DETECTION AND MANAGEMENT OF VIRUSES ASSOCIATED WITH PASSION FRUIT WOODINESS IN RWANDA

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DECLARATION

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

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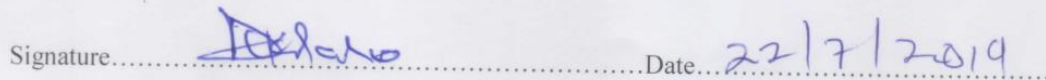

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DEDICATION

I dedicate this work to my beloved family, Mum (Bayenda Diana), Sister Ngabire Jovia, Brothers Gasana James and Mutabazi Geoffrey and Uncle Munyaburanga Emmanuel. Your moral support, patience, encouragement and prayers have made me come this far.

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

CABMV	_	Cowpea Aphid Borne Mosaic Virus
CMV	–	Cucumber Mosaic Virus
ELISA	–	Enzyme-linked immunosorbent assay
GEB	–	General Extraction Buffer
HCA	–	Horticultural Council for Africa
KALRO	–	Kenya Agricultural and Livestock Research Organization
IPM	–	Integrated Pest Management
LAMP	–	Loop-Mediated Isothermal Amplification
LSD	–	Least Significant Difference
MINICOM	–	Rwanda Ministry of Trade and Industry
MINAGRI	–	Rwanda Ministry of Agriculture and Animal Resources
NAEB	–	Rwanda, National Agricultural Export Development Board
NAS	–	Rwanda National Agriculture
NISR	–	Rwanda National Institute of Statistics
RT- PCR	–	Reverse Transcription Polymerase Chain Reaction
PWV	–	Passion fruit woodiness disease
RAB	–	Rwanda Agriculture and Animal Resources Development Board
RCBD	–	Randomized Complete Block Design
RDB	–	Rwanda Development Board
TBE	–	Tris Borate EDTA

USAID	–	United States Agency for International Development
VDI	–	VDI – Virus Disease Incidence
g	–	Gram (s)
Kg	–	Kilograms
M	–	Molar
Kb	–	kilo base (s)
µl	–	microliter (s)
Ml	–	Milliliter (s)
µM	–	micro molar (s)
Min	–	Minute (s)
RNA	–	Ribonucleic acid
Rpm	–	Revolutions per minute
Sec	–	Second (s)
V	–	Volts
KH_2P0_4	–	Potassium di- hydrogen phosphate
$KHP0_4$	–	Potassium hydrogen phosphate
dNTP	–	Deoxy ribonuclotide triphosphate

GENERAL ABSTRACT

Passion fruit (*Passiflora edulis* [SIMs] is an important food and cash crop in Rwanda. However, its production is low due to pests and diseases, poor disease management and lack of clean planting materials. Passion fruit woodiness disease (PWD) is among the diseases that reduce the production of passion fruit in the world including Rwanda. It is caused by one or mixed infections by three potyviruses, Passion fruit woodiness virus (PWV); Cowpea aphid-borne mosaic virus (CABMV) and East Asian Passiflora virus (EAPV). This study was conducted to: (i) determine relative sensitivity and effectiveness of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings and (ii) determine the potential of combining sticky traps and orchard fertilization in managing viruses associated with passion fruit woodiness disease in Rwanda.

Sensitivity and effectiveness of ELISA and RT-PCR techniques for detection of CABMV was compared by testing passion fruit plants grown from seeds saved by farmers, from private nurseries and government sources and serial dilutions over a period two seasons. After germination and growing for two months (four leaf stage), 25 leaf samples from each seed source were collected and tested at three different time periods, 30, 60 and 90 days. Confirmed disease free passion fruit seedlings were inoculated with CABMV and allowed to develop symptoms for two months. Using one confirmed CABMV infected leaf extract seven serial dilutions up to 10,000,000 times were done. The results showed that leaf samples tested at 30 and 60 days using both ELISA and RT-PCR tested negative for CABMV for all three seed sources. However, at 90 days, 32% of samples from farmer's saved seed tested positive for CABMV using RT-PCR method but was negative with the ELISA test. There was a significant difference ($p \leq 0.005$) between the two diagnostic methods in detecting CABMV presence. ELISA detected CABMV

from 32% and 58% of the leaf samples in season 1 and 2, respectively while RT-PCR detected CABMV in 60% and 62% of the leaf samples in season 1 and 2, respectively. Sixty one and 45% of samples tested positive for CABMV using RT-PCR and ELISA respectively. In the dilution experiment, ELISA detected the presence of CABMV up to the forth serial dilutions (10^4) while RT-PCR diagnostic method detected CABMV up to seventh serial dilutions (10^7).

For the second objective, six vector management options namely:1) yellow sticky trap deployment, 2) Inorganic fertilizer for rapid plant growth, 3) yellow sticky traps + inorganic fertilizer, 4) pesticide application, 5) yellow sticky traps + inorganic fertilizer and pesticide application, 6) untreated control were investigated during two cropping cycles of 2017 and 2018. The treatments were replicated three times. Aphid vector populations were recorded once a week for duration of 29 weeks**.** The incidence of passion fruit woodiness virus disease (%) was assessed during each season. Disease severity was also recorded based on severity scores of 1-5. At harvest, passion fruits were harvested and evaluated on six plants per treatment. The results indicated that yellow sticky traps, yellow sticky traps and inorganic fertilizer, and a combination of yellow sticky traps, inorganic fertilizer and pesticide application recorded the highest number of aphid populations trapped compared to inorganic fertilizer, pesticide applications and the untreated control during both cropping seasons. There were no significant differences among aphid vector management options during the long rains cropping cycle. However, there was a significant ($p<0.05$) difference during the short rain period. There were negative correlations between the numbers of aphids, disease incidence and passion fruit woodiness virus severity. The application of pesticides, yellow sticky trap and a combination of yellow sticky + inorganic fertilizer trap resulted in numerically higher marketable yield, as compared to the other treatments. These results indicate that both ELISA and RT-PR are able to index for CABMV

presence on passion fruit seedlings, however, RT-PCR is more sensitive. Therefore RT- PCR could be used in certification program for screening passion fruit viral diseases in nurseries of planting materials while ELISA could be used for disease management. Yellow sticky traps were the most effective strategy for monitoring and management of aphid vectors in passion fruit orchard in the tropical highlands of Rwanda.

CHAPTER ONE

INTRODUCTION

1.1 Horticulture industry in Rwanda

In Rwanda, agriculture sector constitutes the backbone of the economy with a contribution of 33 % to the National GDP (National Bank of Rwanda, 2015). The Government of Rwanda considers agriculture as the engine of economic growth. ((MINICOM, 2013). Agriculture has also been identified as an avenue to reduce poverty levels estimated at 44.9% with a projected reduction up to 30.2% .(NISR, 2012). The sector continues to play a leading role in the socio-economic development of the country, employing over 68% of the population (NISR, 2016). Rwanda considers horticulture as a promising sub-sector for intensive job creation, investment attracting industry, and supporting rural development by reducing poverty, increasing smallholder famers' income and increasing foreign exchange earnings (MINAGRI, 2011).

Survey indicates that the total horticulture gross sales are estimated at US$ 5.33 million per year and that the horticulture sector is steadily becoming an important sub-sector. In the year 2013-2014, it contributed about 5% of agriculture export earnings, 6% of total arable land and 8.3% of total agriculture production Rwanda, (NAEB, 2014). Among vegetables, tomatoes, onions and cabbages account for 28.4%, 14.2% and 12.8% by volume of total horticulture production, respectively. For fruits, pineapple contributes 12.8% of total horticulture production, whereas passion fruit production ranks second at 3.5% followed by tamarillo, which contributes 2.6% by volume of the fresh fruits (MINAGRI and NAEB, 2014). Rwanda currently produces two types of passion fruits; dark purple grown in high altitudes of the country and bright yellow or golden passion fruit grown in the lower parts of the country (NAEB, 2014). The purple variety is grown

at altitude of 900m to 2500m with temperature ranges between 14^0C to 25^0C. The areas have rich loam soil and receive rainfall between 900-1900mm ((RDB, 2010). The yellow variety has generally larger fruit (6cm) than the purple (5cm) but the purple is less acidic, richer in aroma and flavor, and has a higher proportion of juice (35-38%). The purple passion fruit weighs about 35-45g while that of the yellow variety is about 75g (Rwahungu, 2002). Passion fruit juice is an important source of vitamin A, ascorbic acid, riboflavin, niacin, various minerals and flavors that are strongly pleasant (Kilalo, 2012). Seeds of passion fruit are rich in lipids (24.5 g/100 g) and insoluble dietary fibre (64.1 g/100 g).

Rwanda has good climate, soil and altitude for growing passion (NAEB, 2014). The average minimum temperature in Rwanda is between 9 to 16^0C and maximum temperature between 20^0C to 29^0C. The rainfall ranges between 990 and 1590mm per annum (RDB, 2010). The country produces passion fruit both for local and export market (Austin, 2009). Passion fruit is one of the few fruits that are locally processed into juices and other products. The production of passion fruit crops in Rwanda has declined due to diseases caused by bacteria, fungi and viruses (NAEB, 2014). These diseases were first reported in Rwanda by United States Agency for International Development (USAID, 2002) in the Northern Province (Rulindo and Gicumbi districts) and Kigali- Ngali. Today, the diseases are widely distributed in all regions of the country where passion fruit is grown (RAB, 2014).

1.2 Statement of the Problem

Passion fruit (*Passiflora edulis*) is an important crop in of Rwanda and is a significant crop for the small-scale farmers due to the high market value and short maturity duration. It is also an

important source of vitamins and nutrients among the local population. The potential for passion fruit production in the country is high ranging from 15-20 tons /ha due to the favorable climate, soil, ambient temperatures and high altitude. (NAEB 2014)). However, yields of passion fruit in Rwanda are currently 2 tons / ha, which is extremely low (RAB, 2014) compared to the production statistics of other countries such as Kenya, South Africa and Australia which have recorded average production of 8-9, 19 and 24 tons / ha, respectively (Macharia and Mwangi, 2013). The low production has been attributed to high disease and pests, poor disease management tactics and lack of clean and improved planting materials.

Among diseases, plant viruses are considered to be a major cause of low productivity of passion fruit and have tremendous consequence on fruit yield and quality; and also impacts the age of orchards (RAB, 2014). Passion fruit woodiness disease, caused by one or mixed infections from three potyviruses, Passion fruit woodiness virus (PWV); Cowpea aphid-borne mosaic virus (CABMV) and East Asian Passiflora virus (EAPV) is the most important viral disease that affect passion fruit worldwide (Garcêz *et al*., 2015; Novaes and Rezende 2003; Gioria *et al.,* 2002). Both passion fruit woodiness virus (PWV) and cowpea aphid-borne mosaic virus (CABMV) are transmitted in a non-circulative manner by several species of aphids (Bragard *et al.,* 2013; Brault *et al.,* 2010; Ng and Falk 2006). Published data on the efficacy of different management strategies for the control of virus diseases in passion fruit in Rwanda is limited, which makes it hard for effective management of the diseases. The management of passion fruit virus diseases consists of development of tolerant or resistant cultivars, pre-inoculations with mild strains of the virus, and adoption of cultural practices (Alfenas et al. 2005; Novaes and Rzende 2003). In addition to the above, the application of pesticides for control of the aphid vectors has been

widely used (Garcez et al. 2015). Most of the farmers in Rwanda are over-dependent on the use of pesticides for vector control with very limited use of other control strategies. .

1.3 Justification of the study

In Rwanda, passion fruit is important for small scale farmers but its production is reduced by viral, fungal, and bacterial diseases according to (USAID, 2002). The passion fruit diseases have also been reported in the neighboring countries in the region, (Macharia and Mwangi 2013; Kilalo 2012) in Kenya and (Ochwo-ssemakula *at al*., 2012b) in Uganda. Viral diseases are the major limiting factor to the production of passion fruit not just in Kenya but the worldwide (Garcêz *et al*., 2015; Damiri, 2013; Kilalo, 2012). The yield of passion fruit in Rwanda has been reduced from 15 tons /ha (Horticultural Council for Africa (HCA), (2013) to 2 tons per ha as reported by (RAB, 2014) due to lack of quality planting materials and poor disease management. Information on passion fruit diseases in Rwanda is scanty, which makes it hard for effective management of the diseases. Evaluating methods of detection for screening planting materials and aphid management options are important in managing disease. Controlling the vectors transmitting diseases of passion fruit and disseminating disease free planting materials (Lu *et al*., 2012) are important in managing viral diseases. There is no report on comprehensive study that has been carried out in Rwanda comparing the two commonly used diagnostic methods (ELISA and RT-PCR) for screening woodiness disease in passion fruit seedlings. Similarly, the efficacy of cultural practices as a component of vector management options has not been evaluated in the country. Use of clean planting material is important for virus disease control.This requires effective, sensitive and cost effective diagnostic methods.. Equally, evaluating simple to adopt vector management options to minimize the risk of pesticide spray and loss of yield due to

disease are also important. In this study, the efficiency of two diagnostic methods (ELISA and RT-PCR in screening viral infection in seedlings of passion fruit, and the potential of combining yellow sticky traps and orchard fertilization in managing viruses associated with passion fruit woodiness were evaluated.

1.4 Objectives of the study

1.4.1 Overall objective

To improve the production of passion fruit and incomes of small-scale farmers through detection and management of woodiness disease and vectors of the disease

1.4.2 Specific objectives

1. To determine relative sensitivity and effectiveness of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings in Rwanda.
2. To determine the potential of combining sticky traps and orchard fertilization in managing viruses associated with passion fruit woodiness disease in Rwanda.

1.4.3 Hypothesis

1. There is no significance difference in sensitivity between ELISA and RT-PCR for virus diagnosis on passion fruit.
2. Vector Management options have no effect in managing passion fruit woodiness disease in Rwanda.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy, Biology and distribution of Passion fruit

Passion fruit belongs to the family of *Passifloraceae* and genus *Passiflora.* Passion fruit originated from tropical America (Cerqueira *et al.,* 2008). The Passifloraceae family contains 12 genera and about 500 species, of which 400 species belong to the genus Passiflora (Jack, 2013). The most important commercial cultivars from this species are purple and yellow passion fruit which is a hybrid between *P. edulis* Sims and *P. ligularis*. *Passiflora edulis* originated from southern Brazil and was introduced into England in 1810, later spreading to Australia, South Africa and Hawaii in 1880. It was introduced Tanzania in 1896 (Brand *et al*., 1992), and in Kenya, in 1920 in Trans Nzoia and Sotik districts (Kilalo, 2012). In Rwanda, passion fruit has been grown for over 100 years (Rwahungu, 2002) after its introduction by catholic missionaries after 1900. The first nurseries were established in the missionary stations and isolated plants were grown on fence and shade trees in missionary stations (RAB, 2014). However, Passion fruit started to be grown in Rwanda as a commercial crop largely after 1994.

The purple and yellow passion fruit leaves have 10-15cm (4-7 In) length along the margins. The granadilla has leaves of 10-20cm (4-8) along the stem in cross section. The passion fruit flowers are small, equivalent to 4.5cm in diameter, while yellow flowers are about 6cm in diameter. The passion fruit plant is pollinated by honey bees and others like bats, humming birds and bumble bee (Kilalo, 2012).

2.2 Importance and production of Passion fruits in Rwanda

In Rwanda, passion fruit ranks fourth in production and acreage after banana, avocado and

pineapple (RHODA, 2008). About 46% of the crop is grown in the Western, 43% in the Northern, 10% in the Southern and 1% in the Eastern part of the Rwanda (RHODA, 2008). Passion fruit is used for industrial purposes with products like juices, oil, as well as cattle and poultry feeds (Jack, 2013). The crop is an important source of income to the small scale farmers because it provides employment and nutrition for the rural population (Kilalo *et al*., 2013). The juice of passion fruit is also important for vitamins and minerals (Knight and Sauls, 2005). Passion fruit juice is important because it has a longer shelf life than pineapple juice (Austin, 2009). The most important variety of passion fruit grown in Rwanda is the dark purple (*P. edulis* Sims) which is less acidic than golden yellow passion fruit (MINAGRI and NAEB, 2014).

According to (NISR, 2018) report there has been steady increase in the quantity of passion fruit exported from Rwanda from 2013 to 2018 with a large increase in volume in 2017-2018.(Fig. 2.1).

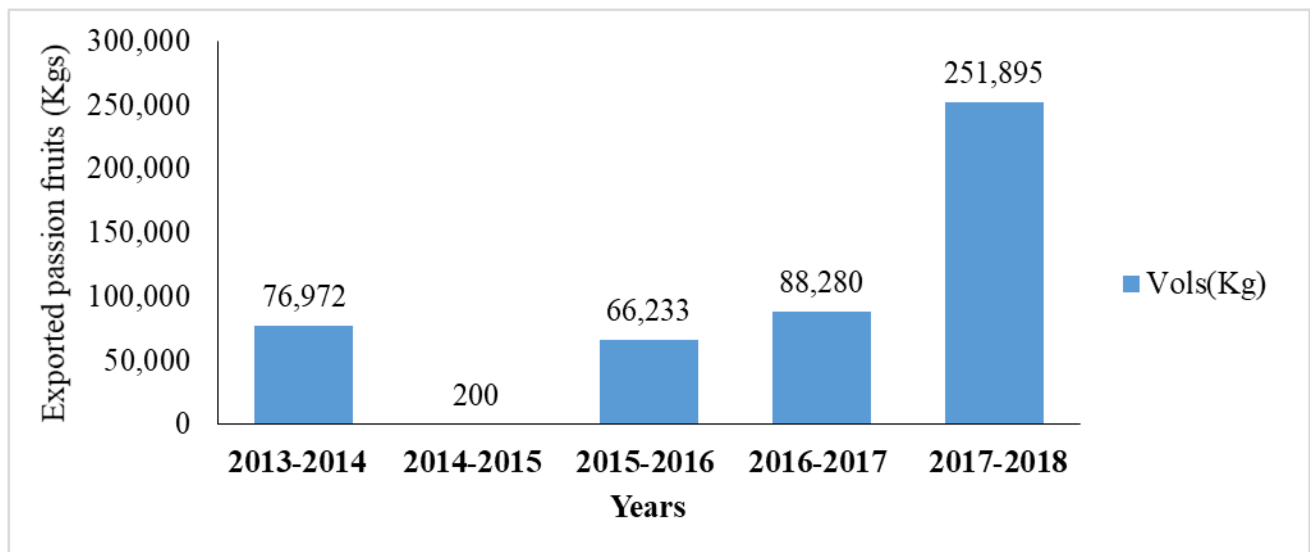


Figure 2.1: Passion fruit exported from Rwanda 2013 – 2018.

2.3 Ecological requirements

The recommended altitudes for cultivation of passion fruits is between 1700m -1900m (Austin,

2009). The optimum temperature for growth of passion fruit is 25^0C to 30^0C. Temperatures beyond 30^0C inhibit the seed set (Jack, 2013). Passion fruit is grown on different soil types, but the best for good growth are sandy- clay with pH of 6.5 to 7.5. The soil should be rich in organic matter, fertile, well drained and low in salt. Passion fruit grows well in both hot and moist climate depending on variety. Yellow passion fruit grows best under tropical conditions while purple grows well under sub-tropical conditions or high altitudes.

2.4 Mineral nutrition of passion fruit

Passion fruit requires both micro and macro nutrients for growth and reproduction at different stages in order to minimize unwanted growth of the plant. Each nutrient has its own specific physiological role in the growth of passion fruit (Limo, 2014). The macro-nutrients N, K and Ca are taken up in the greatest quantities, followed by S, P and Mg. Of the micro-nutrients, Mn and Fe are absorbed in the greatest quantities, followed by Zn, B and Cu.. Many soils have enough natural potassium, but one or two tablespoons of potassium sulphate is needed to provide the required potassium (Rozendaal, 1997). A balanced fertilizer that supplies nitrogen, phosphorus and potassium in approximately equal proportions, as well as essential micronutrients (magnesium, manganese, copper, zinc and iron), is usually adequate on the slightly acid, sandy soils. On the alkaline soils, phosphorus is needed less than nitrogen and potash, but micronutrients must be applied for normal growth and production.

2.5 Constraints in passion fruit production

The main constraints that limit production of passion fruits in Rwanda are pest and diseases, lack of enough clean planting materials, poor post-harvest handling and high cost of clean passion fruit seedlings. Among the diseases that reduces the passion fruit production are fungal diseases

such as Fusarium wilt, Brown spot (*Alternaria passiflorae)*, phytophra spp, Anthracnose (*colleforicham passiflorae),* bacterial disease are bacteriosis caused by *Xanthomonas campestris pv. Passiflorae* (Austin, 2009; Gesimba, 2008). Passion fruit woodiness diseases is a major biotic factors that constrain the production of passion fruit worldwide (USAID, 2002). Over 80% yield losses may occur due to the occurrence of these diseases (Gesimba, 2008). This high losses has been attributed to the lack of resistant clean planting varieties. Other constraints include, high coast of clean planting materials, drought, poor yielding varieties, poor post-harvest handling, poor agronomic practices and inadequate planting materials (Cooke, 2009). Like for many of the horticultural crops, post-harvest management aspect of passion fruit is also not given due attention, thus resulting in loss of large quantity of the harvested produce and deterioration in quality of the produce.

2.6 Passion fruit diseases and pests

The main diseases of passion fruit include: Fusarium wilt *Fusarium oxysporium* which causes wilting and death of plant, with the vascular tissues showing brown discoloration; Blight *Phytophthora nicotianae*, which appears as dark water soaked lesions on leaves, which later spread as infected tissues die. Young shoots also can be infected; Brown spot *Alternaria Passiflora* which attacks leaves and fruits causing brown rings with dead spots, infection on leaves can extend to leaf axils and terms. Alternaria leaf sport (*Alternaria passiflorae*) which causes the brown spot disease on both leaves and fruits. It also reduces the diameter of leaf from 1/16-7/8 inch, and fruits can develop necrotic areas of reddish colour that are 0.5-2 cm in diameter. The fungal disease is favored by wet weather and wind. Another pathogen is Anthracnose (*Glomerella cingulate/Colletotrichum gloesporiodes*), which can cause brown

cankers that kill distal plant parts. Fusarium stem canker *(Fusarium solani*) is also another fungal disease that affects passion fruit production and it destroys the passion fruit plant by increasing wilting (Simone *et al.,* 2000). Bacterial diseases affecting passion fruit production are *Xanthomonas axonopodis pv*. Passiflorae and *Pseudomonas syringae pv*. Passiflorae that affects ripening passion fruits by developing necrotic lesion (Schuber *et al.,* 2014).

The main pests include: Reds spider mites *Brevipalpus sp* – occur on lower leaf surface between the veins and cause leaves to dry, Mealy bugs *Planacoccus sp* are stationary oval shaped, pinkish in colour and covered with waxy thread, Fruit fly *Ceratitis sp* lays eggs on fruit and causes sunken brown spots. Aphids *Aphis sp*, are green in colour and suck sap from tissues, they transmit viruses especially the woodiness virus. Thrips, attacked plant parts shrivel, flowers and young fruits fall prematurely, Stink bug, there are three types of stinkbugs observed in passion fruits in different areas Green vegetable bug-Nezara viridula, Yellow edge stink bug-Nezara pallidocorspasa and Brown stink bug-Boerias maculate they feed by piercing and sucking young fruits Pierced areas appear sunken and lowers fruit quality(Joy and Sherin, 1983). Nematodes are soil inhabitants causing formation of galls or knots on roots, yellowing of leaves, stunting and eventual wilting of the affected plants (Cooke, 2009). Others are nematode associated diseases like *Meloidogne javanica* which also affects passion fruits by injecting the roots with toxic substances and phytoplasma that courses chlorosis on leaves and form abnormal flowers (Joy and Sherin, 1983). Integrated pest management (IPM) is necessary to control pests and diseases of passion fruit, since most of the farmers growing passion fruits in Rwanda are still using chemical control which is not acceptable for the European market.

2.7 Viral diseases of Passion fruit

Viruses affecting passion fruit that include *passion fruit ring spot virus*, *passion fruit mottle potyvirus*, *passion fruit woodiness virus (PWV), cow pea aphid borne mosaic virus (CABMV), Ugandan Passiflora virus*, *Passion fruit virus Y*, *Passion fruit purple granadilla mosaic virus* and *Malaysian Passiflora virus, passion fruit yellow mosaic virus (PaYMV), passiflorae leaf mottle virus, passion fruit cucumber woody virus (CWV), and passion fruit vein clearing virus* (Bragard *et al.,* 2013; Kilalo, 2012); Joy P.P., Sherin 1983). Passiflora virus Y and Passion fruit Mottle Virus (PaMV) cause mild mottling on leaves (Cooke, 2009). PaMV induces skin mottling on fruits and plant infected with East Asian Passiflora virus (EAPV) also show faded fruits (Fischer and Rezende, 2008). Passion fruit plants affected by *Cucumber Mosaic virus* (CMV) show symptoms such as plant stunting, Chlorosis and wilting. Leaves also display brown lesions on stalks (Dragich, *et al*., 2014).

2.8 Passion Fruit Woodiness Disease

Passion fruit woodiness diseases (PWD), is caused by one or a mix of potyviruses,*Passion fruit woodiness virus* (PWV), Cow *pea aphid borne mosaic virus* (CABMV), *Cucumber mosaic virus* (CMV) and *East Asian Passiflora virus* (EAPV). These viruses have been implicated in woodiness disease of Passiflora in Australia, Brazil, Colariccio and South Africa (Brand *et al*., 1992). Passion fruit woodiness disease is characterized by malformation. Passion fruit plants infected by passion fruit woodiness mosaic virus shows woody and deformed fruits and hardening of fruits (Ochwo-ssemakula *et al*., 2012; Chang *et al*., 1996). The younger leaf also shows mottling, and chlorotic spots (Joy and Sherin, 1983). The woodiness disease is a major threat to passion fruit production worldwide (Gillings and Bowyer, 2012). In Kenya and Uganda

Passion fruit woodiness disease was reported as major limiting factors for production of passion fruit (Kilalo, 2013) and (Ochwo-ssemakula *et al.*, 2012) respectively. Woodiness disease is transmitted by several species of aphids, grafting and mechanical inoculation using knifes, scissors and nail during cultural practices (Joy *et al.,* 2000).

2.9 Factors favoring development of passion fruit viruses

The abiotic factors such as temperature, rainfall are some of the factors that affect virus spread. Passion fruit viruses can be transmitted by mechanical, soil (direct root), plant debris and biotic vectors like insects, aphids, mites and nematodes (Kumar, 2010). The aphids are the major vectors that transmit virus. Thisoccurs at high rate when temperature is high, ranging from 21 to 25^0C. This is best for growth of passion fruit, but temperature ranging between 18 to 33^0C can affect the growth of passion fruit and flavour and influence pests and diseases. For the intermediate temperature ranging between 23 to 28^0C passion fruit grows within 60 days, but at low or high temperature, the growth period is 75 days Joy, (2010). Other factors favoring spread include alternate hosts, rainfall and others.

2.10 Passion fruit diseases management

The fungal diseases of passion fruits can be managed by applying fungicides, removing affected fruits, using less susceptible passion fruit varieties and planting grasses under the vines to reduce soil contamination (Cooke, 2009). The bacterial diseases can be controlled by using grafted cultivars and resistant seedlings to reduce soil contamination while nematodes can be reduced by using nematode resistant root stock (KALRO, 2006). The viral diseases of passion fruits can be managed by using tolerant varieties and removing infected passion fruit plants from virus free

planting materials because it is transmitted quickly by aphids (Cooke, 2009). Furthermore, management of alternative hosts through weeding, disinfection of tools used for pruning and controlling pests and aphids is done (Ferron and Deguine, 2005). Passion fruit viruses are also found in bananas and pumpkins hence avoiding planting it near the crop is recommended.

The virus can also be transmitted between plants by parasitic seed plant and mechanical inoculation through pruning tools (Johnson, 2013).

There are different methods used for monitoring pests, but the common one is yellow sticky traps. These traps monitor pest species like whiteflies, leaf miners and aphids. Yellow sticky traps have also been used to control white flies (Lu *et al*., 2012) and aphids (Horowitz, 1984).

2.11 Techniques for detecting passion fruit viruses

Different techniques are used for indexing of different strains of passion fruit are serological, molecular, Electron microscope, and Loop mediated isothermal amplification (LAMP) techniques (Boonham *et al*., 2014).

2.11.1 Loop-mediated isothermal amplification (LAMP) method

Loop-mediated isothermal amplification (LAMP) is a powerful innovative e gene amplification technique used as a simple rapid diagnostic tool for early detection and identification of viral diseases. Amplification can be achieved in 1 hour under isothermal conditions with a set of six primers in a single tube. Amplification can be detected by aturbidimeter, colour change or by Agarose Gel Electrophoresis (AGE) (Satyagopal, *et al.*, 2015). LAMP uses three pairs of primers: internal, external and loop primer to generate amplification product which contain a

single stranded loop region were primer can bind without temperate denaturation (Boonham *et al.,* 2014) at temperature around 65^{0}C. The internal primer produces self -complementary into amplification product, while external primer causes displacement of the extension products of the internal primers, (Boonham *et al*., 2014).

LAMP is a relatively cheap assay for DNA amplification plant disease diagnostics. The visual and rear time end point analysis is idea and easy to interpret, less skill and basic equipment can be used to get the results, the time taken for amplification is relatively short. This method can be used by regulatory and extension for rapid passion fruit viruses detection (Boonham *et al*., 2014).

2.11.2 Serological method

Serological test of plant viruses, it is the most widely used due to its sensitivity, simplicity and adaptability (Kumar, 2010). Enzyme- Linked Immunosorbent Assay (ELISA) requires little or no special equipment and is suitable for use in developing countries. It is the most practical and least expensive for small scale use and is more efficient on large scale. In ELISA test specific antibodies bind to the specific antigens and induce an enzymatic reaction that results in colour changes to yellow for positive sample (Dragich *et al.,* 2014).

2.11.3 Molecular method

Molecular method such as polymerase chain reaction (PCR) is another technique which is most sensitive for detection of plant viruses (Kilalo, 2012). Nucleic acid (RNA or DNA) can be extracted using either Trizol method (Romário *et al*., 2015), CTAB method (Munguti *et al.,* 2016; Chang *et al*., 1996). The molecular method uses a pair of primers, the forward and reverse primer and synthetic oligonucleotide primers for targeting dsDNA strand, the hybridization

primer for DNA polymerase creates complementary DNA strand (Kashif, 2009). PCR occurs in three (3) cycles separation of cDNA 95^0C, primer annealing and between 48 -75^0C and extension at 72 -78^0C. Although PCR is more sensitive than ELISA, it cannot distinguish virus species and strains for those in the potyviridae family. PCR test is also able to detect virus in very low concentration. Whereas ELISA method can be used to detect many samples less expensively compared to PCR the procedure takes a long time (Kilalo, 2012).

CHAPTER THREE

Relative sensitivity and reliability of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings in Rwanda.

Abstract

Passion fruit is an important food and cash crop in Rwanda. However, its production is hampered by potyviruses that cause woodiness disease. There is poor diagnosis and detection of these viruses in passion fruit seedlings and orchards making their management difficult. The study was conducted to compare enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT- PCR) for diagnosis of the potyviruses associated with woodiness disease. Seed from three seed sources, farmers saved seed, private seed suppliers and public institutions were planted in vector free greenhouse at Rubona research station. After germination and growing for two months (four leaf stage), 25 leaf samples from each seed source were tested at three different time periods: 30, 60 and 90 days. In addition, confirmed disease free passion fruit seedlings were inoculated with cow pea aphid borne mosaic virus (CABMV) and allowed to develop symptoms for two months. Fifty leaf samples from the infected plants were tested for CABMV by serial diluting the plant extract up to 10,000,000 (10^{-7}) to compare the sensitivity of the two diagnostic methods. There were significant ($p<0.007$) difference between the two diagnostic methods in detecting CAMBV presence in leaf samples. Leaf samples tested at 30 and 60 days using both ELISA and RT-PCR tested negative for CABMV for all the three seed sources but at 90 days, 32% of the samples from farmers seed source tested positive for CABMV with RT-PCR but not with ELISA. Of 100 artificially inoculated leaf samples, 33% and 45% tested positive for general potyvirus and CABMV using ELISA test, respectively, while 61% tested positive for CABMV using RT-PCR. ELISA detected the presence of CABMV up to a

dilution of 1000 times while RT-PCR detected CABMV presence up to a dilution of 1,000,000 times. In conclusion both ELISA and RT-PCR diagnostic methods were able to detect CABMV. However, RT- PCR was more sensitive for detecting low levels of virus in plant tissue and it can be used in certification program for screening seedlings in passion fruit nurseries while ELSA can be used for disease management.

3.1 Introduction

Passion fruit is one of the most important food and cash crops in Rwanda (NAEB, 2014; HCA, 2013; Mutabazi, 2011). The most common commercial cultivars are purple and yellow passion fruit. The yellow cultivar, is a hybrid between *P.edulis* Sims and *P.ligularis* that originated from southern Brazil (Brand *et al*., 1992). In Rwanda, the Purple cultivar is grown in high altitudes of the country while the yellow or golden passion fruit is grown in midland of the country. The most commonly produced is purple variety (NAEB, 2014). The production of passion fruit in Rwanda has been on the decline because of the viral, fungal, and bacterial diseases (NAEB, 2014; USAID, 2002). Among the diseases, viral diseases are the most limiting. These viruses are reported to be the most limiting to the production of passion fruit worldwide (Garcêz *et al*., 2015; Damiri, 2013; Kilalo, 2012; Ochwo-ssemakula *et al.*, 2012; Fischer and Rezende, 2008; Gioria *et al.,* 2002; Brand, 1992).

In Africa and Brazil, woodiness disease in passion fruit crop is caused by *cowpea aphid borne mosaic virus* (CABMV) (Romário *et al*., 2015; Garcêz *et al.,* 2015; Ochwo-ssemakula *et al*., 2012); Alfenas *et al.,* 2005; Mc kern *et al.,* 1994). However, in other countries such as Australia, the most common causal agent is passion fruit woodiness virus (PWV) (Sokhandan *et al.,* 1997).

CABMV is transmitted by aphids in the field (Bragard *et al.,* 2015; Kilalo *et al.,* 2013; Brault *et al.,* 2010). In Rwanda, passion fruit woodiness diseases was first reported in the Northern part of the country (USAID, 2002). Today, the disease is widely distributed in all regions of the country (MINAGRI and NAEB, 2014; RHODA, 2008). This is mainly due to lack of clean passion fruit planting material, quick and accurate diagnosis and effective methods for pest and disease control (Bowyer, 2012; Cooke, 2009; Simone, 2000).Viral diseases can be spread through different ways such as grafted seedlings, germplasm and vectors like aphids (Garcêz *et al*., 2015). Therefore, the objective of this study was to compare the relative sensitivity and reliability of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings in Rwanda.

3.2. Materials and methods

3.2.1 Study site

The study was conducted at Rubona Research station, located in Huye district, southern province. The station is at an altitude of 1826M, Latitude of 22^{0}06’28’’ - 24^{0}12’09’’and Longitude of 29^{0}26’22’’ - 29^{0}33’43’’. The trial was conducted between October, 2017 and March, 2018. The average temperature was 19°C with annual rainfall range from 1400mm to1600mm per annum.

3.2.2 Experimental layout, seed source seedling establishment and initial virus detection

The passion fruit seeds of dark purple variety *Passiflora edulis var. edulis* were used. The seeds were obtained from three seed sources; farmer saved seeds, private seed companies and public institutions. The collected seeds were planted on 30th October 2017 in vector free greenhouse.

Leaf samples were collected from twenty five (25) plants per seed source at the 4-6 leaf stage and divided into equal halves. One half of the leaf extract was used for ELISA and another half was used for RT-PCR. The leaf samples were tested after 30 day, 60 days and 90 days. The experiments were laid out in a Completely Randomized Design (CRD).

Purple variety fruits were obtained from local market and then fermented to get seeds. The dried seeds were grown in vector free greenhouse in two separate experiments and then seeded on plastic trays and allowed to germinate for about two weeks. The seedlings were then transplanted in the pots (one seedling per pot) and allowed to grow up to the 4-6 leaf stage (after two months). The germinated seedlings were sampled and tested using RT-PCR before inoculation to confirm whether the seedlings were free from CABMV. Inoculated passion fruit leaf samples were established and tested in different time period. First inoculated plants were established in October 2017 and tested in December 2017. The second inoculated plants were established April 2018 and tested in June 2018.

3.2.3 Virus inoculums preparation

The passion fruit leaf samples with disease symptom were collected from farmer's field in northern region of Rwanda. All collected passion fruit leaf samples were having virus like symptoms. The most common symptoms were hard cracked fruits, mosaic, chlorosis, molting, vein clearing and malformation of fruits (Figure 3.1). The leaf samples were tested using RT-PCR to confirm the presence of CABMV. The inoculum was prepared by grinding 0.3 g of symptomatic leaf tissues from farmer's field within 2ml of both potassium hydrogen phosphate monobasic ($KHPO_4$) and potassium hydrogen phosphate (K_2HPO_4 of pH 7) dibasic buffer, using

mortar and pestle plus100 mg of carborundum. The prepared sap was used to inoculate health passion fruit seedlings using cotton swabs. Inoculated plants were kept in a greenhouse for at least two month for symptom observation.

Figure 3.1. Symptoms of passion fruit woodiness on fruit (A) and on leaves (B)
(A) Hard cracked misshapen fruits. (B). Mosaic on leaves, Chlorosis and molting

3.2.4 Sample collection and testing

Once the virus symptoms developed after two month of inoculation, a total of 50 inoculated leaf samples were collected and tested using both ELISA and RT- PCR and this was repeated twice. Leaf samples for testing using RT-PCR were collected in zip lock bags put in a cool box with ice packs and transported to RAB seed plant pathology laboratory located at Kigali – Rubilizi station and stored at -20^{0}C until analyzed. For samples indexed using ELISA method, leaf samples were preserved at 4^{0}C until analyzed.

3.2.5 Dilution experiment

One confirmed CABMV infected leaf extract was diluted seven times up to 10,000,000,000X to compare the sensitivity of ELISA and RT-PCR diagnostic methods for CABMV. *Cowpea aphid borne Mosaic virus* (CABMV) positive control for RT- PCR was obtained from DSMZ (German collection) while the negative control used water / buffer for ELISA. The positive and negative control for ELISA were supplied together with ELISA kits.

3.2.6 Analysis with ELISA and RT-PCR

3.2.6.1 Serological virus detection using ELISA

The detection of virus in passion fruit was performed with double antibody sandwich linked immunoassay (DAS- ELISA). The general procedure followed the manufacturer's protocol and with modifications as described by Kilalo (2012); Adams *et al.* (2009); Berniak *et al.* (2009). The polyclonal antibodies (AS-0417) used for detection of *Cowpea aphid borne Mosaic virus* were purchased from Germany collection of microorganisms and cell culture (DSMZ). The controls consisted of negative and positive controls supplied together with the ELISA kits while polyclonal antibodies for detection of potyvirus were purchased from AGDIA (USA). Samples were considered as positive if their absorbance was greater than or equal to double the average reading of the negative control.

ELISA tests for the samples were carried out in duplicates. For detection of CABMV and potyviruses, 0.3 g of leaf sample were weighed and extracted in 2 ml of general extraction buffer. 100 µl of leaf sample were placed in wells and the entire plates incubated overnight at 4^{0}C. All buffers used were prepared according to the manufacturers' recommendations. Each

step of ELISA was incubated at $37^{0}C$ for 4 hours. After incubation the plates were washed using PBS-T washing buffer three times, with an interval of 3 min for each wash. The plates were further treated with 100 µl of conjugate solution per well and incubated. The plate was washed again eight times with an interval of 3 min. 5g of p-Nitrophyyl phosphate (P-NPP) were dissolved in 10 ml of substrate buffer. 100ul of the solution were added in each well and incubated at room temperature ($25^{0}C$) for 1 hour. The colour change yellow was observed and the absorbance determined using ELISA plate reader (DR- 200Bs Micro plate Reader) at 405nm wavelength spectrophotometer.

3.2.6.2 RT- PCR Virus detection

RT –PCR was conducted by collecting passion fruit leaf samples and extracting total RNA using Trizol protocol according to (Chomczynski and McKay, 1995). 0.05g of leaf samples were weighed and added to 600 µl of Trizol reagent and ground until the leaf extract was released. The mixture was centrifuged for 1 min at 12000rpm from which 500 µl of the supernatant was removed and added to 500 µl of 96% ethanol. The mixture was vortexed for 1 min and transferred into Zymo Colum collection tube. It was then centrifuged for 1 min at 12000 rpm. 400 µl of pre- wash buffer was added and the mixture further centrifuged for 1 min at 12000rpm. The flow through was discarded. This step was repeated to ensure completely removal of residuals. 700 µl of RNA wash buffer was added to Zymo Colum collection tube and centrifuge for 2 min to further clean the RNA trapped in the membrane. The flow through was discarded. The Zymo Colum collection tube was transferred into an RNAase free tube and 50 µl of RNase free water added directly to the Column matrix and centrifuged for 1 min at 12000 rpm. The eluted RNA was transferred to a 0.5 ml Eppendorf tube and stored at $-20^{0}C$.

One step RT-PCR was conducted for each sample as follows: The master mix that included 12.865 µl of water with 5 µl of 5X Go Taq green buffer, 1.25 µl of 0.1M DTT, 2 µl of 10 µM concentration Forward and Reverse Primers, 0.5 µl of 10 mM dNTP, 0.1 µl of RNAse OUT (40 U/µl), 0.035 µl Superscript II (200 U/µl), 0.25 µl GoTaq DNA Polymerase (5 U/µl) and 1 µl of RNA to make a final volume of 25 µl. A negative control that included all the components except the RNA was included and to make 25 µl of reaction mixture an extra 1 µl of water was added. RT-PCR amplification was done using two primer pairs. These included a forward and reverse specific CABMV primers, CABMVF: 5'-CACCAGAGCATCAAAGACACAGCTCA-3'and CABMVR: 5'CAGTGTTCTCACTAGTTGTTGCCAC -3', respectively expected to yield 626 bp product from the coat protein (CP) gene. The following cycling conditions were used for primer pair CABMVF/CABMVR: 95 ^{0}C for 5 min, 30 cycles of 94 ^{0}C for 30 sec, 58 ^{0}C for 1 min, 72 ^{0}C for 45 sec, followed by a 10 min extension at 72 ^{0}C.

To visualize the PCR products obtained after amplification, electrophoresis was carried out. The gel preparation was done by adding 1. 24 g of agarose powder to 100 ml of 0.5X TBE buffer (pH 8.3). The mixture was boiled using a microwave oven until agarose powder dissolved in the buffer. The mixture was allowed to slightly cool (to about 55^{0}C) and 1 ul of Ethidium bromide added. It was immediately poured into a gel tray with fixed combs to create wells for electrolysis of the samples. The gel was allowed to cool and solidify. The solidified gel was placed into electrophoresis chamber and 10 µl of each PCR product loaded into each well. The first and last wells of the cast gel were loaded with 100 bp standard DNA ladder to estimate the size of PCR amplified product being analyzed. Electrolysis was conducted in 0.5X TBE buffer for 45 min at 120 Volts. The PCR products were visualized and photographed under ultraviolet (UV) light (UVB 310nm).

3.2.7 Statistical Analysis

The data were analyzed using R software. CABMV infection rates as detected by RT-PCR and ELISA were compared using paired T-test at 95% confidence level.

3.3 Results

3.3.1 Screening of passion fruit woodiness disease from the three different seed sources

At 30 and 60 day periods, both EISA and RT-PCR diagnostic methods did not detect CABMV from the three different seed sources (Table 3.1). However, at the 90 day period, the ELISA test did not detect CABMV in the leaf samples but RT-PCR detected CABMV from 32% of the samples from farmer seed source and nothing from the rest of the seed sources (Table 3.1; Fig.3.2)

Table 3. 1 Detection of CABMV from the three different seed sources using ELISA and RT-PCR at 30, 60 and 90 days after planting

Seed Sources	**Days of Testing**	**Detection (%)**	
		ELISA	**RT-PCR**
Farmer	30	0	0
	60	0	0
	90	0	32
Private	30	0	0
	60	0	0
	90	0	0
Government	30	0	0
	60	0	0
	90	0	0

3.3.2 Indexing of inoculated passion fruit seedlings for CABMV

The results from tested passion fruit leaf samples for detection of CABMV using ELISA and RT-PCR revealed that forty five and sixty one percent of plants were infected by CABMV, respectively. Out of the forty six asymptomatic leaf samples tested, 22% and 65% tested Positive

for CABMV with ELISA and RT-PCR, respectively. Eighteen samples that had unclear symptoms of woodiness, 38.8% and 100% tested positive for CABMV with ELISA and RT-PCR, respectively. The detection levels of CABMV significantly varied between the two methods in the season 1 ($p < 0.002$). However, in season 2 the CABMV detection levels by the two methods did not vary but RT-PCR method had higher detection levels at 62% (Table 3.2). Overall the test rate for RT- PCR was significantly higher ($p < 0.007$) than that of ELISA. Figure 3.3 shows that the materials that were used for inoculating the plants were infected with CABMV.

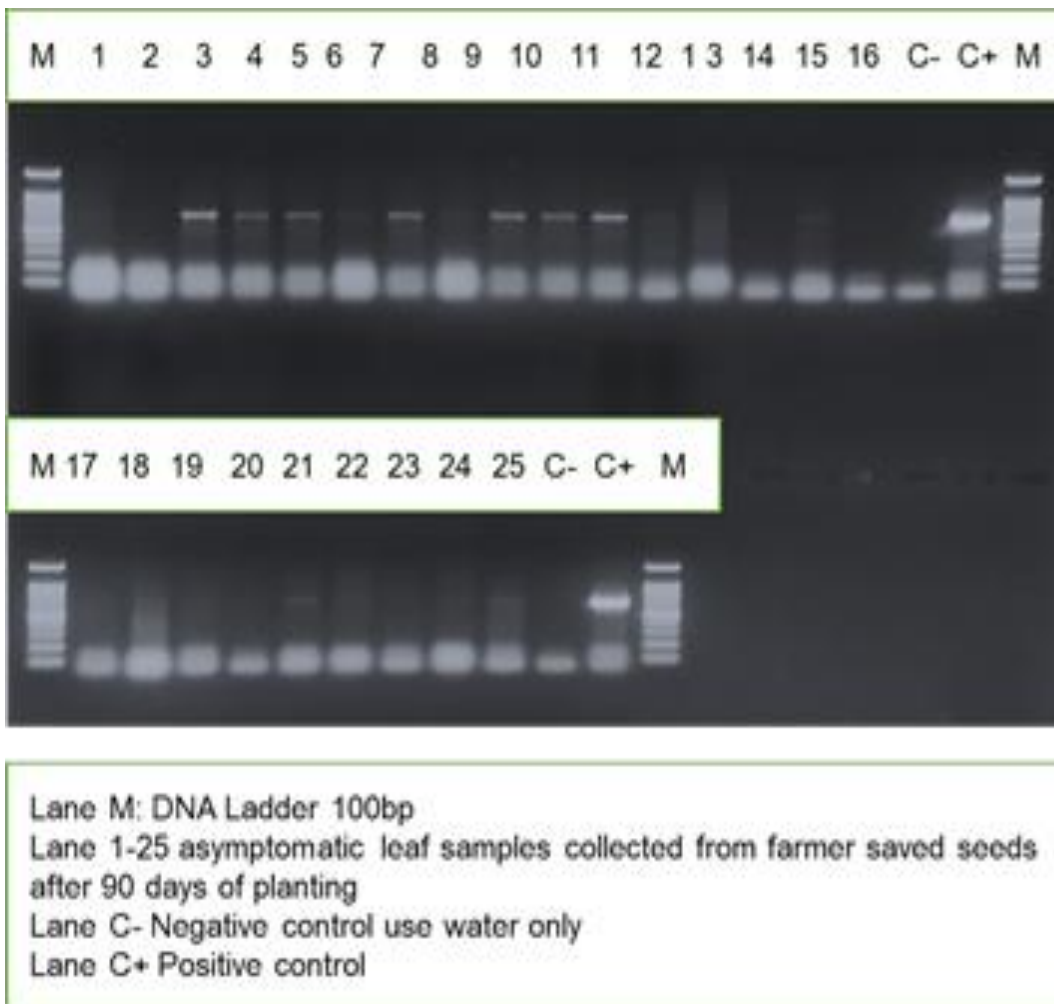
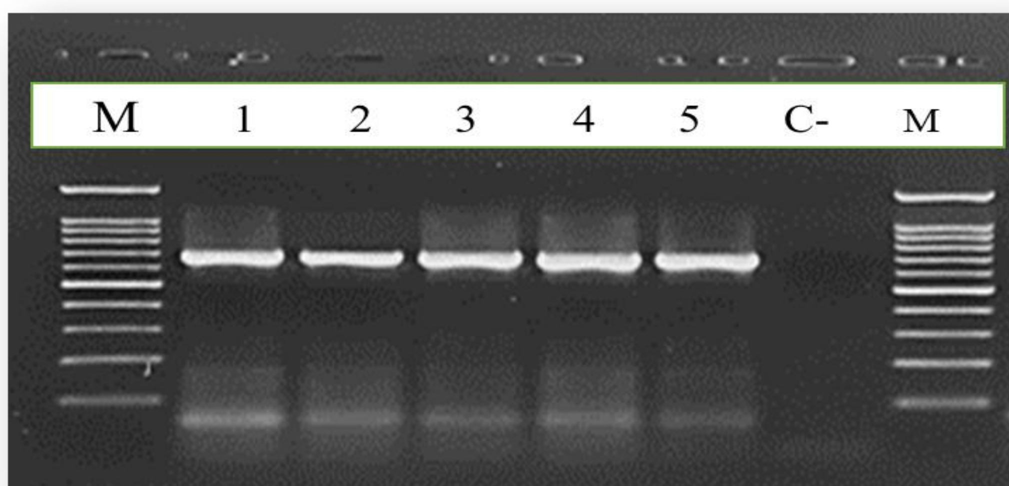


Figure 3.2 Amplification products for RT- PCR for detection of CABMV using specific primers on farmers saved seed 90 days after planting.

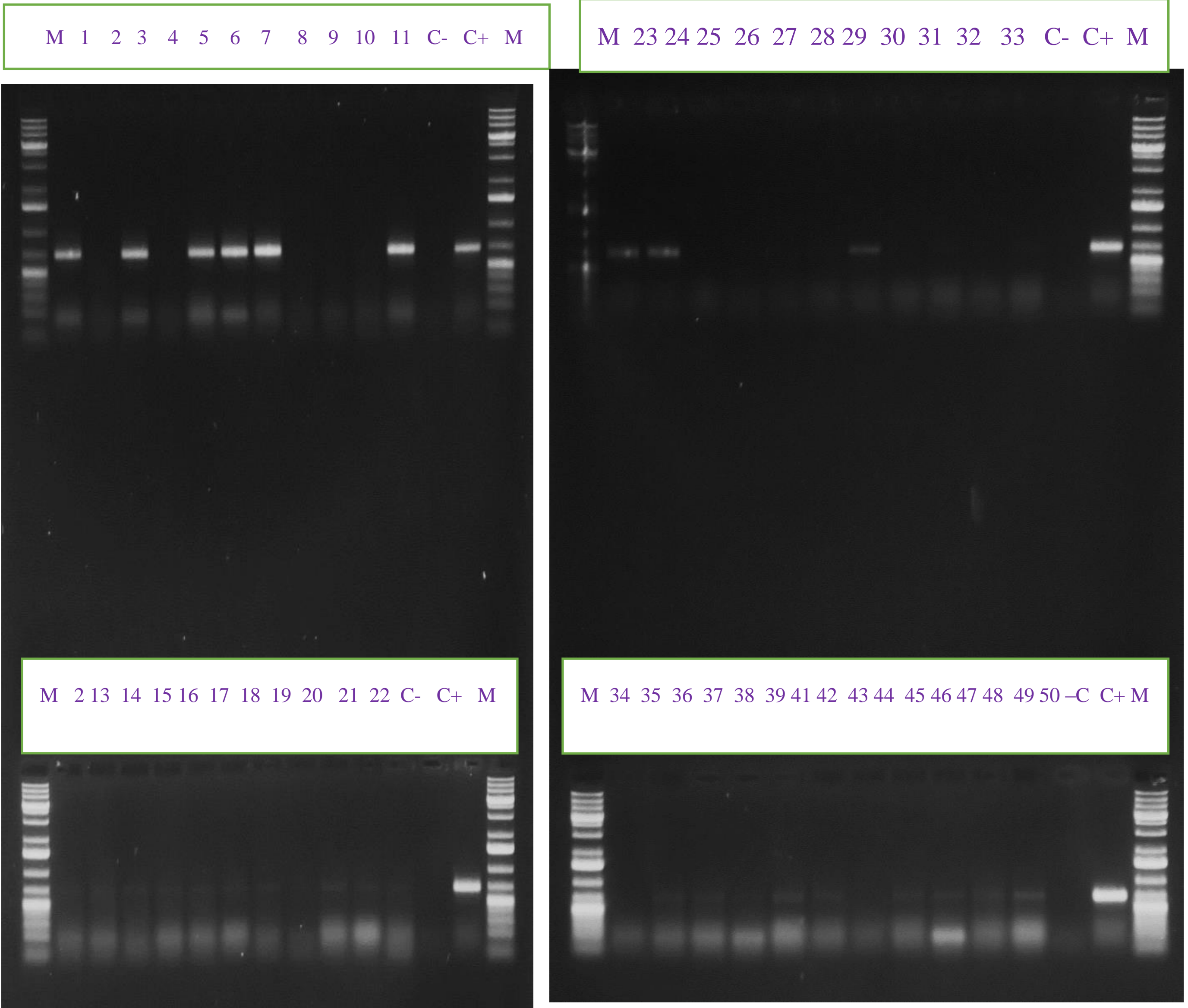
Table 3.2 CABMV and potyvirus detection levels in inoculated passion fruit leaf samples using ELISA (For both CABMV and potyvirus) and RT- PCR on CABMV only (percentage) for included plants in Rwanda in 2017 (season 1) and 2018 (season 2).

	Detection (%)			
Methods	**Season 1**		**Season 2**	
	CABMV	**Potyvirus**	**CABMV**	**Potyvirus**
ELISA	32	58	45	33
RT-PCR	60		61	
P- value	*0.0002*		*0.007*	



Lane M: DNA Ladder 100bp
Lane 1- 5 Symptomatic leaf passion fruit leaf samples collected from farmers field

Figure 3.3: CABMV positive symptomatic leaf samples from farmer's field used as source of inoculum.



Lane M: DNA Ladder **1kb plus**
1 symptomatic, **2**, Asymptomatic**, 3** symptomatic, **4,** Asymptomatic, **5-7** Symptomatic**, 8-10** Asymptomatic, **11**, Symptomatic, **12- 18** confusion, **19-** Asymptomatic, **20-22** Symptomatic, **C-** Negative control and **C+** Positive Control , Primer size **628bp,** Lane, **23** Symptomatic, **24**, Symptomatic, **25- 28** Asymptomatic, **29** Symptomatic, **30-33** Asymptomatic**, 34** Asymptomatic, **35-44** Confusion, Lane **45-47** Asymptomatic, **48-50**, Symptomatic.

Figure 3.4: RT-PCR test results for the fifty inoculated CABMV passion fruit leaf samples (Season 1).

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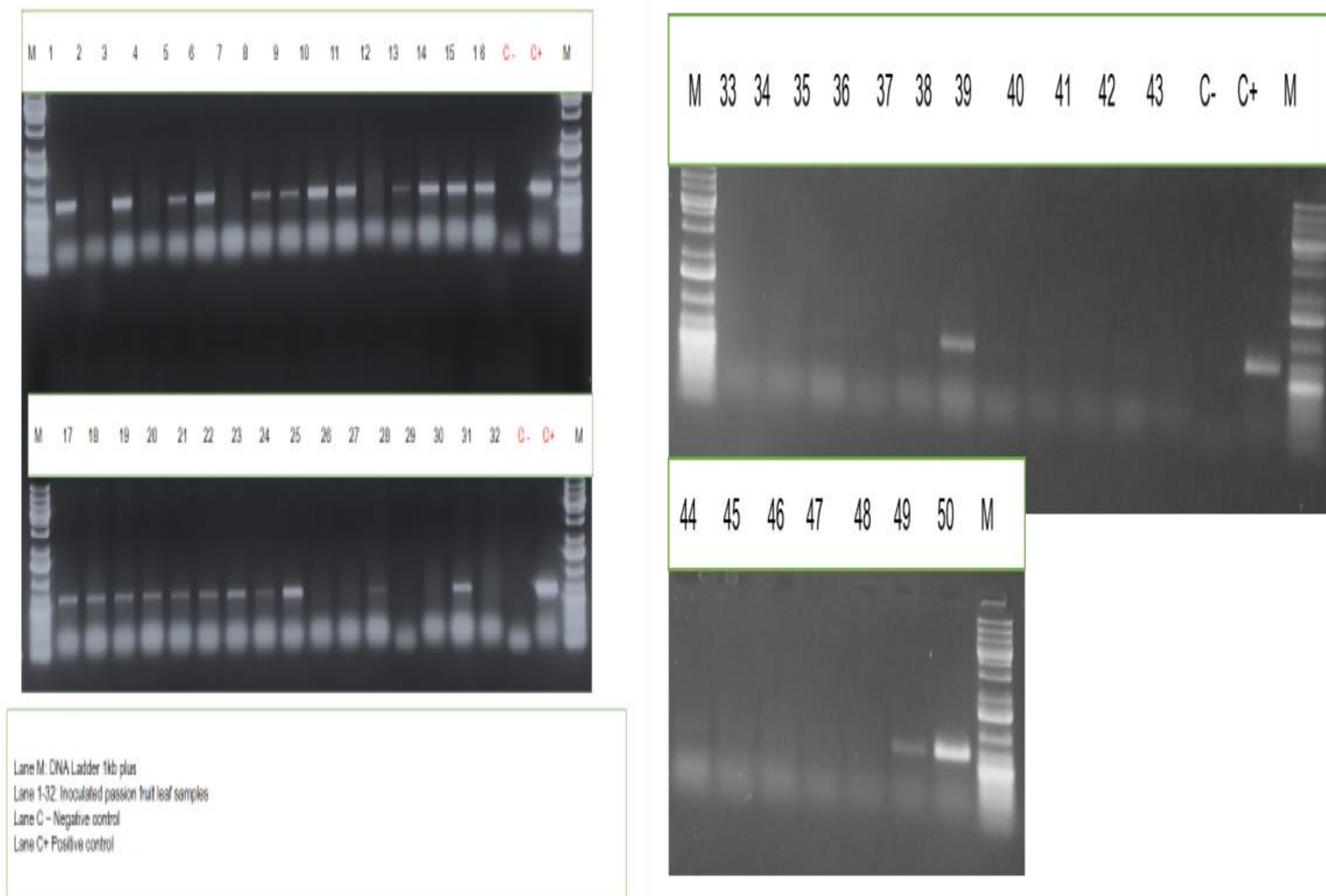

Figure 3.5: RT- PCR test results for the fifty inoculated passion fruit leaf samples (Season 2)

3.3.3: Sensitivity of ELISA and RT-PCR in serial dilution experiment

ELISA gave positive results for CABMV only for the first four dilutions (1X, 10X, 100X and 1000X) after which it tested negative for other dilutions more than 1000 times (Table 3.3) while RT- PCR gave positive results for CABMV up to six serial dilutions (1.000.000 times) (Figure 3.6 B).The higher the concentration of the virus the higher the absorbance levels (Fig. 3.6 **A**). The higher the virus concentration the stronger the bands observed on running the amplified PCR product (Fig.3.6 **B**).

Table 3.3: Detection of CABMV by ELISA and Reverse transcription polymerase chain reaction (RT-PCR) on serial dilutions.

Sample code	Serial dilution	ELISA	RT-PCR
01	1X	+	+
02	10X	+	+
03	100X	+	+
04	1000X	+	+
05	10.000X	-	+
06	100.000X	_	+
07	1.000.000X	_	+
08	10.000.000X	_	_

+ = Positive samples and **-** = Negative samples

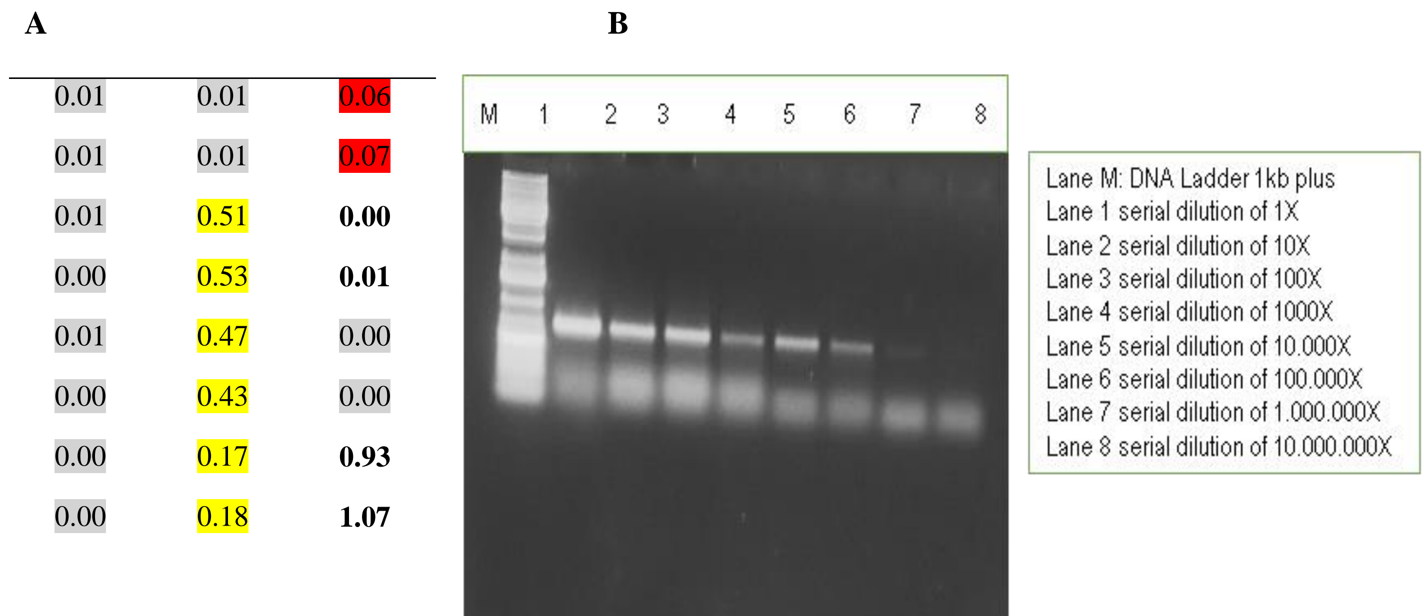

Figure 3.6: Sensitivity of ELISA (**A**) and RT- PCR (**B**) for detection of CABMV in the different serial dilutions.

Note: [yellow] Diluted samples that tested positive, (Positive control **0.93 and 107**)

[red] Diluted samples that tested Negative, (Negative control **0.00 and 0.01**)

[grey] Buffer use only

3.4 Discussions

The results reveal that ELISA and RT-PCR methods were able to detect virus infection from asymptomatic, symptomatic leaf samples and those with unclear symptoms. However, RT-PCR had a higher sensitivity and specificity for capturing samples that had returned as false negatives by ELISA test. The results show that RT-PCR was able to detect the virus at low level and ELISA consistently detected few samples as being positive compared to RT-PCR. All the two methods could detect on 30 and 60 days after planting. This may be explained by the fact that at 30 and 60 days the viruses could have been developing in plant tissues hence the low titer values that could not be detected by both ELISA and RT-PCR. At 90 days virus titers in plants had probably increased, but just enough to only be detected by RT-PCR and not ELISA which produced false negative results. These findings are similar to those observed by (Mayer, 2016) who reported that the accuracy of diagnostic methods increases as plants age while studying the detection of potato virus Y (PVY). Several authors have reported that RT- PCR is more sensitive than ELISA for plant virus detection (Guan *et al*., 2017; Kilalo, 2012; Ahangaran *et al*. 2009). (Cerqueira-Silva *et al*., 2008; hang *et al.,* 1996; Kinard *et al.* 1996) obtained similar results and reported that ELISA diagnostic method failed to detect because of low virus level in the plant tissue or it could also be affected by inhibitor compounds found in the plant sap. However, (Bariana, 1994) reported that the negative virus results was not due to failure of ELISA detection method but rather it might have been because of inhibitors in the sample extract. (Hanna *et al.,* 2009) reported that the quality and specificity of antibodies used on ELISA may also be critical for obtaining reliable results while (Raj *et al*. 2002 and Hu *et al*. 1995) suggested that the application of monoclonal antibodies might increase the reliability and specificity of ELISA tests. Other studies have shown no differences between ELISA and RT-PCR for viruses such as

Tomato spotted wilt virus (TSWV) in peanut showed a 37.5% infection level with ELISA compared to 45.8% using RT-PCR (Lucie *et al*., 2016; Romário *et al*., 2015; Dang *et al*., 2009; Layan *et al.,* 2006).

These results show that RT-PCR could be the best assay for screening viruses on seedlings of passion fruit, and that CABMV might be a seed borne disease since the tested samples from seed sources were planted in vector free greenhouse, and pesticide were applied against the vectors. More studies are needed to confirm how CABMV multiplies within the plant over time for detection to take place.

Both diagnostic methods (ELISA and RT-PCR) detected CABMV at different levels of dilution where ELISA detected up to 10^3 and RT-PCR detected up to 10^6 dilution. According to (Layan *et al.,* 2006) while comparing the two methods in Turkey, RT-PCR had a higher sensitivity although both ELISA and RT-PCR detected the viruses in symptomatic samples. (Bariana, 1994) reported that both ELISA and RT-PCR were effective for detecting seed borne legume viruses in symptomatic leaf samples but RT-PCR was 10^5 more sensitive than ELISA on asymptomatic leaf samples. (El-Kewey *et al*., 2007; Bashir *et al*., 2002; Tsuchizaki *et al*., 1970) reported that all symptomatic cow pea leaf samples were positive for CABMV when tested using ELISA. However, ELISA may also be convenient as a low cost diagnostic method and where many samples are to be indexed over a short period. This findings compares with those of (Usta and Murat, 2005) who detected *Prunus necrotic ring spot virus* (PNRV) up to dilution of 1:390,000 by RT-PCR and 1: 10,000 with ELISA. (Navarro *et al.,* 1998) reported a detection limits of CABMV with RT-PCR to be higher than ELISA. The present results also correspond to those of

(Berniak *et al.,* 2009; Dang *et al*., 2009; Ahangaran *et al*., 2009) who reported that RT-PCR had a higher sensitivity and can provide more reliable results than ELISA. Mechanisms of virus spread within the plant may act at different stages of virus infection.

CHAPTER FOUR

Efficacy of combining sticky traps and orchard fertilization in managing viruses associated with passion fruit woodiness disease in Rwanda

Abstract

The viruses associated with woodiness disease of passion fruit are transmitted by aphid vectors. Farmers in Rwanda use pesticides to manage aphid vectors but the pesticides have not been effective. This study evaluated six vector management options namely; 1) yellow sticky trap deployment, 2) inorganic fertilizer for rapid tree growth, 3) yellow sticky traps + inorganic fertilizer, 4) pesticide application, 5) a combination of the above 4 treatments and 6) un-treated control during two cropping cycles in 2017 and 2018 in Rwanda. Aphid vector populations were evaluated once a week for 29 weeks using yellow sticky traps and water-pan**.** The disease incidence was assessed as a percentage and severity scored using a scale of 1-5. Yields were evaluated on six plants for each treatment. The results show that sticky traps, a sticky trap and inorganic fertilizer and a combination of sticky traps, inorganic fertilizer and pesticide recorded a high number of aphids compared to pesticide spray alone or the control during both seasons.. There were no significant differences between vector management options during the long rain period. However, there was a significant ($p<0.05$) difference between aphid management options during the short rain period. There was a negative correlation between the number of aphids trapped on sticky trap, disease incidence and disease severity. Pesticide spray produced a higher marketable yield but not significantly ($P < 0.003$) different from yellow sticky trap treatment. This implies that whatever management option is used, yellow sticky traps should be a central component for virus vector management.

4.0 Introduction

Passion fruit *(Passiflora edulis* Sims) is an important crop for small-scale farmers in Rwanda. It is a source of food and income due to high market value and short maturity period. It is ranked second after pineapple and both contribute 2.05 million FRW per year (NAEB, 2014). The country has potential to produce 15-20 tons /ha of passion fruit due to the good climate, soil and altitude, factors that flavour its production (HCA, 2013). However, the production of passion fruit is currently low at about 2 tons per ha (RAB, 2014) compared to Kenya, South Africa and Australia having 8-9, 19 and 24 tons per ha, respectively. According to (Mwangi. 2016 and Macharia, 2013) low yields are due to high disease incidences, poor disease management and lack of planting material. Among the pathogens limiting production, viruses are considered to be a major cause of low productivity in passion fruit affecting yield and quality and age of orchards (Boonham *et al*., 2014; Moreira, *et al*., 2008; Gioria, *et al*., 2002; Simone, 2000). Passion fruit woodiness disease, is caused by one or mixed infection of the potyvirus such as *Passion fruit woodiness virus* (PWV); *Cowpea aphid-borne mosaic virus* (CABMV); *Ugandan Passiflora virus* (UPFV), or *East Asian Passiflora virus* (EAPV) (Kilalo *et al.,* 2013; Ochwo-ssemakula *et al*., 2012).

Passion fruit woodiness is the most important viral disease that affects this crop worldwide (Ochwo-ssemakula *et al*., 2012; Iwai, 2006; Brand *et al*., 1992). Passion fruit woodiness disease reduces the quality and production of passion fruit not only in Rwanda but also in the whole world (Mayer, 2016; Novaes and Rezende, 2003; USAID, 2002). The plant viruses are transmitted in different ways, through seeds, grafting and mechanical transmission (Bowyer, 2012; Gesimba, 2008; Fischer and Rezende, 2008). Both *passion fruit woodiness virus* (PWV)

and *cowpea aphid-borne mosaic virus* (CABMV) are transmitted in a non-circulative manner by several species of aphids (Olango *et al*., 2014 and Chang *et al*., 1996). Different aphid species, *Aphis gossypii* (Cotton aphid), *Aphis fabae* (Bean aphid) and *Myzus persicae* (green peach aphid) and *Aphis craccivora* among others (Gobiye *et al.,* 2016; Garcêz *et al*., 2015) are the most important in transmitting plant viruses, especially in passion fruit (Mayer, 2016 and Fanigliulo *et al.,* 2006).

Management of viral diseases in passion fruit include selection of tolerant varieties, pre-immunization with mild strains of the target virus and adoption of cultural practices (Alireza *et al*., 2017 and Alfenas *et al*., 2005) In many cases, there has been over reliance of pesticides for the control of vectors and little or no use of cultural practices (Lewis *et al.,* 2014 and Fanigliulo *et al*., 2009). Pesticides have been effectively used in the management of insect vectors (Koch, 2016; Martin *et al*., 2004 and Perring *et al*., 1999). However, other researchers have reported that insecticides are rarely effective in reducing some species of aphids (Mwangi, 2016; Jack, 2013 and Kalleshwaraswamy *et al.,* 2012). According to (Olango *et al*., 2014 and Moreau and Isman, 2012) there are alternative methods of controlling insect pests which use a combination of different methods and they could be a better way over controlling insect vectors.

Yellow sticky trap and other cultural practices may also function as tools of monitoring insect pests including aphids (Gobiye *et al*., 2016; Vänninen, 2013; Moreau and Isman, 2012; Lu *et al.,* 2012 and Schuber *et al*., 2009). In Rwanda the spread of viral diseases is partly due to lack of knowledge for their management using different cultural practices, including management of insect vectors (Jaeger, 2001 and Mutabazi, 2011). Most of the farmers in Rwanda use pesticides

to control aphid transmitted plant viruses in passion fruit crops due to lack of other management methods (MINAGRI and NAEB, 2014; RHODA, 2008 and USAID, 2002). Information on management of passion fruit diseases in Rwanda is scanty, which makes it hard for effective management of the diseases. The aim of this study was to evaluate vector management options for managing passion fruit viruses associated with woodiness disease.

4.1 Materials and Methods

4.1.1 Description of the study area

The study was conducted at Rubona Research station, located in Huye district, southern province. The station is at an altitude of 1826M, Latitude of $22^0$06'28'' - $24^0$12'09''and Longitude of $29^0$26'22'' - $29^0$33'43''. The trial was conducted between September, 2017 and March, 2018. The average temperature is 19^oC and annual rainfall ranges from 1400mm to1600mm per annum, and the soil is sandy-clay. Long rains were received from December, 2017 to March 2018 while short rain period was received from September to November, 2017.

4.1.2 Experimental Design and Treatments

The purple variety *Passiflora edulis var. edulis* was used in this study. The passion fruit was established in plots measuring 15x 4m at spacing of 3x 2m. The treatments were assigned randomly and all the recommended agronomic practices including, fertilization and weeding were done. At planting 20kg of manure was applied per plot. Six treatments consisting of: 1) Untreated (control), 2) yellow sticky trap alone, 3) inorganic fertilizer (NPK 17:17:17), 4) yellow sticky trap + inorganic fertilizer (NPK 17:17:17), 5) Insecticides, Profenofos (*O*-4-bromo-2-chlorophenyl *O*-ethyl S-propyl phosphorothioate) 40% + Cypemethirin [cyano-(3-phenoxyphenyl)methyl]-2,2-dimethyl cyclopropane-1-carboxylate (4% EC) and 6) combinations

of yellow sticky traps + inorganic fertilizer + insecticide applications. The six treatments were laid out in a Randomized Complete Block Design (RCBD), with each plot measuring 15m x4m with four replications.

4.1.3 Assessment of aphid population in the field

The population of aphids in the field was assessed using water pan trap and yellow sticky traps. The traps were placed on wooden frames 1.5m high above the ground. The yellow sticky trap used had 22.9 x27.9 cm diameter with glue on both sides. In each plot, two yellow sticky traps were placed equidistantly on every variation, and the number of aphids was recorded once a week for the period of 29 weeks. The water pan traps used were round yellow basins, with 20cm length and 30cm diameter. They were half-filled with clean tap water replaced every week immediately after counting aphids. The specimens were preserved in 70% ethyl alcohol for identification and counting. These were later combined /merged to one cumulative sample to give the total sample collected per month per plot. The aphid species were determined based on the morphological features like, body colour and its shape as described by Martin, (1983). The surrounding vegetation and crops were recorded for the two growing seasons. The aphids collected were separated, identified and counted in the laboratory using a stereomicroscope. Apart from the traps, direct sampling was done on the vines.

4.1.4 Determination of disease incidence and Severity

The disease incidence for each treatment was evaluated in percentage using viral disease incidence (VDI) formula: VDI =Number of symptomatic plants/Total number of assessed plants)*100 (Rossouw *et al.*, 2009). The disease incidence in each treatment was evaluated by

counting the number of symptomatic plants with typical symptoms of passion fruit woodiness virus disease i.e. mosaics on leaves, intervene chlorosis, yellowing between leaf veins with occurrences and spread to younger leaves, leaf distortion and hard cracked misshapen passion fruits. Disease severity was assessed using the disease scoring scale of 1-5 on six tagged plants per treatment (1=asymptomatic, 2=mild symptoms, leave distortion, 3= moderate symptoms, leaf distortion and stunting, 4= severe symptoms of disease on leaves, 5= more than 75 % on infected Assiri *et al*., (2017); Gobiye *et al*., (2016).

4.1.5 Evaluation of passion fruit yield

The passion fruit yield was estimated on six plants per treatment / plot and separated into marketable fruits (healthy fruits, not having any disease symptoms and of normal size) and non-marketable fruits (deformed fruits having disease symptoms and deformed, and small size (abnormality). The total yield fruits was determined using digital hook type weighing scale in kilograms (Kg).

4.1.6 Statistical Analysis

Statistical analyses were conducted using R software. Data on weekly numbers of aphids per trap were analyzed for homoscedasticity using the Levine's test. The data was log transformed prior to ANOVA to meet assumptions of normality, which was assessed using normal quintile plot (normal Q–Q plot). For comparison of means of aphids among vector management options, analysis of variance (ANOVA) was conducted. Significant means among treatments were compared using Turkey's HSD pairwise comparisons. Pearson correlation analysis was used to relate the number of aphids with the disease severity and disease incidence.

4.2 Results

A total of 191.5mm of rainfall was recorded during the experiment most of which was received in November 2017 (191.5mm). In 2017, September to October season was relatively warm with an average temperature of 20.5^{0}C. The lowest temperatures (14.7^{0}C) were experienced in November during the long rain season and the highest in September (26.1 ^{0}C) during short rain period. There were very minor variations in average ambient temperatures during the experiment (Figure 4.1). The average temperature during the pre-flowering phase was 19.3^{0}C. At flowering phase of plant growth, the mean temperature was 15.1^{0}C. During the fruit harvesting the minimum and maximum temperature was 15.6^{0}C and 25.4^{0}C, respectively (Figure 4.1). Overall, average temperature during the experiment was 20^{0}C.

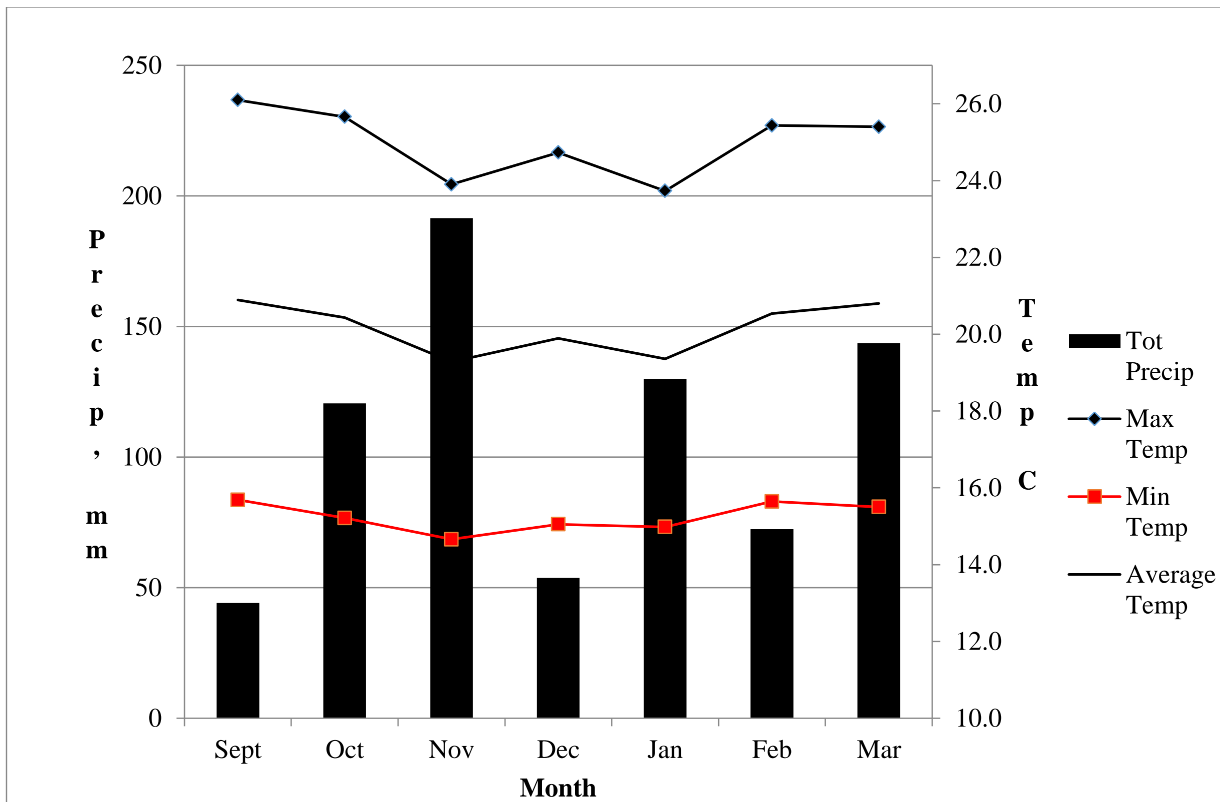

Figure 4.1: Monthly mean temperatures and rainfall values at Rubona, Southern Province, Rwanda during field experiments from Sept 2017 to March 2018.

4.2.1 Effect of management options on aphid population

The aphid species identified on the traps were *Aphis fabae* (black in colour) Bean aphid and *Aphis gossypii* (green in colour) cotton aphids. Higher number of aphids were collected during short rain period from September to October 2017 when the mean temperature was high. During that time the rainfall did not exceed 120.6mm (Figure 4.1). It was observed that the aphid population in all treatments was high during short rain season where the mean temperature and rainfall was 20.6^{0}C and 44.1mm, respectively. During long rain season where the mean temperature and rainfall was 19.3^{0}C and 191.5mm, respectively, aphid populations were low. There were no significant differences between vector management options during the long rain period. However, the pesticide only treatment recorded the least number of vectors trapped while the yellow sticky trap treatment recorded the highest number of aphid vectors trapped. In the short rain season (season 2) there was a significant ($p<0.05$) between aphid management options during the short rain period. The untreated control and sticky trap combined with pesticide and inorganic fertilizer had least number of vectors trapped whereas the sticky trap treatment recorded the highest number of vectors trapped. The results show that sticky traps, a sticky trap and inorganic fertilizer and a combination of sticky traps, inorganic fertilizer and pesticide recorded had high populations of aphids trapped compared with pesticide spray alone or the control during both short and long rain periods (Table 4.1).

At the beginning of monitoring, the aphid vectors populations were high but as time passed on the populations decreased to a level within a mouth where all the vector management options did not differ in their effect on the aphids. The sticky traps trapped the most aphids but this decreased with time. Similarly, the rest of the vector management options had the vectors trapped in varied proportions while the untreated control had least aphid trapped.   Thee aphid

populations decreased in all the treatments to a level where there was no difference in effect on the aphids between the vector management options (Fig. 4.2).

Table 4.1: Effect of different vector management options on number of aphids on passion fruit orchard in field experiment carried out at Rubona, Rwanda in 2017-2018.

Treatments	**Season1 Aphid numbers**	**Season 2 Aphid numbers**	**Seasonal Mean Aphid numbers**
Un-treated (control)	4.12a	1.40a	2.76a
Sticky trap (yellow)	16.06b	1.94a	9.00b
Inorganic fertilizer	4.27a	1.62a	2.95a
Sticky trap + inorganic fertilizer	12.73b	1.57a	7.15b
Profenofos (40%) +Cypemethirin	3.31a	1.68a	2.50a
Sticky trap +fertilizer Profenofos+ Cypemethirin	11.12b	1.40a	6.26a

Means with different letters are significantly different (P<0.05).

At the beginning of monitoring, the aphid vectors populations were high but as time passed on the populations decreased to a level within a mouth where all the vector management options did not differ in their effect on the aphids. The sticky traps trapped the most aphids but this decreased with time. Similarly, the rest of the vector management options had the vectors trapped in varied proportions while the untreated control had least aphid trapped. Thee aphid populations decreased in all the treatments to a level where there was no difference in effect on the aphids between the vector management options (Fig. 4.2).

4.2.2 Correlation of aphid population with weather parameters

The correlation of aphid population with weather parameters (Rain fall, maximum, minimum, temperature and average temperature) are presented in Table 4.2. There was a significant (p=0.05) negative correlation between aphid population, and rain fall. There was no significant correlation of aphid populations with maximum and minimum temperatures.

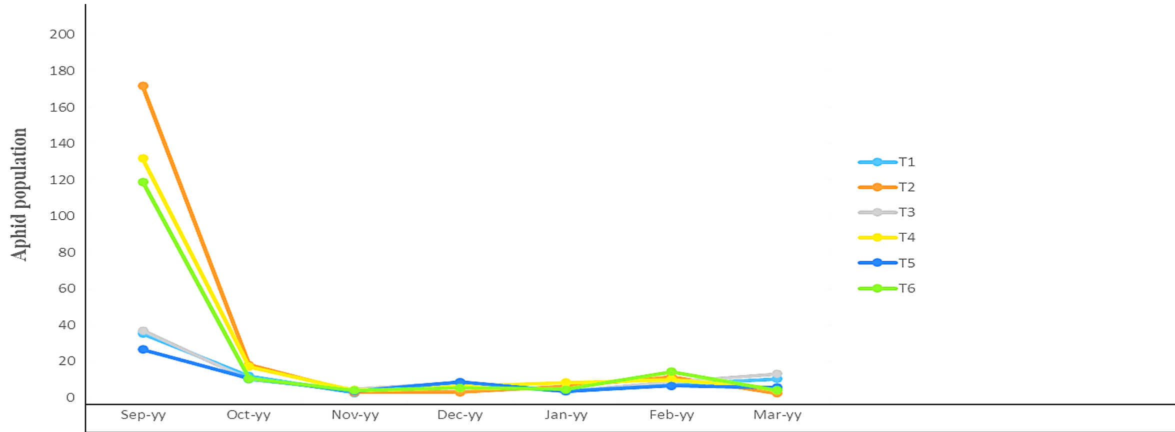

Figure 4.2: Average monthly aphid populations at Rubona, southern province Rwanda during field experiments from Sept 2017 to March 2018. T1: Untreated control, T2: Yellow sticky trap, **T3**: Inorganic fertilizer, **T4**: Sticky trap + inorganic fertilizer, **T5**: Profenofos (40%) +Cypemethirin and **T6**: Sticky trap +fertilizer+ Profenofos / Cypemethirin.
Add the X and Y axis lines

Table 4.2: Correlation between aphids' population, rainfall and temperature on passion fruit orchard at Rubona Research Station, Rwanda in 2017(season1) and 2018 (Season 2).

	Aphids number	**Precipitation**	**Max temp.**	**Min temp.**	**Ave. temp.**
Aphids number	1				
Precipitation	-0.563**	1			
Max temperature	0.508	-0.449	1		
Min temperature	0.226	-0.136	0.591	1	
average temperature	0.454	-0.376	0.95	0.814	1

*Significance level '**' 0.05*

4.2.3 Effect of management options on disease incidence and severity

The vector management strategies had variable effects on passion fruit disease incidence and severity (Table 4.3). Disease incidence was highest in the sticky traps + fertilizer treatment and

lowest in the combination treatment of sticky traps + fertilizer + insecticides. However, its effect was not different from that of the sticky trap treatment by itself (Table 4. 3). Disease incidence in the second season was higher than in the first season (Table 4.3). Overall, passion fruit woodiness virus severity was low in the two cropping seasons and ranged from 1.44-3.01%. Vector management options significantly ($p < 0.05$) affected the severity of the woodiness disease on passion fruit. However, the vector management options effect was variable in the two seasons. However, the sticky trap alone consistently recorded the least aphid populations in the two seasons (Table 4.

Table 4.3: Effect of vector management strategies on the incidence and disease severity of passion fruit woodiness virus on passion fruit orchard at Rubona Research Station, Rwanda in 2017(season1) and 2018 (Season 2).

Treatments	Season 1[b] Disease incidence (%)	Season 2[b] Disease incidence (%)	Season 1[c] Disease severity (%)	Season 2[c] Disease Severity (%)
Un-treated (control)	11ab	32d	1.65a	3.01a
Sticky trap (yellow)	9a	10a	1.56c	1.63c
Inorganic fertilizer	15b	25c	1.90a	2.57a
Sticky trap + inorg. fertilizer	23c	28cd	2.42b	2.72b
Profenofos (40%) + Cypemethirin	10ab	20c	1.60a	2.22a
Sticky trap +fertilize + Profenofos / Cypemethirin	7a	34d	1.44b	3.04b

1. [a]Cultural and insecticide management treatments applied for monitoring of aphid populations in a passion fruit orchard.
2. [b]Cropping season 1 (Long rains) refer to December 2017 to March 2018 and cropping season 2 (short rains) refer to September to November 2017. Numbers with different letters among treatments are significantly different ($P<0.05$).
3. [c]Average aphid population density for two growing seasons. Numbers with different letters among treatments are significantly different ($P<0.05$).

There was a negative correlation between the number of aphids trapped on the sticky trap with disease incidence and with disease severity. Disease severity has a positive correlation with disease incidence (Table 4.4).

Table 4.4: Correlation between number of aphid caught, disease severity, and disease incidence in field experiment conducted at Rubona, Rwanda 2017-2018

Data collected	Number of Aphids caught	Disease severity	Disease Incidence
Number of Aphids	1.000		
Disease severity	-0.263*	1.000	
Disease incidence	- 0.261*	0.998***	1.000

*Significance level '***' 0.01 '*' 0.05*

4.2.4 Effect of management options on passion fruit yield

The vector management options significantly (P < 0.05) increased the yield of passion fruit compered to untreated control. Pesticide spray alone had the highest fruit and marketable yield but the yield was not significantly (P < 0.201) different from that recorded in yellow sticky trap treatment alone and that from the sticky trap + inorganic fertilizer. Untreated control had the least fruit and marketable yield (Table 4.5). The non – marketable fruit yield was not different (P < 0.201) among the vector management options. However, the non-marketable proportion was higher were the high were recorded**.**

Table 4.5: Marketable and Non – Marketable yield (Kg /ha) of passion fruit harvested from plants subjected to various aphid vector management strategies at Rubona Research Station, Rwanda.

Treatments	**Mean yield/Treatment (Kg/Ha)**	**Non – Marketable yield (Kg/ Ha)**
Profenofos (40%) +Cypemethirin	2992 a	500 a
Sticky trap (yellow)	2756 a	650 a
Sticky trap + inorganic fertilizer	2411ab	642 a
Inorganic fertilizer	2028 bc	567 a
Sticky trap +fertilizer+ Profenofos + Cypemethirin	2012 bc	309 a
Un-treated (control)	1369 c	175 a

Means with different letters are significantly different (P<0.05).

4.3 Discussion

High populations of aphids were recorded during the short rain period from September to October 2017 when the temperatures were relatively high. The high temperate ranging between 22^0C to 26^0C could have favored the growth of aphids whereas low temperatures ranging between 14^0C to 16^0C and heavy rain fall could have been unfavorable to aphid growth (Bulletin 2013and Fanigliulo *et al.,* 2009). According to Carvalho *et al*. (2002) temperatures within the 22–24 °C range and low rainfall are favorable for most aphid species in passion fruit. The same trend was observed by Schröder and Krüger (2014) on maize field in South Africa and Fanigliulo *et al.* (2009) on tomato field in Southern Italy. It has also been reported that the absence of weeds during dry season may have a role in diffusion of aphids while presence of weeds at the beginning reduces the number of aphid's (Munyua *et al.*, 2007 and Dugravot *et al*., 2007). According to Krishi *et al*. (2012), Schuber *et al*. (2007) and Hedge *et al.* (2004) weather might be a factor that limits the growth of aphids in passion fruit. The same authors

observed that during heavy rain fall the population of aphids decreased, which is in agreement with the results obtained in this study. Fajinmi *et al*. (2011) reported that the environment might be another abiotic factor affecting the dynamics of aphid population and that the decrease of aphid population may not only be due to heavy rain fall but it could also happen because of other conditions such as, high temperature, nutritional stress and overcrowding. Another author Kelm *et al*. (2002) reported that chemical composition of plant tissue may indirectly influence aphid development. According to Fajinmi *et al*. (2011), Fishpool *et al.* (1995), Dangelet *et al*. (1981) and Glding *et al.* (1936) high temperature and long rain season may reduce aphid populations. In eastern Uganda, Legg *et al*. (1994) reported that there were high number of aphid population observed during high temperature. High aphid populations were observed in the passion fruit crops at vegetative stage. This is in agreement with results of Legonbon *et al*. (2002) who reported that the vegetative stage of the crops such as potatoes play an important role in the dynamics of aphid populations. According to Guan *et al.* (2017) and Assiri *et al.* (2017) this may also happen due to the prevailing growing conditions, variety of crop, age of crop, level of soil fertility and altitude.

The high population density of aphid vectors recorded in treatments with yellow sticky traps alone or in combination with other techniques (pesticide application and inorganic fertilizer application), indicates that yellow sticky traps are an effective strategy for monitoring and management of aphid vectors in passion fruit orchard in the tropical highlands of Rwanda (Table 4.2). The management of insect vectors of plant viruses by use of yellow sticky traps has been previously reported in passion fruit crop (Garcez *et al*. 2015). The assessment of aphid population dynamics has previously been done using yellow sticky traps and insecticides

(Ochwo-Ssemakula *et al*., 2011) consistent with the current research. This study findings are also consistent with previous research reports in which yellow sticky traps were reported to be significant in monitoring aphid populations in horticultural crops such as passion fruit and potatoes in Uganda (Ochwo-ssemakula *et al*., 2012) and in Kenya (Mayer, 2016; Kilalo *et al.* 2013). The high population density of aphids recorded in sticky traps suggests that the yellow sticky traps can be used to reduce aphid vector movement resulting in low disease spread. The low population density of aphids recorded in treatments where insecticides were applied suggests that pesticides can be utilized effectively to control aphid populations in passion fruit orchard. However, the low aphid population levels in treatments with inorganic fertilizer and untreated plots is contrary to our expectations of recording high aphid populations since the plants were not protected. Therefore, the use of inorganic fertilizer may not impact passion fruit vector populations.

The differences in disease incidence and severity between the two cropping seasons may be attributed partially to aphid population dynamics in Seasons 1 and 2. In the short rainy season (Season 1 – Sept to Nov 2017), the aphid population was high, but disease incidence and severity were low. This could probably be explained by the lag time between aphid infestation, virus acquisition and transmission onto the passion fruit plants, multiplication and the spread of the virus within plant tissues prior to symptom expression. The correlation coefficient analysis of the data showed negative correlation between the number of aphids on the traps with disease incidence and severity values (Table 4. 4). Although the high aphid populations were observed in the passion fruit crops at vegetative stage, the sticky traps effectively attracted the aphids away from the plants and could have contributed to the low disease transmission and incidence on

passion fruit plants, hence low symptom expressions. It could also be explained that the prevailing growing conditions in the geographical region (ambient temperature, rainfall, and humidity), crop cultivars, plant age, level of soil fertility and altitude could influence vector populations and transmission efficiency as well as symptom expression (Assiri *et al*., 2017).

A combination of two insecticides consisting of Profenofos 40% + Cypemethirin 4% were used as foliar spray fortnightly. This treatment resulted in the least number of aphids in both seasons. This observation may be due to the effectiveness of the insecticides in reducing aphid populations in the experimental plots. Profenofos is an acetylcholine inhibitor insecticide which is non-systemic with contact mode of action as well as stomach activity against many insect vectors. In addition, this insecticide has homicidal effects on insect eggs. In a previous study on chrysanthemum aphid and other aphid species, application of profenofos resulted in 100% mortality of the aphids on plants (Reddy and Latha, 2012). Similarly, cypermethrin is a sodium channel modulator with contact activity for morbidity of insects and has long duration of activity. Other researchers have documented insecticide applications as crucial in the control of whitefly and aphid vectors in different agro-ecosystems (Roy *et al.,* 2017). Although insecticides have beneficial impacts on vector control, they have problems associated with development of resistance, effects on non-target species, development of secondary pests and elimination of natural enemies in some agro-ecosystems (Perring *et al*. 1999).

The high fruit yield recorded in treatments where insecticides and sticky traps were used indicate that the combinations of insecticides and sticky trap treatments could have contributed to the low disease incidence and severity as vector populations were reduced. Therefore, this

effect could have resulted in positive attributes to the fruit quality, and hence high marketable yield according to (Elbakidze *et al*. 2011) reported that application of chemical fertilizers and pesticides in agricultural systems has increased in the past decades as a means for improving crop yields.

CHAPTER FIVE

General Discussion, Conclusions and Recommendations

5.0 Discussion

Passion fruit is an important crop for small scale farmers for providing high income and for food in the world. Different researchers have reported that its production and quality have been reduced by woodiness disease (Graces et al., 2015; Romário et al., 2015; Ochwo-ssemakula et al., 2012a). In Rwanda this disease was first reported by (USAID, 2002) in northern part of the country. Today the disease is distributed in all regions where passion fruit is grown. The study was conducted to compare the sensitivity and reliability of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings and to evaluate the effective vector management options that would enhance passion fruit productivity. The study was conducted to determine relative sensitivity and effectiveness of ELISA and RT-PCR diagnostic methods for screening viral infections in passion fruit seedlings and to determine the potential of combining sticky traps and orchard fertilization in managing viruses associated with passion fruit woodiness disease in Rwanda.

On determining relative sensitivity and effectiveness of two diagnostic methods, RT-PCR was a more sensitive method for detecting CABMV at very low concentrations as exemplified by 10^6 serial dilutions. ELISA was capable of detecting CABMV in infected leaf material but at 10^3 serial dilution implying that there is a likelihood of returning false negatives. This findings suggest that RT-PCR is a more sensitive method and can be used for screening woodiness viral diseases of passion fruit in the nurseries within research institution and private companies. This is because it showed a high capacity of detecting 65% of asymptomatic passion fruit leaf sample

while ELISA detected 22%. These findings agree with those previously reported by Kumar, (2010), Kashif, (2009) and Fischer and Rezende 2008). RT-PCR is more sensitive and can be used in a certification program while ELISA can be used for detection of CABMV from farmer's field for purposes of disease management (Damiri, 2013; Dang *et al*., 2009; Berniak *et al*., 2009; Ahangaran *et al*., 2009; Usta and Murat, 2005).

On determining vector management options of the aphid vectors, yellow sticky traps, sticky traps plus insecticides (profenofos + cypemethrin) and sticky traps plus fertilizer were the most promising measures for capturing aphid vectors. It has been reported previously by Lu *et al.* (2012and Gerling and Horowitz, (1984) that yellow sticky traps were significant for monitoring aphid populations in different crops. Overall, passion fruit woodiness virus severity was low in the two cropping seasons and ranged from 1.44-3.01%. The differences in disease incidence and severity between the two seasons may be due to high number of aphid population present at the beginning of the experiment which continued reducing as the vector management options were in place for six mouth. There was a negative correlation between the number of aphids on the traps with disease incidence and severity values. These might be due to the sticky traps effectively attracted the aphids away from the plants and could have contributed to the low disease transmission on passion fruit plants, and hence low symptom expressions. It could also be because over the prevailing growing conditions in the geographical region (ambient temperature, rainfall, humidity), crop cultivars, plant age, level of soil fertility and altitude could influence vector populations and transmission efficiency as well as symptom expression (Guan *et al.* 2017). The effect of insecticide and yellow sticky trap were significant for increasing passion fruit yield. A phenomena described by Elbakidze *et al*. (2011) and

Fanigliulo et al. (2009),who reported that pesticide spray may reduce the disease incidence and increase yield in tomatoes. The insecticide spray and yellow sticky trap had produced high marketable fruit yields (Kg/Ha). This implies that, farmers may either use yellow sticky traps or insecticide spray as vector management options because they were found to capture more aphids and also produced high marketable fruit yield. Research findings have indicated that while pesticide applications and yellow sticky trap may be vital for insect control, in situations where plant viruses are transmitted in a non-persistent and non-circulatory manner, vector control may not impact disease levels and yield (Fischer and Rezende, 2008).

5.1 Conclusions

This study has demonstrated that RT- PCR and ELISA are capable of detecting CABMV while screening seedlings of passion fruit at different levels of dilution. However, RT-PCR diagnostic method is more sensitive for CABMV detection and has the potential for use in a certification program. ELISA diagnostic method is simple, cheap and requires fewer resources for detection of viruses while running many samples within a short period compared to RT-PCR.

The study also revealed that the use of yellow sticky trap or a combination of yellow sticky trap with inorganic fertilizer and pesticide as vector management options have the potential to avoid the risk of early virus infection. These options can be used as an alternative or in combination pesticide spray by the farmers. These vector management options have the potential to improve farmer yields with minimum intervention with pesticides.

5.2 Recommendations

1. The study supports the use of RT- PCR in certification program for screening woodiness disease viruses in nurseries of planting materials, and use of ELISA for detection of CABMV from farmer's field. RT-PCR diagnostic method is recommended for screening woodiness disease in passion fruit nurseries in research institution and private companies,
2. From this study yellow sticky trap alone or a combination of yellow sticky and inorganic fertilizer has a potential of managing aphid vectors, therefore the options can be recommended for farmer use.
3. More research is needed to identify the aphid species transmitting passion fruit viral disease in Rwanda,
4. Further studies are needed to determine the distribution of passion fruit viral diseases in major growing regions of Rwanda,
5. Further studies are needed to identify the diversity of strains, if any, of CABMV present in Rwanda because they have a bearing in the sensitivity of the choice of diagnostic method used.
6. More studies are needed to confirm how CABMV multiplies within the plant over time for detection to take place.

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APPENDICES

Appendix I: DAS-ELISA Protocol for detection of CABMV

Items required

- ELISA Plate, Plate Reade, Grinding bags, incubation box, water bath, paper towel, 75% ethanol and mortar and pestle

- Buffered used in ELISA are,

- Coating buffer, General Extraction buffer, PBS (pH 7.4) buffered saline, PBS- Tween (PBST), Conjugate buffer and Substrate buffer.

Procedures

- Dilute specific antibody in coating buffer (recommended dilution see delivery note and tube); i.e. 20µl in 20 ml buffer at a recommended dilution of 1:1000 or 40µl in 20 ml buffer at a recommended dilution of 1:500. Add 100µl to each well of the microliter plate.
- Cover the plates and incubate at 37 °C for 2- 4 h.
- Wash plate with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.
- Extract samples 1:20 (w/v) in extraction buffer. Add 200 µl aliquots of the test sample to duplicate wells.
- Cover the plates and incubate overnight at 4 °C.
- Wash three times as in step 3.
- Add 200 µl enzymes conjugate, recommended dilution is given in the delivery note, in

conjugate buffer.

- Cover the plates and incubate at 37 °C for 2- 4 hours.
- Wash three times as in step 3.
- Add 100 µl aliquots of freshly prepared substrate (1 mg /ml para- nitro phenyl-phosphate in Substrate buffer) to each well.
- Cover the plate and incubate at 37°C for 30-60 min, or as long as necessary to obtain clear reactions.

Appendix II: RNA Extraction Protocol

Items required

3 Eppendorf tube, Zymo Colum tube, 95% ethanol, Trizol reagent (Invitrogen), Grinding bags, mortar and pestle, Ice, RNA Pre- wash buffer, RNA wash buffer, RNA / DNA free water, fume hood machine, Liquid nitrogen, powder free gloves, Aerosol- barrier tips, micropipettes, refrigerated centrifuge, centrifuge tube and vortex mixer.

Procedures

4 Take 0.05g of leaf Field sample.

5 Add 600ul of Trizol reagent grind until solved.

6 Remove 500ul of juice sample into Eppendorf tube.

7 Centrifuge for 1 min at 12000 RTM.

8 Remove 500ul of juice sample without removing residues.

9 Add 500ul of ethanol to 500ul of juice sample.

10 Mix well by voltex.

11 Put it in zymo Colum in zym collection tube.

12 Centrifuge for 1 min in 12000 RTM.

13 Add 400ul of Pre- wash buffer and centrifuge for 1 min Discard the flow – through.

14 Repeat step number 10.

15 Add 700ul of RNA wash buffer to Colum and centrifuge for1 min. Discard the flow - through to ensure complete removal of the wash buffer.

16 Centrifuge the Colum for an addition 2 min I an amplified collection tube.

17 Transfer the Colum carefully into an RNase free tube.

18 Add 50ul of RNase- free water directly to the Colum matrix and centrifuge for 1 min.

19 Then the eluted RNA can be used immediately or stored at -20°C.

Appendix III: RT- PCR Protocol for detection of CABMV

Component	µl per reactions
H2O	12.865
5X GoTaq Buffer	5
0.1 M DTT	1.25
Forward Primer (10 µM)	2
Reverse Primer (10 µM)	2
10 mM dNTP	0.5
RNAse OUT (40 U/µl)	0.1
SuperscriptII Rtase (200 U/µl)	0.035
GoTaq DNA Polymerase (5 U/µl)	0.25
Total	24

Note: Take 24ul of master mix +1ul of pure RNA.

RT- PCR Conditions

94^0 C for 5 min,

94^0 C for 30 Sec

58^0 C for 1 min

72^0 C for 45 min, 72^0 C for 10 min and Holding at 4^0 C

Run Gel preparation

0.8 g of Agarose

8 ml of 5X TEB buffer, 72 ml of sterile distilled water and the PCR products on 1.24g of agarose in 100ml of 0.5X TBE buffer for 45 min at 12ov.

Appendix IV: Sample details for inoculated plants

ELISA and RT PCR results for inoculated samples.

SAMPLE CODE	SAMPLE VARIETY	Symptoms	ELISA Results		RT- PCR Results
			Potyvirus	CABMV	CABMV
1	Purple	Symptomatic	+	-	+
2	Purple	Symptomatic	+	-	-
3	Purple	Asymptomatic	+	-	+
4	Purple	Asymptomatic	+	-	-
5	Purple	Symptomatic	+	-	+
6	Purple	Asymptomatic	+	-	+
7	Purple	Symptomatic	+	-	-
8	Purple	Symptomatic	+	-	+
9	Purple	Symptomatic	-	+	+
10	Purple	Asymptomatic	-	+	+
11	Purple	Symptomatic	+	+	+
12	Purple	Asymptomatic	+	-	-
13	Purple	Symptomatic	-	+	+
14	Purple	Symptomatic	-	+	+
15	Purple	Symptomatic	-	+	+
16	Purple	Symptomatic	+	+	+
17	Purple	Symptomatic	-	+	+
18	Purple	Symptomatic	-	+	+
19	Purple	Symptomatic	+	+	+
20	Purple	Symptomatic	+	+	+
21	Purple	Symptomatic	-	+	+
22	Purple	Asymptomatic	-	-	+
23	Purple	Symptomatic	-	+	+
24	Purple	Symptomatic	-	+	+
25	Purple	Symptomatic	-	+	+
26	Purple	Asymptomatic	-	+	-
27	Purple	Asymptomatic	-	+	-
28	Purple	Asymptomatic	-	-	-
29	Purple	Symptomatic	-	+	+
30	Purple	Asymptomatic	-	+	-
31	Purple	Asymptomatic	-	-	+
32	Purple	Asymptomatic	-	+	-
33	Purple	Asymptomatic	-	+	-

34	Purple	Asymptomatic	-	+	-
35	Purple	Symptomatic	-	+	+
36	Purple	Asymptomatic	-	+	-
37	Purple	Asymptomatic	-	-	+
38	Purple	Symptomatic	-	+	+
39	Purple	Asymptomatic	-	-	+
40	Purple	Asymptomatic	-	-	-
41	Purple	Asymptomatic	-	+	-
42	Purple	Symptomatic	-	+	+
43	Purple	Asymptomatic	-	+	-
44	Purple	Symptomatic	-	+	+
45	Purple	Asymptomatic	-	-	-
46	Purple	Asymptomatic	-	-	-
	Purple	Asymptomatic	-	-	-
48	Purple	Asymptomatic	-	-	-
49	Purple	Asymptomatic	-	-	+
50	Purple	Asymptomatic	-	-	+

ELISA and RT PCR results for 50 inoculated samples