determined. The viruses are widespread across the counties and could be moving to new areas not yet reported. Co-infections of the viruses have been reported. Papaya ringspot shows peculiarity in prevalence and symptoms development depending on weather conditions. Sometimes, masking of the symptoms in the infected plants occurs depending upon the seasons (Mowlick & Akther, 2008; Stevens, 1983). As such, there is a need for monitoring and surveillance of the viruses to establish if there are differences in symptoms and prevalence between different times of the year. Meanwhile, management measures such as the use of virus-free planting materials, roguing of infected plants and restricted movements of seedlings from one region to another and certification for the production of clean seedlings should be put in place to prevent the disease from spreading to those regions not yet infested. Further, the effects of these viruses' co-infection on papaya plants will need to be determined. Further, there is a need to determine if there are other viruses that could be causing the symptoms in samples where no viruses were detected.

CHAPTER SIX

MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF MOROCCAN WATERMELON MOSAIC VIRUS ISOLATES

Abstract

The potyvirus Moroccan watermelon mosaic virus (MWMV) naturally infects and severely threatens the production of cucurbits and papaya. In this study, MWMV isolated from pumpkin (*Cucurbita moschata*) intercropped with MWMV-infected papaya plants were identified and characterized through next-generation and Sanger sequencing approaches. The role of pumpkin and papaya host plants infected with MWMV in the spread of the virus in fourteen plant species through sap inoculations was evaluated. Complete MWMV genome sequences were obtained from two pumpkin samples through NGS and validated using Sanger sequencing. The isolates share 83.4-83.7 % nucleotide (nt) and 92.3-95.1 % amino acid (aa) sequence identities in the coat protein and 79.5-79.9 % nt and 89.2-89.7 % aa identities in the polyprotein with papaya isolates of MWMV. Phylogenetic analysis using complete polyprotein nucleotide sequences revealed the clustering of both pumpkin isolates of MWMV with corresponding sequences of cucurbit isolates of the virus from other parts of Africa and the Mediterranean regions, distinct from a clade formed by papaya isolates. Through sap inoculation, a pumpkin isolate of MWMV was pathogenic on zucchini (*C. pepo*), watermelon (*Citrullus lanatus*), and cucumber (*Cucumis sativus*), but not on other plant species tested. Conversely, the papaya isolate of MWMV infected zucchini but was non-pathogenic on other plant species used as differential hosts. The results suggest the occurrence of two strains of MWMV in Kenya having different biological characteristics associated with the host specificity.

6.1 Introduction

Moroccan watermelon mosaic virus (MWMV) is a member of the genus Potyvirus (McKern et al., 1993), one of the large plant viral groups comprising many economically important viruses. At the molecular level, MWMV forms part of the

papaya ringspot virus (PRSV) cluster (Yakoubi et al., 2008). The virus has a single-stranded positive-sense 9.7 kb RNA genome with a single open reading frame that is translated into a large polyprotein which is cleaved by the virus-encoded proteases into individual functional proteins (Wylie et al., 2017). It was first described in Morocco in 1974 as a strain of watermelon mosaic virus based on host range, having been reported in all commercial cucurbit-producing regions of the country as causing severe damage to cucurbits (Fischer & Lockhart, 1974). Using serological techniques, MWMV was reclassified as a distinct potyvirus species (Purcifull & Hiebert 1979). Quiot-Douine et al. (1990) established MWMV to be distantly related to the PRSV Potyvirus subgroup based on its biological and serological properties. Using tryptic peptide profiles, McKern et al. (1993) supported the classification of MWMV as a distinct species. Subsequently, sequence analysis of the coat protein gene and whole-genome established MWMV as a distinct member of the genus *Potyvirus* (Lecoq et al., 2001; Yakoubi et al., 2008). Since then, the virus has been detected in many other parts of Africa including Niger (Yakoubi et al., 2008), South Africa (Ibaba et al., 2016) Sudan (Lecoq et al., 2001), Zimbabwe, Cameroon (Yakoubi et al., 2008), Congo (Arocha et al., 2008; Yakoubi et al., 2008), Tunisia (Yakoubi et al., 2008), Tanzania (Menzel et al., 2011), Nigeria (Owolabi et al., 2012), and Kenya (Kidanemariam et al., 2019; Read et al., 2020). Outside Africa, the MWMV has been reported in Mediterranean countries including Italy (Roggero et al., 1998), Portugal (Yakoubi et al., 2008), France (Lecoq et al., 2007), Greece (Malandraki et al., 2014), Iraq (Bananej et al., 2018) and Spain (Miras et al., 2019).

The MWMV naturally infects and poses a serious production threat to cucurbits and *Carica papaya* L. (Lecoq et al., 2001; Arocha et al., 2008; Lecoq et al., 2007; Yakoubi et al., 2008; Ibaba et al., 2016; Kidanemariam et al., 2019; Mumo et al., 2020; Read et al., 2020). The virus also infects members of *Chenopodiaceae* through sap inoculation (Yakoubi et al., 2008). The virus is transmitted to cucurbits in a non-persistent manner by a range of aphid species including *Myzus persicae*, M. *persicae* subsp. *nicotianae*, *Aphis gossypii, A. spiraecola*, *A. fabae*, and *A. nerii* (Chatzivassiliou et al., 2016; Owolabi & Ekpiken, 2014). However, the aphid-mediated transmission of MWMV to papaya is not established yet. But like in the cucurbits, MWMV is most likely transmitted in papaya in a non-persistent manner by

several species of aphids, due to presence of highly conserved “RITC” “CSC” “PTR” motifs in the helper component-protease (HC-pro) and a ‘DAG’ motif in the coat protein of papaya isolate of the virus (Mumo et al., 2020). These motifs are associated with aphid transmission in potyviruses (Huet et al., 1994; López-Moya et al., 1999). It was reported that MWMV transmitting aphid species heavily colonize cucurbits (Chatzivassiliou et al., 2016) but not papaya (Martins et al., 2016).

Symptoms of MWMV infection in papaya include mottling, mosaic, shoe stringing, curling and puckering on the leaves, and ringed spots on the fruits of different sizes and shapes. Other symptoms in papaya due to MWMV infection include numerous water-soaked or oil-streaked lesions on the upper part of the plants’ stems and leaf petioles. As the disease progresses, the infected papaya plants become stunted and rosette with fibrous internal trunks (Arocha et al., 2008; Mumo et al., 2020). On the other hand, cucurbits infected with MWMV show mosaic, severe filiform and striking interveinal chlorosis, with raised dark green blisters on the leaves. Early infection of cucurbitaceous plants leads to severe stunting resulting in minimal fruit yield or complete crop failure. Infected fruits are misshapen with blistered surfaces (Fischer & Lockhart 1974; Lecoq et al., 2001; Yakoubi et al., 2008; Ibaba et al., 2016).

While sampling for papaya ringspot in Kenya in a previous study (Mumo et al., 2020), papaya crops were observed to be intercropped with pumpkin. The intercropped pumpkin plants frequently showed symptoms resembling those due to MWMV infection. Results of the previous study revealed that papaya plants displaying ringspot symptoms were infected with MWMV (Mumo et al., 2020). The goal of this objective was to identify and characterize viruses present in the symptomatic pumpkin plants using high throughput NGS and Sanger sequencing approaches. Further, the potential role of pumpkin as an inoculum source for MWMV spread to papaya and vice versa was investigated through sap inoculation experiments. These findings improve the understanding of MWMV epidemiology and support the improvement of disease management approaches.

6.2 Materials and methods

6.2.1 Sample collection

Leaf tissue samples were collected from two diseased pumpkin plants displaying symptoms of mosaic, puckering, vein clearing, and vein banding symptoms (Plate 6.1) in two different MWMV-infected papaya fields. The fields were in Meru and Kiambu counties in Kenya. Papaya isolates of MWMV from both fields were sequenced in our previous study and deposited in GenBank with accession numbers MH595741 for isolate Mer (Meru county) and MH595742 for isolate Kia (Kiambu county) (Mumo et al., 2020). The leaf samples were preserved in *RNAlater*® solution (Invitrogen™) and transported to the Biosciences eastern and central Africa–International Livestock Research Institute (BecA-ILRI) Hub laboratories in Nairobi, Kenya for next-generation sequencing on the Illumina MiSeq platform.

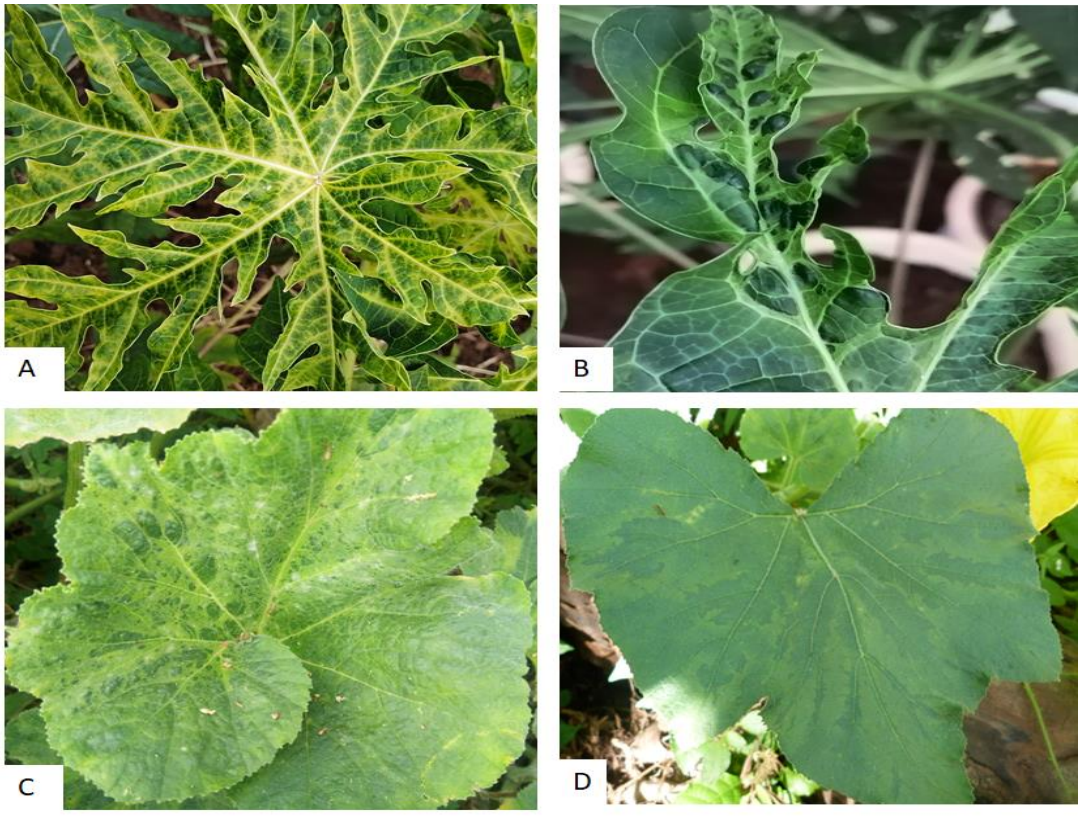

Plate 6.1: MWMV-infected papaya and pumpkin leaf samples. Vein clearing and mosaic A; puckering, mosaic and leaf distortion B; and pumpkin crops intercropped with MWMV infected papaya showing mosaic, puckering and vein clearing C and

mosaic and vein banding D. Papaya and pumpkin samples A and C were collected from the same farm in Kiambu county while B and D were from the same farm in Meru county.

6.2.2. RNA extraction, sequencing and bioinformatics analysis

Total RNA was extracted from the two pumpkin leaf samples using RNaesy® Plant Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and used for cDNA library preparations. The libraries were prepared using the Illumina TruSeq® RNA sample preparation protocol (Illumina, San Diego, CA, United States) and sequenced on the Illumina MiSeq system at the BecA-ILRI Hub, Nairobi, Kenya. The obtained 35-151 bp paired-end reads were checked for quality using FastQC, and low quality reads and sequencing adapters were removed using Trimmomatic v. 0.33 (Bolger et al., 2014). The host genome was removed by mapping all the reads to the *Cucurbita maxima* genome (GenBank accession number GCA_002738345.1) using Bowtie2 v. 2.2.8 (Langmead & Steven 2013). The remaining non-host reads (unmapped) were then assembled *de novo* to obtain contigs using metaSPAdes V 3.9.0 (Nurk et al., 2017) with default settings. The Krona web-based tool (Ondov et al., 2011) was used for identification and visualization of the assembled virus contigs. The resultant contigs were compared with other sequences in the NCBI non-redundant database (http://www.ncbi.nlm.nih.gov/) (Benson et al., 2012) and the Plant Virus Genome database (Camacho et al., 2009) using BLASTn search. The top hit accession was used for virus identification. Reference assemblies were performed by mapping the *de novo* sequences against the most similar existing viral genomes using the read mapping module of CLC genomics workbench version 5.5.1 (CLC Bio, Aarhus, Denmark). The *de novo* sequences and consensus sequences from reference mapping were then compared through visual inspection of individual mappings to ensure no artifacts were incorporated due to sequencing errors or errors during genome assembly. *De novo* sequences were however preferred over the consensus of reference assembly as a precautionary measure in case the viruses identified had considerably diverged from similar viral genome sequences in GenBank database.

6.2.3. Validation of assembled *de novo* sequences through RT-PCR and Sanger sequencing

To validate the assembled virus specific contigs, primers were designed from Illumina generated sequences using Primer 3 (Untergasser et al., 2012), and evaluated for specificity using NCBI primer-BLAST tool (Ye et al., 2012). The primer sequences designed are shown in Table 6.1. The primers were used for RT-PCR and the amplified products were shipped to Macrogen, (Netherlands, Europe) for Sanger sequencing. The obtained sequences were assembled using CLC Genomics Workbench (version 5.5.1) and compared with the *de novo* assembled sequences from the Illumina MiSeq through alignment and visual inspections. The Sanger sequences were also used for BLASTn search in NCBI non-redundant database.

Table 6.1: List of primers used in the detection for *Moroccan watermelon mosaic virus* isolates from pumpkin in Kenya

Primer [a]	Sequence (5'-3')	Size (bp)	Target Gene	Location (nt) [b]
MWMV18-F	TGCTGTTGGTAGTGGCAAAT	613	cylindrical inclusions (CI)	4016-4035 (Ken-pump) 4060-4079 (Ken-Mer)
MWMV18-R	TTCTGTTCGCCCAACTTTCA			4609-4628 (Ken-pump) 4653-4672 (Ken-Mer)
MWMV20-F	AAACACAAGGGCCACTCAAA	455	coat protein (CP)	8969-8988 (Ken-Pump) 9013-9032 (Ken-Mer)
MWMV20-R	ACAATCGAGTGTTTGCACCT			9404-9423 (Ken-pump) 9448-9467 (Ken-Mer)

[a] F, sense primer; R, antisense primer. [b] The targeting nucleotide (nt) locations according to the complete genome sequence of MWMV MH713899 (isolate Ken-pump) and MT497462 (isolate Ken-Mer).

6.2.4. Sequence and phylogenetic analysis

Open reading frames (ORFs) were determined using ORF finder. The sequence alignment was carried out using in-built program in CLC Genomics Workbench and protein sequence identities were computed using the Sequence Identity And Similarity (SIAS) tool http://imed.med.ucm.es/Tools/sias.html. Phylogenetic analysis

were carried out in MEGA 6 (Tamura et al., 2013) based on complete polyprotein nucleotide sequences of isolates of MWMV and other potyviruses. The phylogenetic tree was constructed using maximum likelihood method based on the Jones–Taylor–Thornton (JTT) matrix (Jones et al., 1992), as determined in the program Modeltest (Posada & Crandall 1998), using 1000 replicates for bootstrap analysis. Identification of potential genome recombination sites of the aligned complete genome sequences of isolates of MWMV and several other potyviruses was performed using RDP4 package (Martin et al., 2015), with default setting. Some of the known recombinant sequences of sugarcane mosaic virus (AF494510, AY149118, AY042184, GU474635, AM110759 and EU091075) were included as controls during recombination analysis (Padhi & Ramu 2011). To determine nucleotide diversity and mutations within MWMV sequences, 22 full genomes available in GenBank, (10 genomes isolated from papaya and 12 genomes isolates from cucurbits including two from this study), were analyzed using DNASP V6.11.0 (Rozas et al., 2017).

6.2.5. Sap inoculation of MWMV isolates

The tests were conducted in a greenhouse at Jomo Kenyatta University of Agriculture and Technology. To rule out the possibility of infection by other viruses in the diseased papaya plant, the sample was tested for the presence of Cowpea mild mottle virus and papaya mottle associated viruses using the virus's specific primers designed in the previous study (Mumo et al. 2020). The pumpkin sample used in this study was infected with one virus from sequencing results. From the field, the viruses were immediately propagated and maintained in their natural hosts, papaya and pumpkin plants, in an insect-free greenhouse. Papaya and pumpkin plants were grown in 5 kg pots containing a steam-sterilized substrate 2 h at 121 ^{o}C composed of a mixture of soil and organic matter. To propagate the viruses, infected plant leaves were ground in 10 volumes (1 g per 10 ml) of cold inoculation buffer (0.01M potassium phosphate, pH, 7.5, 4^{o}C) plus 40 g of 600 mesh carborundum in 1.5 L of inoculum. The crude viral inoculum prepared from infected papaya and pumpkin was rubbed gently with cheesecloth on the two youngest fully expanded leaves of four weeks-old papaya and two weeks-old pumpkin plants after transplanting, respectively. Inoculated leaves were rinsed with sterile water to wash off excess

inoculums. The inoculated plants were observed daily for symptom development. After the development of clear symptoms similar to those observed in the field (28 days after inoculation), the plants were tested for viral infection using viruses' specific primers. The previously reported forward (5′-TCTCAGCTAGCACGCAACAA-3′) and reverse (5′-CGGTGTTGAGCCAAACGAAG-3′) primer pair based on papaya isolates of MWMV (Mumo et al., 2020) was used to target a 315 bp fragment in cylindrical inclusion (CI) region of the papaya isolate. For the pumpkin MWMV isolate, the primer pair MWMV18-F/MWMV18-R (Table 6.1) was used to amplify the 613 bp fragment corresponding to the CI region. Infected papaya and pumpkin plants maintained in the greenhouse were used as a source of viral inoculum for subsequent tests.

Fourteen (14) plant species belonging to four families were mechanically inoculated using the viruses isolated from pumpkin and papaya in the greenhouse. The test plants included *Datura metal*, *D. stramonium*, *Nicotiana clevalendii*, *N. tabacum*, *N. glutanosa*, *N. bentamiana*, *Cucurbita pepo* (Squash or courgette), *C. moschata* (pumpkin), *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *Phaseolus vulgaris* (common bean), *Vigna unguiculata* (cowpea), *V. radiate* (mung bean) and *Carica papaya* L. Seedlings of the test plants were established from seeds in pots containing steam-sterilized soil and maintained in an insect-free screen house. Youngest papaya and pumpkin leaves with clear viral symptoms were used as a source of viral inoculum. The inoculation process was carried out as described in the propagation of viruses. Controls included the same plant species inoculated with buffer without viral inoculum and also non-inoculated plants. The inoculated leaves were rinsed thoroughly with sterile water and monitored daily for virus symptom development, which was recorded weekly until 35 days post-inoculation. Two independent inoculations were conducted using ten plants of each crop for each test. All plants without symptoms were tested 14 and 35 days after inoculation by RT-PCR using virus isolate specific primers. For symptomatic hosts, only two plants of each species were tested.

6.3 Results

6.3.1 Detection of MWMV in pumpkin plants intercropped with MWMV-infected papaya

The presence of MWMV was confirmed through homology search against the NCBI non-redundant database of the longest *de novo* assembled contigs of 9,729 and 9,754 bases with a coverage depth of 1828 and 1571, respectively, from the two pumpkin samples. Based on pairwise sequence comparisons, the contigs from pumpkin samples shared 99 % sequence identity. The contigs shared 81 % sequence identities with MWMV isolates previously reported from papaya in Kenya, GenBank accession numbers MH595736-46 (Mumo et al., 2020). The RT-PCR screening of the pumpkin samples yielded the expected fragment sizes of 613 bp and 455 bp for cylindrical inclusion (CI) and coat protein (CP), respectively. Sanger sequencing of the amplified viral amplicons yielded sequences that were 100% identical at the nt/aa levels to those generated *de novo* based on pairwise comparisons, confirming that the genome sequence assembly of MWMV isolated from pumpkins was accurate. There were no other virus sequences found in the pumpkin samples subjected to NGS.

The MWMV viral genome sequences obtained from pumpkins were deposited in GenBank under accession numbers MH713899 and MT497462. The untranslated regions (UTRs) ends were not verified through rapid amplification of cDNA ends (RACE) analysis but they were almost similar in length to the corresponding sequences of published isolates of MWMV. The MH713899 genome is organized into 158 nt 5′UTR, followed by 9,369 nt polyprotein encoding sequences from which all the proteins of the virus are derived, and 192 nt long 3´UTR. The MT497462 genome, on the other hand, is organized into 202 nt in 5′UTR, 9,369 nt of the polyprotein, and 180 nt 3′UTR. The MWMV polyprotein of each pumpkin isolate in this study contains 3,122 amino acids (aa), compared to 3,124 aa for published sequences of isolates of papaya and other cucurbits and 3,121 aa for MWMV isolate of *Cucurbita pepo* from Burkina Faso (MN688647). The variability in the N-terminal region of the coat protein of MWMV isolates from pumpkin samples, compared to those of papaya, was most evident having a 6 nt, deletion. On the other hand,

MWMV isolate from Burkina Faso had a 6 nt insertion in the same region. Several conserved motifs reported in MWMV isolates in papaya and other cucurbits were also found in MWMV isolates from pumpkins. These include a domain containing 5 repeats of CAA motifs (CAACACAACACAACAACATTCAA) in the 5′UTR, the nucleotide triphosphate (NTP)-binding motif "GAVGSGKST" in N terminal region of cylindrical inclusion, three active sites of RNA-dependent RNA polymerase motifs: the "YCDADGS", "GNNSGQPSTVVDNTLMV", and "NGDDL" in the nuclear inclusion b (NIb). Other motifs like ''RITC'', "PTR'' both in HC-Pro and "DAG" in the coat protein reported in papaya MWMV isolates were also found in the isolates from pumpkins.

6.3.2 Sequence identities and phylogenetic analysis

The genome sequences of the two pumpkin MWMV isolates were almost identical to each other, sharing more than 97 % nt and aa sequence identities in all encoded proteins (Table 6.2). However, the pumpkin isolates shared only 79.5-79.9 % nt (89.2-89.7 % aa) identity in the polyprotein and 83.4-83.7 % nt (92.3-95.1 % aa) identity in the CP region with MWMV isolates from papaya. The P1 was the most variable protein between MWMV isolates from papaya and pumpkins, sharing 69.1-69.5 % nt and 64.1-65.5 % aa sequence identities (Table 6.2). Sequence identities of more than 80 % nt (90 % aa) in the polyprotein and 83 % nt (more than 91 % aa) in the CP were observed between pumpkin MWMV isolates from this study and virus isolates from *Cucurbita pepo* in Burkina Faso (MN688647), Tunisia (EF579955) and South Africa (KU315176) (Table 6.2).

Table 6.2: Polyprotein and gene-specific nucleotide (nt) and amino acid (aa) sequence identities (%) within *Moroccan watermelon mosaic virus* (MWMV) isolates from pumpkin in Kenya (MH713899 and MT497462) and between them and global MWMV sequences

Genome segment	MH713899 vs. MT497462[a]		MH713899/MT497462 vs. MH595736-46[a]		MH713899/MT49746 2vs. MG800832[a]		MH713899/MT49746 2vs. MN688647[a]		MH713899/MT4974 62vs. KU315176[a]		MH713899/MT497462 vs. EF579955[a]	
	nt	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**
Polyprotein	99.4	99.3	79.7-79.9	89.2-89.7	87.9	94.4-94.5	81.4-81.5	89.2-89.3	80.6-80.8	90.8-90.9	80.4-80.5	90.3-90.4
P1	99	98.3	69.1-69.5	64.1-65.5	82.3-82.5	78.8-79.1	74.1-74.5	69.9	73.2-73.7	68.1-68.7	72.7-73.4	66.9-67.5
Hc-Pro	99.8	99.8	78.6-79.3	90.4-91.9	88	98.2-98.5	86.2	95.6	82.5-82.6	93.7	82.8-82.9	94.3
P3	99.4	99.1	81.8-82.5	88.2-89.3	88.1-88.2	92.2	80.7-80.9	88.3	82.0	89	80.1	86.7
6K1	100	100	82.6-83.2	92.3	89.0	92.2	81.9	98.1	83.9	92.3	81.3	92.3
CI	99.3	99.5	80.9-81.3	94.8-95.6	89.7-89.8	98	79.3-79.4	82.7	80.5-80.8	95.9	81.1	95.4
6K2	99.4	100	78.8-79.4	87.7-89.5	85.5-86.5	89.2	77.1-77.6	86.0	76.5-77.1	80.7	78.8-79.4	80.7
Vpg	98.6	97.9	78.7-79.6	86.8-87.9	86.5-87.2	93.1-95.3	83.7-85.1	95.8-97.9	79.8-81.2	91.6-93.7	79.6-80.6	91.1-93.2
NIa	99.7	100	79.2-79.9	92.3-93.2	88.5	96.6	83.8	96.6	79.5-80.0	94.1	81.6-81.8	94.6
NIb	99.5	99.6	82.3-82.7	94.2-94.9	87.6-88.1	97.9	84.2	95.3	82.5-82.6	96.1	81.1	95.7
CP	99.3	98.9	83.4-83.7	92.3-95.1	90.2-90.4	94.3-95.4	86.2	95.4-96.4	83.7	92.1-93.2	83.5	91.8-92.8

[a] MH713899 and MT497462 (pumpkin isolates sequenced in this study); MH595736-46 (papaya isolates from Kenya); MG800832 (pumpkin isolate from Kenya); MN688647(squash isolate from Burkina Faso; KU315176 (squash isolate from South Africa); EF579955 (squash isolate from Tunisia).

Analysis of the aligned complete genome nucleotide sequences using seven different algorithms showed no evidence of recombination involving pumpkin MWMV isolates (data not shown). Based on the full-length genome sequence, sequences of MWMV isolates from cucurbits were more diverse (π =0.14495) than those from papaya (π = 0.01939). The MWMV isolates from papaya had a lower number of mutations (927) compared to those recorded in MWMV isolates from cucurbits (3,809). The average number of nucleotide substitutions per site between MWMV isolates from cucurbits and those from papaya was 0.20398.

Phylogenetic inferences based on polyprotein nucleotide sequences of selected potyviruses revealed clustering of MWMV isolates from cucurbits in one group while those from papaya formed into a separate cluster with strong bootstrap support of 100 % (Figure 6.1). As suggested by the phylogenetic analysis, MWMV from papaya and those from cucurbits shared a common ancestor as supported by the 100 % bootstrap value. The MWMV isolates from pumpkin sequenced in this study clustered together in one subgroup, with the closest isolate being MG800832, previously sequenced from the pumpkin in Kenya (Kidanemariam et al., 2019). The clustering pattern of MWMV sequences from cucurbits correlated well with their geographical origins, with isolates from South Africa (KU315175 and KU315176) forming a separate cluster, those from North Africa and the Mediterranean region (EF579955, LN810061, and KY762266) forming another cluster, and those from and East and West Africa (MN688647, MG800832, MH713899 and MT497462) clustering in a different subgroup (Figure 6.1).

6.3.3 Host range of MWMV isolates from pumpkin and papaya

The infectivity of the MWMV was determined using a pumpkin (MH595741) and a papaya (MH713899) isolate of the virus (Mumo et al., 2020). The pumpkin MWMV isolate systemically infected and induced symptoms in zucchini, watermelon, cucumber, and pumpkin plants following sap inoculation. In general, symptoms, which started appearing twelve days after inoculation included mosaic, raised dark green patches, vein clearing and leaf distortion. However, the MWMV-pumpkin

isolate did not produce visible symptoms on plants from the fourteen species used as differential hosts including papaya (Plate 6.2; Table 6.3).

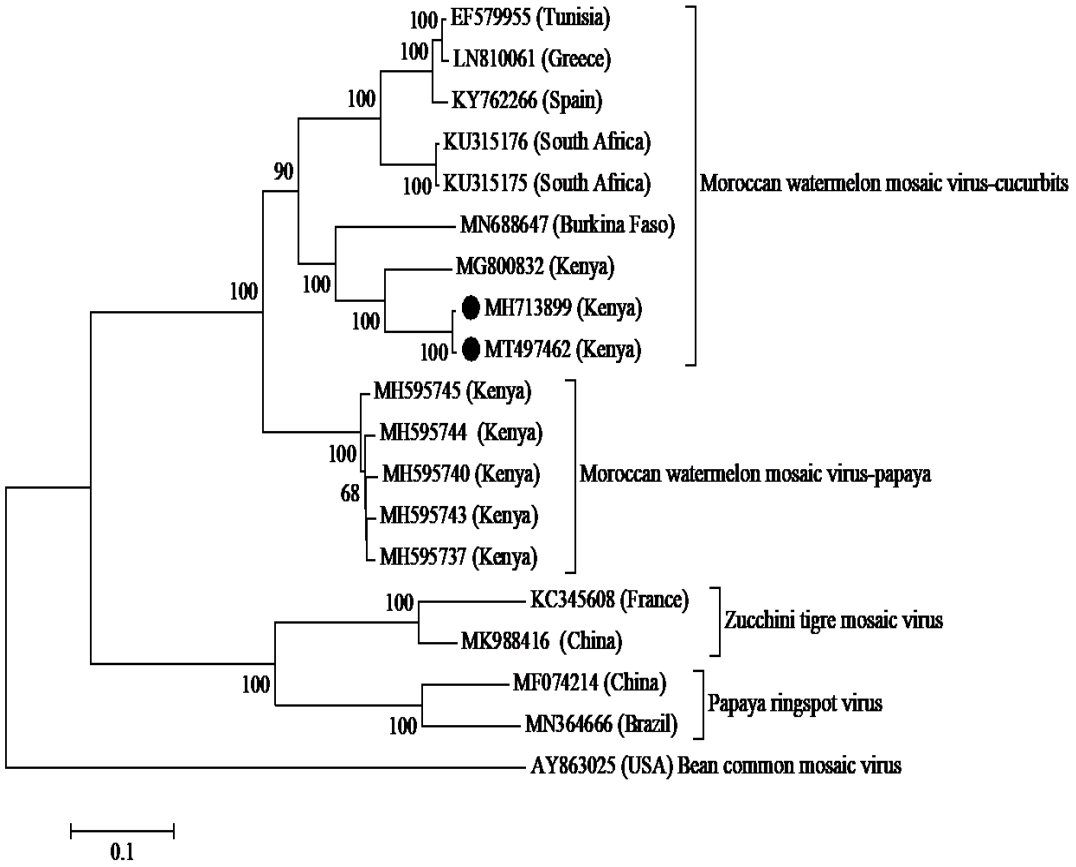

Figure 6.1: Rooted phylogenetic tree depicting the evolutionary relationships among *Moroccan watermelon mosaic virus* isolates from papaya and cucurbits based on analyses of complete polyprotein nucleotide sequences of the virus and corresponding sequences of isolates of other potyviruses. The tree was generated in MEGA 6 (Tamura et al., 2013) using the Maximum Likelihood method based on JTT matrix-based model (Jones et al., 1992). The scale bar is given in the number of nucleotide substitutions per site. Phylogeny was inferred following 1,000 bootstrap replications, and the node values show percentage bootstrap support. The isolates sequenced from the pumpkin in this study are shown with black circles (•). *Moroccan watermelon mosaic virus* –Cucurbits refers to MWMV isolates from

cucurbits while *Moroccan watermelon mosaic virus* –papaya refers to MWMV isolates from papaya.

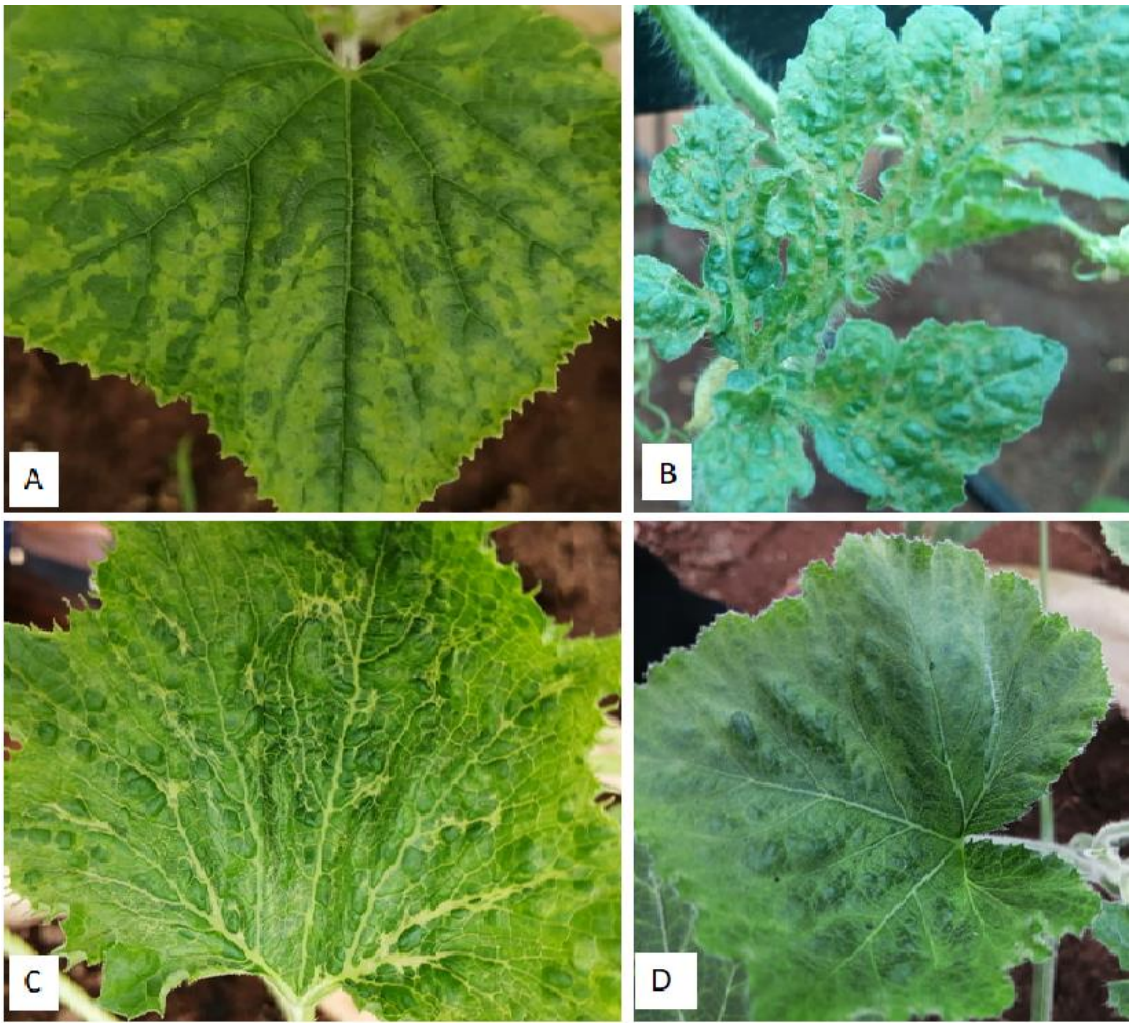

Plate 6.2: Symptoms induced by MWMV isolate from pumpkin on cucurbits plants twenty-eight days after sap inoculation. A: mottling on cucumber plants B: puckering and leaf distortion on watermelon; C: vein clearing, puckering and leaf distortion on Zucchini plants and D: puckering and mosaic on pumpkin leaves. Symptoms were recorded fourteen days after inoculation.

The papaya MWMV isolate on the other hand, induced typical viral symptoms on papaya plants similar to those observed in the field ranging from vein clearing, mottling, ringspots on leaves, leaf distortion, shoe stringing and water-soaked marks

on the stem. The symptoms in the inoculated papaya started appearing 10 days after inoculation. The isolate also caused chlorotic spots on zucchini plants (Plate 6.3). However, no visible symptoms were observed on pumpkin, watermelon, cucumber and other plant species tested.

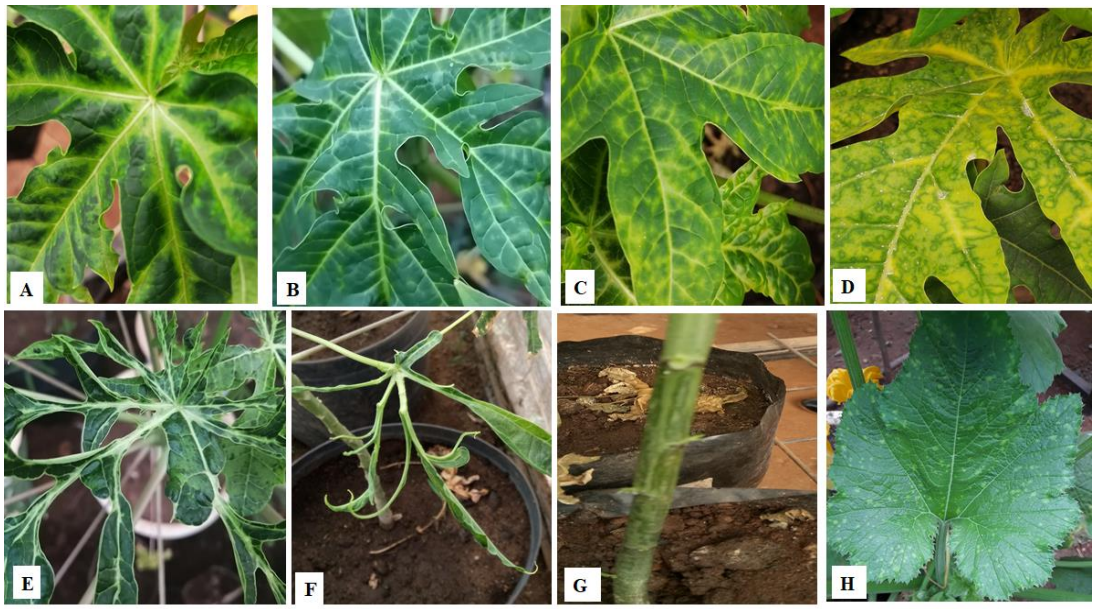

Plate 6.3: Symptoms induced by MWMV-P in papaya and zucchini. A: vein clearing; B: mild mottling; C: severe mottling and vein clearing; D: ringspot of leaves; E: leaf distortion; F: shoe stringing; G: water-soaked marks on the stem and H: chlorotic spots on zucchini

The presence or absence of the MWMV on the test plants was further confirmed through RT-PCR (Plate 6.4). Pumpkin, watermelon, zucchini and cucumber plants inoculated with the pumpkin MWMV isolate tested positive for the virus but the papaya plants inoculated with the same isolate tested negative (Plate 6.4). Papaya and zucchini plants inoculated with papaya MWMV isolate tested positive whereas other plant species inoculated with the same isolate tested negative (Plate 6.4; Table 6.3).

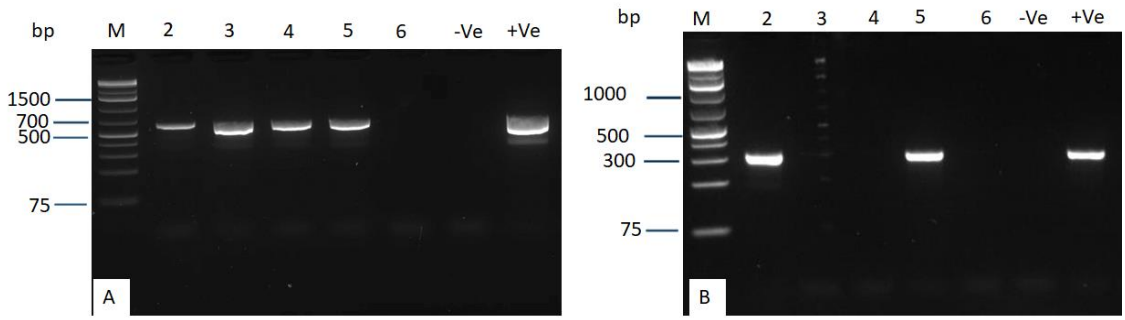

Plate 6.4: Agarose gel electrophoresis analysis of PCR products for diagnostic studies of MWMV infections on test plants. A band at 615 bp in A shows MWMV isolated from pumpkin infections on pumpkin (2), zucchini (3), watermelon (4), cucumber (5) and papaya (6). A band at 315 bp in B shows MWMV isolated from papaya infections; papaya (2), pumpkin (3), cucumber (4), zucchini (5) and watermelon (6). M indicates the O'GeneRuler™ 1 kb plus DNA ladder. +ve is positive control, -ve is negative control.

Table 6.2: Reaction of several cucurbits and papaya plants to isolates of *Moroccan watermelon mosaic virus* obtained from pumpkin and papaya based on sap inoculations.

Source of virus isolate	Test plants	Symptoms[a]	No. of plants showing symptoms/No. of plants inoculated	No. of plants infected /No. of plants tested (RT-PCR)
Pumpkin	Zucchini	LD, LC, PU, VC	10/10	2/2
	Pumpkin	LD, Mo, MO, PU	10/10	2/2
	watermelon	PU, LD	10/10	2/2
	cucumber	LD, Mo, PU	10/10	2/2
	papaya	NS	0/10	0/10
Papaya	Zucchini	CS	10/10	2/2
	Pumpkin	NS	0/10	0/10
	watermelon	NS	0/10	0/10
	cucumber	NS	0/10	0/10
	papaya	LD, RS, WS,VC, MO, SS	10/10	2/2

[a] Symptom description; CS: chlorotic spots; LD: leaf distortion; LC: leaf curl; ML: Mottling; MO: Mosaic PU: puckering; RS: ringspots on the leaves; SS: shoe stringing of leaves; WS: water-soaked marks on the stem/petioles; VC: vein clearing and NS: no symptoms. No symptoms were induced on other test plants. The results presented here were confirmed in a separate experiment.

6.4 Discussion

Intercropping in general has many advantages including efficient utilization of land resources, enhanced returns per unit area, and insurance against crop failure (Malézieux et al., 2009). The practice, however, may facilitate disease spread as intercrops can serve as alternate hosts or reservoirs of pathogens, a crucial role in the perpetuation of several diseases in different crop species (Ara et al., 2012; Martins et al., 2016; Ocimati et al., 2018).

In this study, MWMV was detected and characterized from field samples of pumpkin intercropped with papaya. The pumpkin MWMV isolate has similar genome composition as previously reported MWMV isolates of papaya from Kenya (Mumo et al., 2020) and *Cucurbita pepo* from Tunisia (Yakoubi et al., 2008). The observed deletion of six nucleotides (two amino acids) in the N terminal region of the coat protein of the genome sequences of the pumpkin MWMV isolates is reminiscent of reported nucleotide deletions in the N terminal region of a snake cucumber (*C. melo* var flexuosus) MWMV isolate from Sudan (Lecoq et al., 2001). The biological significance of these CP nucleotide deletions is unknown and should be the subject of future studies.

Sap inoculation experiment showed the existence of two strains of MWMV associated with plant specificity. The MWMV strain infecting pumpkin systemically infected several cucurbits species but not papaya plants. On the other hand, the strain infecting papaya infected both papaya and zucchini plants. Although in the experiment, insect transmission of the viruses was not evaluated to mimic the natural infection process, it is evident that there is the existence of MWMV host plant specificity. The MWMV can be transmitted mechanically or by aphids (Yakoubi et al. 2008; Owolabi et al. 2012). More efficient aphid transmission occurs in a non-persistent manner (Yakoubi et al., 2008), where aphids acquire and inoculate virus particles in the epidermal cells within a few seconds because virus particles bind rapidly but loosely to receptors within an aphid's probing mouthparts (stylet) and are released during salivation (Uzest et al., 2010; Groen et al., 2017) Similar results in papaya ringspot virus (PRSV) (a virus closely related to MWMV), have been reported, where, based on biological properties, there are two strains; PRSV-P

(isolated from papaya) that infects several cucurbits whereas PRSV-W (isolated from cucurbits) that is unable to infect papaya (Shukla & Ward 1988; Gonsalves, 1998). The two PRSV biotypes cannot be distinguished based on divergence in their CP sequences (Bateson et al., 1994; Ventura et al., 2004). For instance, Bateson et al. (1994), studying seven Australian isolates (four P-type and three W-type), found that they shared a high degree of sequence homology in the CP gene, ranging from 98.1 to 98.9 %. On the other hand, Silva-Rosales et al. (2000), studying three Mexican P-type isolates from geographically close areas observed a lower degree of nucleotide sequence homology, ranging from 93.4 to 98.4 % at the CP. Sequences of MWMV infecting papaya and those of pumpkin could be distinguished based on sequence divergence in the CP.

Genomes of plant viruses in the genus *Potyvirus* encode large polyproteins that are cleaved by virus-encoded proteases into ten mature proteins namely P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP (Adams et al., 2005b; Revers & García 2015). P1 is the most variable protein both in size and sequence (Adams et al., 2005b; Valli et al., 2007), and it is alleged that P1 diversification has contributed to the successful adaptation of potyviruses to a wide range of host species (Valli et al., 2007; Salvador et al., 2008). In this study P1 was the least conserved protein between MWMV isolates of papaya and pumpkins in Kenya sharing 69.1-69.5 % nt and 64.1-65.5 % aa sequence identities between them. Similar sequence divergence was observed between MWMV in pumpkins in Kenya and those in *Cucurbita pepo* from Burkina Faso, Tunisia and South Africa. Therefore, whereas the pattern of P1 divergence between papaya and pumpkin isolates of MWMV in Kenya would suggest a possible association with host specificity, this association is less clear when P1 sequences of global isolates of the virus are considered. Further, recombination and mutations are the main forces driving plant virus evolution and host adaptation (García-Arenal et al., 2003; Valli et al., 2007; Nagy, 2008) and are common in potyviruses (Padhi & Ramu 2011; Sebestye & Bala 2015; Moradi et al., 2016). No recombination was detected possibly ruling out its involvement in the adaptation of MWMV to either papaya or pumpkin. Analysis of the whole genome sequences of the MWMV population from different regions of the Africa and Mediterranean showed that the level of nucleotide diversity and number of mutations were lower in

MWMV isolates from papaya compared to isolates from cucurbits. Although MWMV genomes from papaya are only available from Kenya, the highest diversity and mutations observed within MWMV isolates from cucurbits suggest that movement of the virus around the world in cucurbits and then mutation to infect papaya could be a factor in the molecular epidemiology of MWMV.

Phylogenetic inferences among the polyprotein regions showed that MWMV isolates from papaya and those from cucurbits are strains of the same virus sharing a common ancestor. MWMV infects several cucurbit species and has a wide distribution in Africa and the Mediterranean region (Lecoq et al., 2001; Yakoubi et al., 2008; Owolabi et al., 2012; Kidanemariam et al., 2019) and this probably implies that different cucurbit hosts together with local and long-distance movement of the virus may have resulted in variability within MWMV populations. Further, MWMV infection in cucurbits was reported more than three decades (Fischer & Lockhart 1974) before that in papaya (Arocha et al., 2008) indicating that the papaya MWMV isolate might have originated from a cucurbit-infecting isolate from where host speciation occurred.

From the results of this study, it is evident that the MWMV isolated from papaya and MWMV isolated from pumpkin in Kenya are naturally adapted to papaya and pumpkin, respectively. The MWMV isolated from papaya can be transmitted to zucchini and not to other cucurbits through sap inoculations. Since the MWMV strain infecting papaya could infect zucchini, this represents a potential inoculum source when papaya and zucchini are intercropped. Further, in the future, more practical questions need to be evaluated including: (1) Can aphids spread the virus from papaya to cucurbits and then from cucurbits back to papaya? (2) Can they also spread the virus from cucurbits to papaya and then from papaya back to cucurbits? (3) Are there differences in the transmission efficiencies of the virus? Answering these questions will be an important step in the development of appropriate MWMV management strategies for both papaya and cucurbitaceous crops.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Discussion

Papaya is an important fruit crop in Kenya, grown in several counties by small and large-scale farmers for local and export markets. Areas under papaya production in Kenya over years have increased but surprisingly the increase seems not to match the expected papaya fruits production increase in the country. This discrepancy has been attributed to various production constraints, with disease infections playing a major role (HCDA, 2016; Rimberia & Wamocho, 2014). Papaya ringspot is a major threat to papaya production in Kenya, and worldwide in general (Rimberia & Wamocho, 2014; Sharma & Tripathi, 2014). The disease is reported to be spread in many regions where papaya is grown irrespective of the climatic conditions. The disease impact is devastating to the extent that it has forced farmers, especially in severely affected regions, to stop growing papaya, on a global scale leading to about 50 % or more decline in crop production. In Kenya, the disease impact is for example so severe that many papaya growers have abandoned growing the fruit crop in favour of other crops. The disease further challenges papaya production necessitating a speedy intervention in disease management.

To implement a successful disease management program, adequate knowledge, perception and management practices of the disease problem by farmers, are required (Lwin et al., 2012; Schreinemachers et al., 2015). This study indicates that accurate disease diagnosis distinguishing between infected plants from the non-infected ones that only show virus-like symptoms is a key step in the adoption of a good crop disease management system as pointed out by Jeong et al. (2014) and Pearson et al. (2006). Disease incidence, severity as well as prevalence and distribution, are other important aspects needed in the development of an effective disease management scheme in crop production.

In this study, farmers' knowledge, perception and management practices of papaya ringspot in Kenya's major papaya growing regions were profiled. The report showed

that farmers' knowledge, perception and management of disease in the country are limited. For example, only about 40 % of the study respondents were able to identify the disease. Also, about half of respondents aware of the disease did not know its cause, despite some using chemical sprays as its management/control measure.

Next-generation sequencing technology has improved and increased the rate of plant virus discovery, diagnostics, and evolutionary studies (Roossinck et al., 2017). For example, the technology has been useful in the identification of plant viruses with or without prior knowledge of the viral type present, and in revealing the presence of novel and unsuspected agents. Also, the approach has been helpful for the detection of viral co-infection in many plants (Akinyemi et al., 2016; Blawid et al., 2017; Candresse et al., 2014; Roossinck, 2015). This study established the power of NGS where MWMV was determined to be responsible for papaya ringspot in Kenya and not PRSV as previously thought. The technology, indeed, helped to uncover other viruses including CpMMV, a *Carlavirus* and two novel yet divergent *Carlaviruses* putatively named PaMV and PAMMV in symptomatic and asymptomatic papaya leaf samples.

Disease severity, incidence and distribution of the viruses associated with the papaya ringspot in Kenya are vital for the development of the disease evidence-based management options. A survey conducted in 22 counties in five major papaya production regions established mild disease severity (2.0) across the surveyed counties. Generally, the study data showed that the disease is widely distributed, with an average incidence of 21.1% and 65.5 % disease prevalence among the counties surveyed. In terms of the distribution of viruses associated with the disease, MWMV was the most widely recorded in 11 counties. The PaMV was the second most prevalent virus recorded in 9 of 22 counties, while CpMMV was the least prevalent recorded in three counties. Occurrences of two viruses, MWMV and PaMV, were reported in five counties; while that of PaMV and CpMMV were detected in three counties.

While sampling for papaya ringspot exhibiting plants, papaya crops in most sampled fields were intercropped with pumpkins. Surprisingly, the papaya intercropped pumpkin plants frequently showed viral-like disease infection symptoms. An investigation to establish the virus infecting pumpkin found that they were infected with MWMV. Sap inoculation experiments to establish if MWMV-infecting pumpkin could infect papaya and vice versa concluded that MWMV-pumpkin was adapted to pumpkin and other cucurbits plants including watermelon, cucumber and zucchini. The MWMV-infecting papaya was adapted to papaya and could not infect pumpkin but infected zucchini.

7.2 Conclusions

Papaya ringspot continues to impact negatively the papaya industry in Kenya. The disease is associated with MWMV and not PRSV although symptoms portrayed on infected papaya plants by the two viruses are similar. Results of this study showed that farmers' knowledge and management of the disease in surveyed areas are currently limited. However, given the rate at which papaya planting materials are exchanged between farmers in different counties in Kenya (Asudi, 2010), it is likely that viruses detected in diseased papaya plants, although currently are restricted in specific counties, more likely will quickly spread to other papaya areas in the region. As such, management options should be coined for individual viruses present in a given region for better disease management. Primers for the detection of these viruses developed in this study thus may aid in monitoring and surveillance the disease regularly and probably discover new infections, thus preventing the future spread of the viruses as well as innovating new ways of combating and reducing the effects of the viruses on papaya. Furthermore, it is evident from this study that the MWMV isolated from papaya and MWMV isolated from the pumpkin in Kenya are naturally adapted to papaya and pumpkin, respectively. The MWMV isolated from papaya can be mechanically transmitted to zucchini and not to other cucurbits through sap inoculations. However, since the MWMV strain infecting papaya could infect zucchini, this may represent a potential inoculum source when papaya and zucchini are intercropped. As a precaution, care should be taken to remove zucchini plants in the vicinity of papaya and pumpkin fields for the effective management of

papaya ringspot. Overall, the findings of this study have greatly expanded knowledge about the management of the disease in papaya and pumpkin crops in Kenya, and perhaps elsewhere.

7.3 Recommendations

1. As a precautionary measure, papaya seedlings should be tested before planting for virus presence using developed primers from this study.

2. The need to include farm-level training to increase farmers' awareness and knowledge about the disease, identification, and good management practices, which will in return boost the country's papaya fruit production is inevitable. Indeed, farm training by extension services has been shown to improve farmers' knowledge of plant viruses in Tamil Nadu, India.

3. Since the sequencing strategy used in this study targeted viruses with poly-A tail as per the libraries preparation protocol, the possibility of more and new viruses falling outside this detection approach infecting papaya crop cannot be ruled out. As such, additional viral metagenomics studies could help in understanding the complete viruses' diversity infecting papaya in Kenya in the future.

4. Further, more work is also needed for the complete classification of PaMV and PaMMV and to understand the risk they pose to the papaya fruit crop.

5. In the future, measures such as the use of virus-free planting materials, restricted movements of seedlings from one region to another, and certification for the production of clean seedlings should be put in place to prevent the disease from spreading to those regions not infected.

6. Since the MWMV strain infecting papaya could infect zucchini, this represents a potential inoculum source when papaya and zucchini are intercropped. However, insect-vector transmission will need to be carried out in the future to help in understanding the epidemiology of the disease for the development of better management options.

REFERENCES

Adam, R. I., Sindi, K., & Badstue, L. (2015). Farmers' knowledge, perceptions and management of diseases affecting sweet potatoes in the Lake Victoria Zone region, Tanzania. *Crop Protection*, *72*, 97–107.

Adams, M. J., Antoniw, J. F., & Fauquet, C. M. (2005a). Molecular criteria for genus and species discrimination within the family Potyviridae. *Archives of Virology*, *150*(3), 459–479.

Adams, Michael J., Antoniw, J. F., & Beaudoin, F. (2005b). Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. *Molecular Plant Pathology*, *6*(4), 471–487.

Adams MJ, Candresse T, H. J. et al. (2012). Family Betaflexiviridae. In L. E. King AMQ, Adams MJ, Carstens EB (Ed.), *Virus Taxonomy, Classification and Nomenclature of viruses Ninth Report of the International Committee on Taxonomy of Viruses.* (pp. 920–41). San Diego, USA: Elsevier Academic Press. https://doi.org/10.1016/B978-0-12-249951-7.50001-8

Akinyemi, I. A., Wang, F., Zhou, B., Qi, S., & Wu, Q. (2016). Ecogenomic survey of plant viruses infecting Tobacco by Next generation sequencing. *Virology Journal*, *13*(1), 1–12.

Alviar, A. N., Filomena C. Sta.Cruz & Hautea D. M. (2012). Assessing the Responses of Tolerant papaya (Carica papaya L.) Varieties to Papaya Ringspot Virus (PRSV) Infection and Establishment of symptom severity Rating Scale for resistance screening. *Philippine Journal of Crop Science*, *37*(2), 20–28.

Ara, M., Masud, M., & Akanda, A. (2012). Detection of Plant Viruses in Some Ornamental Plants That Act as Alternate Hosts. *The Agriculturists*, *10*(2), 46–54.

Araújo, F. S., Carvalho, C. R., & Clarindo, W. R. (2010). Genome size, base composition and karyotype of Carica papaya L. *The Nucleus*, *53*(1–2), 25–31.

Aravind, G., Bhowmik, D., Duraivel, S., & Harish, G. (2013). Journal of Medicinal Plants Studies Traditional and Medicinal Uses of Carica papaya. *Journal of Medicinal Plants Studies Year Journal of Medicinal Plants Studies*, *1*(1), 7–15.

Arocha, Y., Vigheri, N., Nkoy-Florent, B., Bakwanamaha, K., Bolomphety, B., Kasongo, M., … & Jones, P. (2008). First report of the identification of Moroccan watermelon mosaic virus in papaya in Democratic Republic of Congo. *Plant Pathology*, *57*(2), 387.

Asudi, G. O. ’. (2010). *Collection, Morphological and Molecular Characterization of Papaya.* Unpublished MSc thesis, Juja: Jomo Kenyatta University of Agriculture and Technology.

Asudi, G. O., van den Berg, J., Midega, C. A. O., Pittchar, J., Pickett, J. A., & Khan, Z. R. (2015). Napier grass stunt disease in East Africa: Farmers’ perspectives on disease management. *Crop Protection*, *71*, 116–124.

Atreya, P. L., Lopez-Moya, J. J., Chu, M., Atreya, C. D., & Pirone, T. P. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *Journal of General Virology*, *76*(2), 265–270.

Azad, M. A. K., Amin, L., & Sidik, N. M. (2014). Gene technology for papaya ringspot virus disease management. *The Scientific World Journal*, *2014*. https://doi.org/10.1155/2014/768038

Baker, H. N., Murphy, R., Lopez, E., & Garcia, C. (2012). Conversion of a capture ELISA to a Luminex xMAP assay using a multiplex antibody screening method. *Journal of Visualized Experiments*, (65), 1–7.

Bateson M. F., R. Lines, P. Revill, W.Chaleeprom, C. Ha, A. G. & Dale J. (2002). On the evolution and molecular epidemiology of the potyvirus Papaya ringspot virus. *Journal of General Virology*, *83*(10), 2575–2585.

Bateson, M. F., Henderson, J., Chaleeprom, W., Gibbs, A. J., & Dale, L. (1994). Papaya ringspot potyvirus: Isolate variability and the origin of PRSV type P (Australia). *Journal of General Virology*, *75*(12), 3547–3553.

Bau, H. J., Cheng, Y. H., Yu, T. A., Yang, J. S., Liou, P. C., Hsiao, C. H., … Yeh, S. D. (2004). Field evaluation of transgenic papaya lines carrying the coat protein gene of Papaya ringspot virus in Taiwan. *Plant Disease*, *88*(6), 594–599.

Bayot, R. G., Villegas, V. N., Magdalita, P. M., Jovellana, M. D., Espino, T. M., Exconde, S. B. (1990). Seed transmissibility of papaya ringspot virus. *Philippine Journal of Crop Science*, *15*(2), 107–111.

Benson, D. A., Karsch-Mizrachi, I., Clark, K., Lipman, D. J., Ostell, J., & Sayers, E. W. (2012). GenBank. *Nucleic Acids Research*, *40*(D1), 48–53.

Blanc, S., Ammar, E. D., Garcia-Lampasona, S., Dolja, V. V., Llave, C., Baker, J., & Pirone, T. P. (1998). Mutations in the potyvirus helper component protein: Effects on interactions with virions and aphid stylets. *Journal of General Virology*, *79*(12), 3119–3122.

Blawid, R., Silva, J. M. F., & Nagata, T. (2017). Discovering and sequencing new plant viral genomes by next-generation sequencing: description of a practical pipeline. *Annals of Applied Biology*, *170*(3), 301–314.

Bock, C. H., Poole, G. H., Parker, P. E., & Gottwald, T. R. (2010). Plant disease severity estimated visually, by digital photography and image analysis, and by hyperspectral imaging. *Critical Reviews in Plant Sciences*, *29*(2), 59–107.

Bock, Clive H., Barbedo, J. G. A., Del Ponte, E. M., Bohnenkamp, D., & Mahlein,

A.-K. (2020). From visual estimates to fully automated sensor-based measurements of plant disease severity: status and challenges for improving accuracy. *Phytopathology Research, 2*(1), 020-049.

Bock, Clive H., Chiang, K.-S., & Del Ponte, E. M. (2021). Plant disease severity estimated visually: a century of research, best practices, and opportunities for improving methods and practices to maximize accuracy. *Tropical Plant Pathology*. https://doi.org/10.1007/s40858-021-00439-z

Bock, Clive H., & Nutter, F. W. (2011). Detection and measurement of plant disease symptoms using visible-wavelength photography and image analysis. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *6*(027). https://doi.org/10.1079/PAVSNNR20116027

Bock, K. R. (1982). The identification and partial characterisation of plant viruses in the tropics. *Tropical Pest Management*, *28*(4), 399–411.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120.

Boni, M. F., Posada, D., & Feldman, M. W. (2007). An exact nonparametric method for inferring mosaic structure in sequence triplets. *Genetics*, *176*(2), 1035–1047.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics*, *10*(1), 421.

Candresse, T., Filloux, D., Muhire, B., Julian, C., Galzi, S., Fort, G., … Roumagnac, P. (2014). Appearances can be deceptive: Revealing a hidden viral infection with deep sequencing in a plant quarantine context. *PLoS ONE*, *9*(7), e102945.

Chalak, S., Hasbnis, S., & Supe, V. (2017). Papaya ring spot disease management: A

review. *Journal of Pharmacognosy and Phytochemistry JPP*, *6*(65), 1911–1914. Retrieved from http://www.phytojournal.com/archives/2017/vol6issue5/PartAB/6-5-262-628.pdf

Chan, Y.-K. (2009). Breeding Papaya (Carica papaya L.). *Breeding Plantation Tree Crops: Tropical Species*, 121–159.

Chandra, R., Mishra, M., Pati, R., Agarwal, S., & Jain, R. K. (2010). Shoot tip transformation in papaya (Carica papaya L.). *Acta Horticulturae*, *851*, 219–226.

Chandrashekar, K., Chavan, V. M., Sharma, S. K., & Bhosle, A. B. (2015). Management of PRSV-P in papaya through time of planting and border cropping. *Indian Journal of Horticulture*, *72*(3), 423–425.

Chatzivassiliou, E. K., Papapanagiotou, A. P., Mpenardis, P. D., Perdikis, D. C., & Menexes, G. (2016). Transmission of Moroccan watermelon mosaic virus (MWMV) by Aphids in Greece. *Plant Disease*, *100*(3), 601–606.

Chin, M., Ahmad, M. H., & Tennant, P. (2007). Momordica charantia is a Weed Host Reservoir for Papaya ringspot virus Type P in Jamaica . *Plant Disease*, *91*(11), 1518–1518.

Clark, M. F., & Adams, A. N. (1977). Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, *34*(3), 475–483.

Crane, J. H., Schaffer, B. A., & Mcmillan, R. T. (1995). Comparison of papaya ringspot virus effects on 23 cultivars and 18 selections of papaya (Carica papaya) in South Florida. *Proc. Fla. State Hort. Soc.*, (108), 354–357.

Daagema, A. A., Orafa, P. N., & Igbua, F. Z. (2020). Nutritional Potentials and Uses of Pawpaw (Carica papaya): A Review. *European Journal of Nutrition & Food Safety*, *12*(3), 52–66.

Diallo, H. A., Monger, W., Kouassi, N. K., Yoro, T. D., & Jones, P. (2008). Occurrence of Papaya ringspot virus Infecting Papaya in Ivory Coast. *Plant Viruses*, *2*(1), 52–57.

Dillon, S., Ramage, C., Ashmore, S., & Drew, R. A. (2006). Development of a codominant CAPS marker linked to PRSV-P resistance in highland papaya. *Theoretical and Applied Genetics*, *113*(6), 1159–1169.

Dinoor, A. (1974). Role of Wild and Cultivated Plants in the Epidemiology of Plant Diseases in Israel. *Annual Review of Phytopathology*, *12*(1), 413–436.

Drew, R.A., Magdalita, P., & O'Brien. C.M (1998). Development of Carica Interspecific hybrids. In R. . Drew (Ed.), *Proceedings of the international symposium on tropical and subtropical fruits, Part 2. Acta Horticulture, Netherlands,* (pp. 285–291).

Edward, A. E. & Fredy, H. B. (2015). *An Overview of Global Papaya Production, Trade , and Consumption*. U.S. Department of Agriculture,The Institute of Food and Agricultural Sciences (IFAS). Retrieved from http://edis.ifas.ufl.edu/fe913

Fermin, G. (2018). *Host Range, Host-Virus Interactions, and Virus Transmission*. *Viruses: Molecular Biology, Host Interactions, and Applications to Biotechnology*. Retrieved from https://doi.org/10.1016/B978-0-12-811257-1.00005-X

Fermin, G., Inglessis, V., Garboza, C., Rangel, S., Dagert, M., & Gonsalves, D. (2004). Engineered resistance against Papaya ringspot virus in Venezuelan transgenic papayas. *Plant Disease*, *88*(5), 516–522.

Fischer, H. U. & Lockhart, B. E. L. (1974). Serious losses in cucurbits caused by watermelon mosaic virus in Morocco. *Plant Disease Reporter*, *58*(2), 143–146.

Fitch, M. M. M. (2010). Papaya Ringspot Virus (PRSV) Coat Protein Gene Virus

Resistance in Papaya : Update on Progress Worldwide. *Transgenic Plant Journal*, *4*(Special issue 1).

Fletcher, J. T. (1978). The use of avirulent virus strains to protect plants against the effects of virulent strains. *Annals of Applied Biology*, *89*(1), 110–114.

Gad, L. & Carr .J. P. (2006). Natural resistance mechanisms of plants to viruses. In G. L. and J. P. Carr (Ed.), *Springer* (Vol. №3, p. 547). Springer Netherlands.

Gal-on, A. (2000). A Point Mutation in the FRNK Motif of the Potyvirus Helper Component-Protease Gene Alters Symptom Expression in Cucurbits and Elicits Protection Against the Severe Homologous Virus. *Phytopathology*, (90), 467–473.

García-Arenal, F., Fraile, A., & Malpica, J. M. (2003). Variation and evolution of plant virus populations. *International Microbiology*, *6*(4), 225–232.

Gashaw, G., Alemu, T., & Tesfaye, K. (2014). Evaluation of disease incidence and severity and yield loss of finger millet varieties and mycelial growth inhibition of Pyricularia grisea isolates using biological antagonists and fungicides in vitro condition . *Journal of Applied Biosciences*, *73*, 5883–5901.

Ghoshal, B., & Sanfaçon, H. (2015). Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. *Virology*, *479–480*, 167–179.

Gibbs, M. J., Armstrong, J. S., & Gibbs, A. J. (2000). Sister-scanning: A Monte Carlo procedure for assessing signals in rebombinant sequences. *Bioinformatics*, *16*(7), 573–582.

Gonsalves, D. (1998). Control of papaya ringspot virus in papaya : A Case Study. *Annual Review of Phytopathology*, *36*(1), 415–437.

Gorbalenya, A. E., & Koonin, E. V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Research*, *17*(21), 8413–8438.

Groen, S. C., Wamonje, F. O., Murphy, A. M., & Carr, J. P. (2017). Engineering resistance to virus transmission. *Current Opinion in Virology*, *26*, 20–27.

Hamilton, R. I. Edwardson, J. R.. Francki, R. I. B, Hsu, H.T. Hull, R., Koenig, R. & Milne, R. G. (1981). Guidelines for the Identification and Characterization of Plant Viruses. *Journal of General Virology*, *54*(2), 223–241.

HCDA. (2016). *Horticulture Validated report*. *Nairobi, Kenya*. Nairobi, Kenya. Retrieved from www.agricultureauthority.go.ke/.../Horticulture-Validated-Report-2014-Final-copy.pd...

Hema, M. V, & Prasad, D. T. (2004). Comparison of the coat protein of a South Indian strain of PRSV with other strains from different geographical locations. *Journal of Plant Pathology*, *86*(1), 31–38.

Hong, Y., & Hunt, G. (1996). RNA polymerase activity catalyzed by a potyvirus-encoded RNA-dependent RNA polymerase. *Virology*, *226*(1), 146–151.

Hubert, J., Luzi-kihupi, A., Hébrard, E., & Lyimo, H. J. F. (2016). Farmers' Knowledge and Perceptions of Rice yellow mottle virus in Selected Rice Growing Areas in Tanzania. *International Journal of Science and Research*, *5*(2), 549–559.

Huet, H., Gal-On, A., Meir, E., Lecoq, H., & Raccah, B. (1994). Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility. *Journal of General Virology*, *75*(6), 1407–1414.

Ibaba, J. D., Laing, M. D., & Gubba, A. (2016). Genome sequence analysis of two South African isolates of Moroccan watermelon mosaic virus infecting

cucurbits. *Virus Genes*, *52*(6), 896–899.

Imungi, J. K., & Wabule, M. N. (1990). Some chemical characteristics and availability of vitamin A and vitamin C from Kenyan varieties of papayas (Carica papaya L.). *Ecology of Food and Nutrition*, *24*(2), 115–120.

Islam, W. (2017). Mini Review Management of Plant Virus Diseases; Farmer's Knowledge and our Suggestions. *Hosts and Viruses*, *4*(2), 28–33.

Jain, R. K., Sharma, J., Sivakumar, A. S., Sharma, P. K., Byadgi, A. S., Verma, A. K., & Varma, A. (2004). Variability in the coat protein gene of Papaya ringspot virus isolates from multiple locations in India. *Archives of Virology*, *149*(12), 2435–2442.

Jeong, J.-J., Ju, H.-J., & Noh, J. (2014). A Review of Detection Methods for the Plant Viruses. *Research in Plant Disease*, *20*(3), 173–181.

Jeyanandarajah, P., & Brunt, A. A. (1993). The Natural Occurrence, Transmission, Properties and Possible Affinities of Cowpea Mild Mottle Virus. *Journal of Phytopathology*, *137*(2), 148–156.

Jo, Y., Choi, H., Kim, S. M., Kim, S. L., Lee, B. C., & Cho, W. K. (2017). The pepper virome: Natural co-infection of diverse viruses and their quasispecies. *BMC Genomics*, *18*(1), 1–12.

Jones, D. T., Taylor, W. R., & Thornton, J. M. (1992). The rapid generation of mutation data matricies from protein sequences. *Computer Applications in the Biosciences*, *8*(3), 275–282.

Jones, R.A.C. (2014). Plant virus ecology and epidemiology: historical perspectives, recent progress and future prospects. *Annals of Applied Biology*, *164*(3), 320–347.

Júnior, M. T. S., Nickel, O., & Gonsalves, D. (2005). Development of Virus Resistant Transgenic Papayas Expressing the Coat Protein Gene from a

Brazilian Isolate of Papaya ringspot virus. *Fitopatologia Brasileira*, (30), 357–365.

Kabir, M. A., Begum, F. Faruq, A. N., Lee, J., Tonu, N. N. (2017). Field survey on Papaya viral disease in major papaya growing districts in Bangladesh. *International Journal of Research Science and Management*, *4*(10), 57–66.

Kalleshwaraswamy, C. M., & Kumar, N. K. K. (2008). Transmission Efficiency of Papaya ringspot virus by Three Aphid Species. *Phytopathology*, *98*(5), 541–546.

Kansiime, M. K., Rwomushana, I., Mugambi, I., Makale, F., Lamontagne-Godwin, J., Chacha, D., … & Day, R. (2020). Crop losses and economic impact associated with papaya mealybug (Paracoccus marginatus) infestation in Kenya. *International Journal of Pest Management*, *0*(0), 1–14.

Kertbundit, S., & Juĝíþek, M. (2010). Application of Transgenic Technologies to Papaya: Developments and Biosafety Assessments in Thailand. *Transgenic Plant Journal*, *4*(1), 52–57.

Khan, M., & Damalas, C. A. (2015). Farmers' knowledge about common pests and pesticide safety in conventional cotton production in Pakistan. *Crop Protection*, *77*, 45–51.

Khan, Z. R., Midega, C. A. O., Nyang'au, I. M., Murage, A., Pittchar, J., Agutu, L. O., … Pickett, J. A. (2014). Farmers' knowledge and perceptions of the stunting disease of Napier grass in Western Kenya. *Plant Pathology*, *63*(6), 1426–1435.

Kidanemariam, D. B., Sukal, A. C., Abraham, A. D., Njuguna, J. N., Stomeo, F., Dale, J. L., … James, A. P. (2019). Molecular characterisation of a putative new polerovirus infecting pumpkin (Cucurbita pepo) in Kenya. *Archives of Virology*, *1*(0123456789), 0–4.

Koonin, E. V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *Journal of General Virology*, *72*(9), 2197–2206.

Kreuze, J. F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I., & Simon, R. (2009). Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: A generic method for diagnosis, discovery and sequencing of viruses. *Virology*, *388*(1), 1–7.

Krishna, K. L., Paridhavi, M., & Patel, J. A. (2008). Review on nutritional, medicinal and pharmacological properties of papaya (Carica papaya linn.). *Indian Journal of Natural Products and Resources*, *7*(4), 364–373.

Kulski, J. K. (2016). Next-Generation Sequencing — An Overview of the History, Tools, and "Omic" Applications. In J. Kulski (Ed.), *Next Generation Sequencing: Advances, Applications and Challenges* (pp. 1–60). https://doi.org/http://dx.doi.org/10.5772/61964 5

Kumar, N. K. K., Singh, H. S., & Kalleshwaraswamy, C. M. (2010). Aphid (aphididae: Homoptera) vectors of papaya ringspot virus (PRSV), bionomics, transmission efficiency and factors contributing to epidemiology. *Acta Horticulturae*, *851*, 431–442.

Kumari, S., Trivedi, M., & Mishra, M. (2015). PRSV resistance in papaya (Carica papaya L.) through genetic engineering : A review. *Journal of Applied Horticulture*, *17*(3), 243–248.

Kung, Y., You, B., Raja, J. A. J., Chen, K., & Huang, C. (2015). *Breakdown of Transgenic Resistance by More Virulent Virus Strains and a Potential Solution*. *Scientific Reports* (Vol. 5). https://doi.org/10.1038/srep09804

Kwak, H. R., Kim, J., Kim, M. K., Seo, J. K., Jung, M. N., Kim, J. S., … Choi, H. S. (2015). Molecular characterization of five potyviruses infecting Korean sweet potatoes based on analyses of complete genome sequences. *Plant Pathology Journal*, *31*(4), 388–401.

Langmead, B., & Salzberg, S. L. (2013). Bowtie2. *Nature Methods*, *9*(4), 357–359.

Lawrence, D. M., Rozanov, M. N., & Hillman, B. I. (1995). Autocatalytic processing of the 223-kDa protein of blueberry scorch carlavirus by a papain-like proteinase. *Virology*. https://doi.org/10.1006/viro.1995.1058

Lecoq, H., Végétale, S. D. P., Cedex, M., Dafalla, G., Medani, W., Desbiez, C., & Wipf-scheibel, C. (2001). Biological and Molecular Characterization of Moroccan watermelon mosaic virus and a Potyvirus Isolate from Eastern Sudan. *Plant Disease*, *85*(5), 547–552.

Lecoq, I. Justafré, C. W.-S. & Desbiez, C. (2007). Moroccan watermelon mosaic virus newly reported on zucchini squash in France. *Plant Pathology*, (57), 766.

Lefeuvre, P., Martin, D. P., Elena, S. F., Shepherd, D. N., & Roumagnac, P. (2019). Evolution and ecology of plant viruses. *Nature Reviews Microbiology*, *17*, 632–644.

Legg, J. P., & Thresh, J. M. (2000). Cassava mosaic virus disease in East Africa: A dynamic disease in a changing environment. *Virus Research*, *71*(1–2), 135–149.

Legrand, P. (2015). Biological assays for plant viruses and other graft-transmissible pathogens diagnoses: A review. *EPPO Bulletin*, *45*(2), 240–251.

López-Moya, J. J., Wang, R. Y., & Pirone, T. P. (1999). Context of the coat protein DAG motif affects potyvirus transmissibility by aphids. *Journal of General Virology*, *80*(12), 3281–3288.

Lwin, O. O. M., Yabe, M., & Khai, H. V. (2012). Farmers' Perception, knowledge and pesticide usage practices: A case study of tomato production in inlay lake. *Journal of the Faculty of Agriculture, Kyushu University*, *57*(1), 327–331.

Mansilla, P. J., Moreira, A. G., Mello, A. P. O. A., Rezende, J. A. M., Ventura, J. A., Yuki, V. A., & Levatti, F. J. (2013). Importance of cucurbits in the epidemiology of Papaya ringspot virus type P. *Plant Pathology*, *62*(3), 571–577.

Marais, A., Faure, C., Mustafayev, E., & Candresse, T. (2015). Characterization of new isolates of Apricot vein clearing-associated virus and of a new prunus-infecting virus: Evidence for recombination as a driving force in Betaflexiviridae evolution. *PLoS ONE*, *10*(6), 1–15.

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., … Bryant, S. H. (2011). CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Research*, *39*(SUPPL. 1), 225–229.

Martelli, G. P., Adams, M. J., Kreuze, J. F., & Dolja, V. V. (2007). Family *Flexiviridae* : A Case Study in Virion and Genome Plasticity. *Annual Review of Phytopathology*, *45*(1), 73–100.

Martin, D., & Rybicki, E. (2000). RDP: detection of recombination amongst aligned sequences. Bioinformatics. *Oxford Journals*, *16*(6), 562–563.

Martin, D.P., Posada, D., Crandall, K. A., & Williamson, C. (2005). A Modified Bootscan Algorithm for Automated Identification of Recombinant Sequences and Recombination Breakpoints. *AIDS Research and Human Retroviruses*, *21*(1), 98–102.

Martin, Darren P., Murrell, B., Golden, M., Khoosal, A., & Muhire, B. (2015). RDP4: Detection and analysis of recombination patterns in virus genomes. *Virus Evolution*, *1*(1), 1–5.

Martins, S. D., Aires Ventura, J., de assia AL Paula, R. C., Jos Fornazier, M., Rezende, J. A., Culik, M. P., … & Sousa-Silva, C. R. (2016). Aphid vectors of Papaya ringspot virus and their weed hosts in orchards in the major papaya producing and exporting region of Brazil.Retrieved from

https://doi.org/10.1016/j.cropro.2016.08.030

Martínez, D. R., Sousa, P. De, Duarte, G., Olmedo, J. G., & Figueira, R. (2014). Molecular and biological studies of Papaya ringspot virus isolates from Brazil and Cuba. *American Journal of Agriculture and Forestry*, *2*(5), 209–218.

McKern N. M., Strike P. M. , Barnett O. W., Ward C. W., Shukla, D. D. (1993). watermelon mosaic virus-Morocco is a distinct potyvirus. *Archives of Virology*, (131), 467–473.

Menzel, W., Abang, M. M., & Winter, S. (2011). Characterization of cucumber vein-clearing virus, a whitefly (Bemisia tabaci G.)-transmitted carlavirus. *Archives of Virology*, *156*(12), 2309–2311.

Merga, W. (2018). Measuring and Analysis of Plant Diseases. *International Journal of Research Studies in Agricultural Sciences (IJRSAS)*, *4*(12), 1–8.

Milind, P. (2011). Basketful Benefits of Papaya. *International Research Journal of Pharmacy*, *2*(27), 6–12. Retrieved from http://www.irjponline.com

Ming, R., Hou, S., Feng, Y., Yu, Q., Dionne-laporte, A., Saw, J. H., … Mitchell-olds, T. (2008). The draft genome of the transgenic tropical fruit tree papaya (Carica papaya Linnaeus). *Nature*, *452*, 991–996.

Mishra, Maneesh, R. C. and, & Sangeeta, S. (2007). Genome Mapping and Molecular Breeding in Plants,. In C. Kole (Ed.), *Fruits and Nuts* (Vol. 4, pp. 343–351). Berlin Heidelberg: Springer-Verlag.

Mofunanya, A. A. J., & Edu, E. A. (2015). Physiological and Biochemical Changes in Cucurbita moschata Duch . Ex . Poir Inoculated with a Nigerian Strain of Moroccan Watermelon Mosaic Virus (MWMV): Lagenaria breviflora Isolate. *International Journal of Plant Pathology*, *6*(2), 36–47.

Mohammed, H., Manglli, A., Zicca, S., El Hussein, A., Mohamed, M., & Tomassoli,

L. (2012). First report of Papaya ringspot virus in pumpkin in Sudan. *New Disease Reports*, *26*, 26.

Monci, F., Sánchez-Campos, S., Navas-Castillo, J., & Moriones, E. (2002). A natural recombinant between the geminiviruses Tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology*, *303*(2), 317–326.

Moradi, Z., Mehrvar, M., Nazifi, E., & Zakiaghl, M. (2016). The complete genome sequences of two naturally occurring recombinant isolates of Sugarcane mosaic virus from Iran. *Virus Genes*. *16*, 1302-5

Mowlick, S., Akanda, A. M., & Rahman, A. H. M. A. (2007). Development of Mild Strains of Papaya Ringspot Virus -Papaya Strain. *Journal of Agriculture & Rural Development*, *5*(1&2), 94–97.

Mowlick, S., Akther, M.S., Kundu, B. C. & Akanda, A.M. (2008). Masking behaviour and quantitative assessment of growth and and yield reduction of papaya due to papaya ringspot virus. *Bangladesh Research Publications Journal*, *1*(3), 206–214.

Mumford, R. A., Macarthur, R., & Boonham, N. (2016). The role and challenges of new diagnostic technology in plant biosecurity. *Food Security*, *8*(1), 103–109.

Mumo, N.N., Mamati, G.E., Ateka, E.M., Rimberia, F.K., Asudi, G.O., Boykin, L.M., Machuka, E.M., Njuguna, J.N., Pelle, R., & Stomeo, F. (2020). Metagenomic Analysis of Plant Viruses Associated With Papaya Ringspot Disease in *Carica papaya* L. in Kenya. *Frontiers in Microbiology*, *11*, 205.

Nagaraju, N., Venkatesh, H. M., Warburton, H., Muniyappa, V., Chancellor, T. C. B., & Colvin, J. (2002). Farmers' perceptions and practices for managing tomato leaf curl virus disease in southern India. *International Journal of*

Pest Management, *48*(4), 333–338.

Nagy, P. (2008). Recombination in Plant RNA Viruses. In M.J. Roossinck (Ed.), *Plant Virus Evolution* (pp. 133–156). Springer-Verlag Berlin Heideberg. https://doi.org/10.1007/978-3-540-75763-4

Naidu, R. A., Gowda, S., Satyanarayana, T., Boyko, V., Reddy, A. S., Dawson, W. O., & Reddy, D. V. (1998). Evidence that whitefly-transmitted cowpea mild mottle virus belongs to the genus Carlavirus. *Archives of Virology*, *143*(4), 769–780.

Naidu, R. A., & Hughes, J. d'A. (2003). Methods for the detection of plant virus diseases. *Plant Virology in Sub-Saharan Africa: Proceedings of a Conference Organized by IITA*, 233–260.

Ndunguru, J., & Rajabu, C. A. (2002). Papaya ring spot virus disease in Lake Victoria basin. *Tropical Science*, *42*, 11–16.

Noa-Carrazana, J. C., González-De-León, D., Ruiz-Castro, B. S., Piñero, D., & Silva-Rosales, L. (2006). Distribution of Papaya ringspot virus and Papaya mosaic virus in papaya plants (Carica papaya) in Mexico. *Plant Disease*, *90*(8), 1004–1011.

Nurk, S., Meleshko, D., Korobeynikov, A., & Pevzner, P. A. (2017). MetaSPAdes: A new versatile metagenomic assembler. *Genome Research*, *27*(5), 824–834.

OCDE. (2005). Consensus document on the biology of papaya (Carica papaya), (33), 66. Retrieved from http://www.oecd.org/science/biotrack/46815818.pdf

Ohshima, K., Yamaguchi, Y., Hirota, R., Hamamoto, T., Tomimura, K., Tan, Z., … Gibbs, A. (2002). Molecular evolution of Turnip mosaic virus: Evidence of host adaptation, genetic recombination and geographical spread. *Journal of General Virology*, *83*(6), 1511–1521.

Okon, W. I., Ogri, A. I., Igile, G. O., & Atangwho, I. J. (2017). Nutritional quality of raw and processed unripe *Carica papaya* fruit pulp and its contribution to dietary diversity and food security in some peasant communities in Nigeria. *International Journal of Biological and Chemical Sciences*, *11*(3), 1000.

Omar, A. F., El-Kewey, S. A., Sidaros, S. A., & Shimaa, A. K. (2011). Egyptian isolates of Papaya ringspot virus form a molecularly distinct clade. *Journal of Plant Pathology, 93*(3), 569–576.

Ombwara, F.K., Asudi, G.O. Rimberia, F.K., Ateka, E.M, Wamocho, L. S. (2014). The Distribution and Prevalence of Papaya Ring Spot Virus (PRSV) in Kenyan Papaya. *Acta Horticulturae*, (1022), 119–124.

Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics*, *12*(1), 385.

Orwa et al. (2009). Carica papaya L . Retrieved from http://www.worldagroforestry.org/treedb/AFTPDFS/Carica_papaya.PDF

Owolabi, A. T., & Ekpiken, E. E. (2014). Transmission efficiency of two strains of Moroccan watermelon mosaic virus by two clones of Aphis spiraecola (Patch). *International Journal of Virology*, 253-262.

Owolabi, A. T., Rabenstein, F., Ehrig, F., Maiss Edgar, M., & Vetten, H. J. (2012). Strains of Moroccan watermelon mosaic virus isolated from lagenaria breviflorus and coccinia barteri in calabar, southeastern Nigeria. *International Journal of Virology*, *8*(3), 258–270.

Oyunga, M. A., Grant, F., Omondi, D., Ouedraogo, H., Levin, C., & Low, J. (2016). Prevalence and Predictors of Vitamin a Deficiency Among Infants in Western Kenya Using a Cross-Sectional Analysis. *African Journal of Food, Agriculture, Nutrition and Development*, *16*(1), 10765–10785.

Padhi, A., & Ramu, K. (2011). Genomic evidence of intraspecific recombination in

sugarcane mosaic virus. *Virus Genes*, (42), 282–285.

Padidam, M., Sawyer, S., & Fauquet, C. M. (1999). Possible emergence of new geminiviruses by frequent recombination. *Virology*, *265*(2), 218–225.

Posada, D. & Crandall K. A. (2001). Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. *Proc Natl Acad Sci U S A*, *98*(24), 13757–13762.

Pearson, M. N., Clover, G., Society, R. H., & Guy, P. L. (2006). A review of the plant virus , viroid and mollicute records for New Zealand. *Australasian Plant Pathology*, *35*, 217–252.

Piper, J. K., Handley, M. K., & Kulakow, P. A. (1996). Incidence and severity of viral disease symptoms on eastern gamagrass within monoculture and polycultures. *Agriculture Ecosystems & Environment*, *59*, 139–147.

Purcifull, D. E. & Hiebert, E. (1979). Serological Distinction of Watermelon Mosaic Virus Isolates. *Phytopathology*, *69*, 112–116.

QGIS Development Team. (2019). *QGIS Geographic Information System*;Open Source Geospatial Foundation Project, 2019.

Read, D. A., Muoma, J., & Thompson, G. D. (2020). Metaviromic analysis reveals coinfection of papaya in western Kenya with a unique strain of Moroccan watermelon mosaic virus and a novel member of the family Alphaflexiviridae. *Archives of Virology*, *165*(5), 1231–1234.

Revers, F., & García, J. A. (2015). *Molecular biology of potyviruses*. *Advances in Virus Research* (1st ed., Vol. 92). Elsevier Inc. https://doi.org/10.1016/bs.aivir.2014.11.006

Riesenfeld, C. S., Schloss, P. D., & Handelsman, J. (2004). Metagenomics: Genomic Analysis of Microbial Communities. *Annual Review of Genetics*, *38*(1), 525–552.

Rimberia, F.K., & Wamocho, L. S. (2014). Papaya industry in Kenya: Production, consumption and outlook. *Acta Horticulturae*, (1022), 181–188.

Rimberia, F. K., Ombwara, F. K., Mumo, N., & Ateka, E. M. (2018). *Genetic improvement of papaya (Carica papaya L.). Advances in Plant Breeding Strategies: Fruits*, *3*, 7-21.

Roff, M. N. M., & Lumpur, K. (2007). Disease Rating of Papaya Cultivars to Papaya Ringspot Virus in Malaysia, (September 2004), 277–282.

Romay, G., Lecoq, H., & Desbiez, C. (2014). Zucchini tigre mosaic virus is a distinct potyvirus in the papaya ringspot virus cluster: Molecular and biological insights. *Archives of Virology*, *159*(2), 277–289.

Roossinck, M. J. (2015). Plants, viruses and the environment: Ecology and mutualism. *Virology*, *479–480*, 271–277.

Roossinck, M. J. (2017). Deep sequencing for discovery and evolutionary analysis of plant viruses. *Virus Research*, *239*, 82–86.

Rosario, K., & Breitbart, M. (2011). Exploring the viral world through metagenomics. *Current Opinion in Virology*, *1*(4), 289–297.

Roy, A., Shao, J., Hartung, J.S., Schneider, W., & Brlansky, R.H. (2013). A Case Study on Discovery of Novel Citrus Leprosis Virus Cytoplasmic Type 2 Utilizing Small RNA Libraries by Next Generation Sequencing and Bioinformatic Analyses. *Journal of Data Mining in Genomics & Proteomics*, *4*(2), 0602-0622.

Rozas, J., Ferrer-mata, A., S, J. C., Guirao-rico, S., Librado, P., Ramos-onsins, E., … Gene, D. De. (2017). DnaSP 6 : DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol. Biol. Evol.*, *34*(12), 3299–3302.

Rubio, L., Galipienso, L., & Ferriol, I. (2020). Detection of Plant Viruses and Disease Management: Relevance of Genetic Diversity and Evolution.

Frontiers in Plant Science, *11*(July), 1–23.

Sakuanrungsirikul, S., Sarindu, N., Prasartsee, V., Chaikiatiyos, S., Siriyan, R., Sriwatanakul, M., … Gonsalves, D. (2014). Update on the development of virus-resistant papaya : Virus-resistant transgenic papaya for people in rural communities of Thailand, *26*(4), 307–311.

Salvador, B., Saénz, P., Yangüez, E., Quiot, J. B., Quiot, L., Delgadillo, M. O., … Simón-Mateo, C. (2008). Host-specific effect of P1 exchange between two potyviruses. *Molecular Plant Pathology*, *9*(2), 147–155.

Schreinemachers, P., Balasubramaniam, S., Boopathi, N. M., Ha, C. V., Kenyon, L., Praneetvatakul, S., … Wu, M. H. (2015). Farmers' perceptions and management of plant viruses in vegetables and legumes in tropical and subtropical Asia. *Crop Protection*, *75*, 115–123.

Sebestye, E., & Bala, E. (2015). Recombination analysis of Maize dwarf mosaic virus (MDMV) in the Sugarcane mosaic virus (SCMV) subgroup of potyviruses. *Virus Genes*, (50), 79–86.

Sharma, P., Sahu, A. K., Verma, R. K., Mishra, R., Choudhary, D. K., & Gaur, R. K. (2014). Current status of *Potyvirus* in India. *Archives Of Phytopathology And Plant Protection*, *47*(8), 906–918.

Sharma, S.K., Zote, K. K., Kadam, U. M., Tomar, S. P. S., Dhale, M. G., & Sonawane, A. U. (2010). Integrated management of papaya ringspot virus. *Acta Horticulturae*, (851), 473–480.

Sharma, S. K. & Tripathi, S. (2014). Papaya ringspot virus-P. Overcoming limitations of resistance breeding in *Carica papaya* L. In *Plant Virus-Host Interaction: Molecular Approaches and Viral Evolution* (pp. 177–194). Elsevier. https://doi.org/10.1016/B978-0-12-411584-2.00009-3

Shukla, D. D., & Ward, C. W. (1989). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Archives of*

Virology Virol, *106*, 171–200.

Silva-Rosales,L., N. Becerra-Leor, S. Ruiz-Castro, D. Téliz-Ortiz, and J. C. N.-C. (2000). Coat protein sequence comparisons of three Mexican isolates of papaya ringspot virus with other geographical isolates reveal a close relationship to American and Australian isolates Brief Report. *Archives of Virology*, 835–843.

Singh, S., Awasthi, L. P., Kumar, P., & Jagre, A. (2017). Diagnostic Characteristics of Papaya Ring Spot Virus Isolates Infecting Papaya (Carica papaya L.) in India, *1*(4), 555 - 567.

Singh, S. K., Jha, P. K., & Ray, P. K. (2010). Integrated Management of Papaya Ring Spot Virus (PRSV) in Agro Ecological Conditions of Bihar. *Acta Horticulturae, ISHI*, *851*, 487–494.

Singh, V., Rao, G. P., & Shukla, K. (2005). Response of commercially important papaya cultivars to papaya ringspot virus in eastern U . P . conditions. *Indian Phytopathology*, *58*(2), 212–216.

Singh, V., & Shukla, K. (2011). Influence of inoculation time on severity of virus disease caused by Papaya ringspot virus. *Ann. Pl. Protec. Sci*, *19*(1), 142–146.

Smith, J. M. (1992). Analyzing the mosaic structure of genes. *Journal of Molecular Evolution*, *34*(2), 126–129.

Srinivasulu, M., & Sai Gopal, D. V. R. (2011). Coat protein sequence comparison of south Indian isolates of Papaya ringspot virus with other Indian subcontinent isolates. *Phytopathologia Mediterranea*, *50*(3), 359–369.

Sseruwagi, P., Sserubombwe, W. S., Legg, J. P., Ndunguru, J., & Thresh, J. M. (2004). Methods of surveying the incidence and severity of cassava mosaic disease and whitefly vector populations on cassava in Africa: A review. *Virus Research*, *100*(1), 129–142.

Stevens, W. A. (1983). *Virology of flowering plants*. Boston, MA: Springer.

Strange, R. N., & Scott, P. R. (2005). Plant disease : A Threat to Global Food Security. *Annual Review of Phytopathology*, (43), 83–116.

Syller, J. (2012). Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology*, *13*(2), 204–216.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729.

Teixeira, J. A., Rashid, Z., Tan, D., Dharini, N., Gera, A., Teixeira, M., … & Tennant, P. F. (2007). Papaya (Carica papaya L.) Biology and Biotechnology. *Tree and Forestry Science and Biotechnology*. *1*(1), 47-73.

Tennant, P. F., Fermin, G. A., & Roye, M. E. (2007). Viruses Infecting Papaya (Carica papaya L.): Etiology, Pathogenesis, and Molecular Biology. *Plant Viruses*, *1*(2), 178–188.

Tennant, P., Souza, M. T., Gonsalves, D., Fitch, M. M., Manshardt, R. M., Way, M., & Slightom, J. L. (2005). Line 63-1 : A New Virus-resistant Transgenic Papaya. *HortScience*, *40*(5), 1196–1199.

Tennant, P.F., Gonsalves, C., Ling, K.S., Fitch, M., Manshardt, R., Slightom, J.L. & Gonsalves. D. (1994). Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. *Phytopathology*, (84), 1359–1366.

Thresh, J. M. (1982). Cropping Practices and Virus Spread. *Annual Review of Phytopathology*, *20*(1), 193–216.

Tripathi, S., Suzuki, J., & Gonsalves, D. (2007). Development of Genetically Engineered Resistant Papaya for papaya ringspot virus in a Timely

Manner A Comprehensive and Successful Approach. *Methods in Molecular Biology, Plant-Pathogen Interactions:Methods and Protocols*, *354*, 197–240.

Tripathi, S., Suzuki, J. Y., Ferreira, S. A., & Gonsalves, D. (2008). Papaya ringspot virus-P: Characteristics, pathogenicity, sequence variability and control. *Molecular Plant Pathology*, *9*(3), 269–280.

Uzest, M., Monsion, B., & Jacquot, E. (2010). Aphids as transport devices for plant viruses. *Comptes Rendus Biologies*, *333*, 524–538.

Valli, A., López-Moya, J. J., & García, J. A. (2007). Recombination and gene duplication in the evolutionary diversification of P1 proteins in the family Potyviridae. *Journal of General Virology*, *88*(3), 1016–1028.

Ventura, J. A., Costa, H., & Tatagiba, J. da S. (2004). Papaya Diseases and Integrated Control. In S. A. M. . Naqvi (Ed.), *Diseases of Fruits and Vegetables: Volume II* (pp. 201–268). Dordrecht: Kluwer Academic Publishers.

Villamor, D. E. V., Ho, T., Al Rwahnih, M., Martin, R. R., & Tzanetakis, I. (2019). High Throughput Sequencing in Plant Virus Detection and Discovery. *Phytopathology*, (June), 1–28.

Wamaitha, M. J., Nigam, D., Maina, S., Stomeo, F., Wangai, A., Njuguna, J. N., … & Garcia-Ruiz, H. (2018). Metagenomic analysis of viruses associated with maize lethal necrosis in Kenya. *Virology Journal*, *15*(1), 1–19.

Wamonje, F. O., Michuki, G. N., Braidwood, L. A., Njuguna, J. N., Musembi Mutuku, J., Djikeng, A., … Carr, J. P. (2017). Viral metagenomics of aphids present in bean and maize plots on mixed-use farms in Kenya reveals the presence of three dicistroviruses including a novel Big Sioux River virus-like dicistrovirus. *Virology Journal*, *14*(1), 1–13.

Wang, H.-L., Yeh, S.D.., Chui, R.-J and Gonsalves, D. (1987). Effectiveness of

Cross-Protection by Mild Mutants of Papaya Ringspot Virus for Control of Ringspot Disease of papaya in Taiwan. *Plant Disease, 71,* 491–497.

Webster, C. G., Wylie, S. J., & Jones, M. G. K. (2004). Diagnosis of plant viral pathogens. *Current Science*, *86*(12), 1604–1607.

Wei, X. D., Zou, H. L Chu, L. M., Liao, B., Ye, C. M. & Lan C. Y. (2006). Field released transgenic papaya affects microbial communities and enzyme activities in soil. *Plant Soils*, (285), 347–358.

Woolhouse, M. E. J., Haydon, D. T., & Antia, R. (2005). Emerging pathogens: The epidemiology and evolution of species jumps. *Trends in Ecology and Evolution*, *20*(5), 238–244.

Wu, Q., Ding, S., Zhang, Y., & Zhu, S. (2015). Identification of Viruses and Viroids by Next-Generation Sequencing and Homology- Dependent and Homology- Independent Algorithms. *Annual review of phytopathology*, *53*, 425-444.

Wylie, S. J., Adams, M., Chalam, C., Kreuze, J., López-Moya, J. J., Ohshima, K., … Zerbini, F. M. (2017). ICTV virus taxonomy profile: Potyviridae. *Journal of General Virology*, *98*(3), 352–354.

Xie, X., Chen, W., Fu, Q., Zhang, P., An, T., Cui, A., & An, D. (2016). Molecular variability and distribution of Sugarcane mosaic virus in Shanxi, China. *PLoS ONE*, *11*(3), 1–12.

Yakoubi, S., Desbiez, C., Fakhfakh, H., Wipf-Scheibel, C., Marrakchi, M., & Lecoq, H. (2008). Biological characterization and complete nucleotide sequence of a Tunisian isolate of Moroccan watermelon mosaic virus. *Archives of Virology*, *153*(1), 117–125.

Yamaya, J., Yoshioka, M., Meshi, T., Okada, Y. & Ohno, T. (1988). Cross protection in transgenic tobacco plants expressing a mild strain of tobacco mosaic virus. *Mol Gen Gene, 215,* 173–175.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*, 134.

Yeh, S.-D., & Gonsalves, D. (1984). Evaluation of induced mutants of Papaya ringspot virus for control by cross protection. *Phytopathology*. *4*(9), 1086-1091.

Yeh, S-D. & Gonsalves, D. (1994). Practices and Perspective of Control of Papaya Ringspot Virus by Cross Protection. In K. F. Harris (Ed.), *Advances in Disease Vector Research* (Vol. 10, pp. 237–251). Springer-Verlag New York, Inc., 175 Fifth Avenue, New York, NY 10010, USA.

Yogiraj, V., Goyal, P. K., & Chauhan, C. S. (2014). Carica papaya Linn : An Overview. *International Journal of Herbal Medicine*, *2*(5), 1–8.

Yu, Q., Hou, S., Feltus, F. A., Jones, M. R., Murray, J. E., Veatch, O., … Ming, R. (2008). Low X/Y divergence in four pairs of papaya sex-linked genes. *Plant Journal*, *53*(1), 124–132.

Zambrana-Echevarría, C., de Jesús-Kim, L., Márquez-Karry, R., Siritungayag, D., & Jenkins, D. (2016). Diversity of Papaya ringspot virus isolates in puerto rico. *HortScience*, *51*(4), 362–369.

Zhao, H., Jia, R. Z., Zhang, Y.-L., Zhu, Y. J., Zeng, H.-C., Kong, H., …& Peng, M. (2016). Geographical and Genetic Divergence Among Papaya ringspot virus Populations Within Hainan Province, China . *Phytopathology*, *106*(8), 937–944.

APPENDICES

Appendix I: A questionnaire used in the evaluation of farmers' knowledge, perception and management practices of papaya ringspot in Kenya.

Farmer's personal information

1. Name of the farmer _____Contacts _____

2. Age _____ Gender _____

3. Education level _____

4. Area of residence Sub-location_____ Location_____

Subcounty _____ county _____

GPS coordinates _____

PAPAYA PRODUCTION

5. Farm production: Subsistence ( ) Subsistence + Market ( ) Market ( )

6. What proportion of papaya do you sell 0-25% ( ) 25-50% ( ) 50-75% ( ) >75% ( )

7. Proportion of the farm under papaya cultivation 0-2% ( ) 2-4% ( ) 4-8% ( ) >8% ( )

8. Do you know papaya ringspot? Yes ( ) No ( ) Don't respond ( )

8.1 If yes, do you have it on your farm? Yes ( ) No ( )

8.2 If yes, is it spreading/increasing on your farm? Yes ( ) No ( )

8.3 If yes, how fast is it spreading? Slow ( ) Fast ( )

8.4 Does the disease occur throughout the year? Yes ( ) No ( )

8.5 When is the disease more prevalent? During dry season ( ) during cold season ( ) during long rains ( ) during short rains ( )

9. When are the newly grown plants affected after planting? 1 Month ( ) 2 months ( ) 3 months ( ) >3 months

10. Nature of papaya production in the farm Intercrop ( ) Sole crop ( )

12. Papaya cultivars grown

(a)

(b)

(c)

(d)

13. Source of planting materials

(a)

(b)

(c)

(d)

11. Do you have the following crops on your farm or surrounding farms?

i. Watermelon Yes ( ) No ( )

ii. Pumpkins Yes ( ) No ( )

iii. Cucumber Yes ( ) No ( )

iv. Zucchini (Courgettes) Yes ( ) No ( )

v. Others

12. What control measures do you use to manage the disease? Rouging ( ) Burning () Chemicals ( ) Replanting ( ) others ( ) Do not control ( ).

Appendix II: Disease severity and incidence data entry form

LOCATION				DATE			
County				FIELD No.			
Subcounty				*No. of cultivars*			
Longitude				*Dominant cultivar*			
Latitude				Source of seed			
Altitude (m)				No. of Plants			
Plant No.	Papaya ringspot symptom severity (1-5 scale)	symptoms on leaves	symptoms on fruits	Symptoms on stem	Symptoms on petioles	Plant vigour	Sample No.
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
MEAN							