TRANSMISSION AND DIVERSITY OF PINEAPPLE MEALYBUG WILT DISEASE VIRUSES IN CENTRAL UGANDA

BY

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DECLARATION

I, Mawa Everrest Michael do hereby declare that this is my original work and has never been submitted to any University or institution of higher learning for academic award.

Signed ……

Date……

APPROVAL

This is to certify that this work was carried out under our supervision as University supervisors and it is now ready for submission for examination.

Signed…….

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Date ……

Signed ……

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Date……

DEDICATION

This work is dedicated to my beloved academic mentor Associate Professor Bosco Bua for be ing a constant source of knowledge and inspiration who gave me the opportunity to succeed; my parents Mr. Onziga Lazaro and late Jane Kide for their tireless prayers and unconditional s upport; Aleziyo Roselyne and children Sande, Mande, Venesha, Pearl and Emmanuel.

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ACRONYMS

AAP	Access acquisition period
BP	Base pair
cDNA	Complementary Deoxyribonucleic acid.
CP	Coat protein
CPd	Coat protein duplicate
CRD	Completely randomised design
CsCl	Caesium chloride
CTAB	Cetyltrimethyl ammonium bromide
EDTA.	Ethylenediaminetetraacetic acid
IAP	Inoculation access period
kDa	Kilo Dalton
MAb	Monoclonal antibodies
MTPD	Microtitre plate detection
NAADS	National agricultural advisory services
NaCRRI	National crop resources research institute
NARO	National agricultural research organization
NOGAMU	National organic agriculture movement Uganda
PMWaV	Pineapple mealybug wilt associated virus
PMWD	Pineapple mealybug wilt disease
RT-PCR	Reverse transcriptase polymerase chain reaction
TAE	Tris-acetate EDTA
WB	Washing buffer

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ABSTRACT

Pineapple mealybug wilt disease (PMWD) is one of the latest breakouts of diseases attacking pineapple in Uganda. Although, first reported around 2009, the etiology and epidemiology is not fully elucidated. Pineapple mealybug wilt disease was reportedly devastating in the districts of Mukono and Kayunga with incidence ranging from 15 to 100%, respectively. The causal viruses of PMWD belong to the genus *Ampelovirus*. However, information on the transmission and diversity of the causal viruses of pineapple mealybug wilt disease in Uganda is limited and scanty. Therefore, this study was undertaken to (1) identify and characterise the diversity of viruses associated with PMWD, (2) determine mealybug action threshold for transmission of PMWD and (3) identify sources of resistance to PMWD. Forty four (44) symptomatic pineapple plant leaves were collected from the districts of Masaka, Luwero, Kayunga and Mukono in central Uganda for laboratory analysis. Molecular analysis using polymerase chain reaction (PCR) was done to identify and assess the diversity of pineapple mealybug wilt disease causal viruses. Total plant RNA was extracted using Cetyltrimethyl ammonium bromide (CTAB), the heat-shock protein 70 homolog (Hsp 70h) gene of causal virus was amplified by RT-PCR using specific primer pair 225/226 for PMWaV-1 and 223/224 for PMWaV-2, respectively. Polymerase chain reaction products were separated by electrophoresis and the resulting amplicons were sequenced using Sanger method. The action threshold for transmission of PMWD was assessed using pineapple variety Smooth cayenne inoculated with five levels of viruliferous mealybugs 0, 1, 5, 10 and 15, respectively. The experiment was arranged in a completely randomised design (CRD) with eight replications. Screening for resistance to PMWD was done on five pineapple varieties (Smooth cayenne, Red Spanish, MD2, *Sassilimu* and Victoria) inoculated with 15 viruliferous mealybugs. Data was subjected to AMOVA and ANOVA for genetic diversity and action threshold and resistance, respectively. Results showed that only PMWaV-1 was associated with pineapple mealybug wilt disease in

central Uganda. Accordingly, high PMWD transmission was recorded in 15 mealybugs. There was significant difference ($P<0.05$) in resistance among the pineapple varieties. Red Spanish was highly resistant while *Sassilimu,* MD2 and Smooth cayenne was moderately resistance and Victoria highly susceptible. The results of this study have therefore shown that only PMWaV-1 was associated with PMWD in central Uganda. The mealybug action threshold for the transmission of PMWD is 15. The study also revealed that the sources of resistance to PMWD are variable although Red Spanish was highly resistant. The implications of these findings therefore is that an effective management strategy against PMWD can be instituted based on the identified and characterized causal virus and action threshold of mealybug as well as the sources of resistance identified. However, there is need for additional studies to unravel genetic diversity using more samples as well as the molecular basis for resistance of pineapple varieties to PMWD.

CHAPTER ONE: INTRODUCTION

1.1 Background to the study

Pineapple (*Ananas comosus* L. Merrill) is an important horticultural crop across the globe (Basek e, 2009; Shen and Zheng, 2009; Hernández *et al*., 2010; Bua *et al*., 2013), Pineapples importance is reflected in its ranking among the commercial fruits. Globally, pineapple is the second most im portant tropical fruit after banana (Amar *et al.,* 2015), Aslam *et al* (2019) ranked pineapple amon g the top three important tropical fruits alongside other scholars. According to Coveca (2002), pin eapple production contributed to over 20% in volume of world's tropical fruits. The leading glob al pineapple producers are as shown in (Table 1). According to FAOSTAT (2017), Pineapple is a n increasingly important fruit crop in Uganda being ranked 62nd among the global producer and in the region. In Uganda, the major pineapple growing areas is the central region, areas south of La ke Kyoga specifically the districts of Kayunga, Luwero, Iganga, Kamuli and Masaka in Uganda (Bua *et al.,* 2013).

Table 1: Global leading pineapple producing countries

Countries	**Production (MT)**
Costa Rica	3,056.45
Philippine	2,671.71
Brazil	2,253.90
Thailand	2,123.18
India	1,861.00
Indonesia	7195.99
Nigeria	1642.38
China	1,576.42
Colombia	1,091.04
Mexico	945.21

Source: FAOSTAT, 2017

1.2 Importance of pineapple

Pineapple is a tropical fruit with exceptional juice, flavor and immense health benefits. Pineapple fruit can be eaten as fresh salads but also processed as juice, jam, dried fruits, preserves, cakes, and puddings; or used in sauces (Debnath *et al.,* 2012). Mature pineapple fruit has high sugar content of 14% (brix) which is very important for the normal functioning of the body and brain energy requirement (Joy, 2010; Kamol *et al.,* 2014). Kwikiriza *et al.* (2016) reported that pineapple production in Uganda provides opportunity for the women to earn cash to improve their economic and living conditions. According to Namuwoza and Tushemerirwe (2011), Uganda organic pineapple makes up 75% of the total fruit exports of the country. In fact, about 100 tonnes of pineapple is exported annually from Uganda fetching about 200,000 euros (Amin, 2006). Pineapple also acts as a cover crop that protects the soil against soil erosion and the crop residues latter add manure to the soil control soil microbes (Wang *et al.,* 2003).

According to Hemalath (2013), pineapple fruit supply 16.2% of the daily body requirement of water soluble vitamin C for growth and repair of tissues or body parts. Pineapple produces collagen, some neurotransmitter, cartilage, tendon, ligament, blood vessels, the antioxidant activity has health benefits to reduce cancer and defend the body against free radicals that attack and damage normal body cells and DNA (Joy, 2010). Pineapple is rich in ascorbic acid that protects the body against bacterial and viral infections (Uckiah, 2009; Debnath *et al.,* 2012).The proteolytic enzyme, bromelain, found in the pineapple fruit and stem is useful in the reduction of excessive coagulation of blood, severe inflammation and reduce tumor growth (Jessie, 2018). Pineapple enhances digestion and is also used as for meat tenderizing or marinating (Omotoyinbo *et al.,* 2017). Furthermore, bromelain's medicinal uses include relief for arthritis sufferers, as a digestive aid, in the reduction of blood clotting, as an anti-inflammatory agent, and for skin debridement of

burns. Bromelain also has industrial uses including clarification of beer, production of vegetable oils and the dehydration of eggs and soya milk (Joy, 2010).

1.3 Constraints to pineapple production

Pineapple production in Uganda is constrained by a number of factors including socioeconomic factors such as access to credit, markets, cost of inputs and price of products (Akhilome *et al.,* 2015; Ankwasa, 2018); extension services, environmental or abiotic and biotic factors such as pineapple mealybug wilt disease (PMWD), pineapple heart rot disease (PHRD) and insect pests among others (NARO, 2012; Bua *et al*., 2013; Ocwa *et al.,* 2016). The abiotic factors especially drought, temperature, soil fertility, soil texture, humidity and reduced irrigation directly influence pineapple yield (Sether, 2001; Bartholomew *et al.,* 2003; Kwikiriza *et al*., 2016). For instance, in Ghana, studies on the impacts of climate variability indicated that 82% of the pineapple yield variation was due to rainfall and temperature effects on pineapple production. In fact, pineapple is sensitive to temperature variation and the detrimental temperature conditions for pineapple are temperature below 15.6°C (60°F) and temperature above 32.2°C (90°F) (Portia *et al.,* 2017). The rainfall requirements for effective growth of pineapple range between 1000 mm to 1500 mm well distributed in the year. The biotic factors affecting the pineapple production are insect pests, weeds and diseases. Some of the insect pests attacking pineapple include termites, thrips, and the caretaker ant's most common species of *Pheidole* and *Solenopsis* (Jahn *et al.,* 2003); the mealybug which transmits the pineapple wilt disease viruses in association with the tending ants is the most serious biotic factor. According to Kishore *et al*. (2018), the most serious disease affecting pineapple is pineapple mealybug wilt disease.

Pineapple mealybug wilt disease is a widespread and devastating disease in many pineapple growing areas of the world (Rohrbach *et al*., 1988; Wakman *et al.,* 1995; Gary *et al*., 2003; Borroto, 2010). Pineapple mealybug wilt disease (PMWD) was reported in Uganda in around 2009. According to Bua *et al.* (2013), PMWD is a devastating disease in central Uganda especially around the Lake Victoria crescent districts of Masaka, Luwero, and survey conducted in Kayunga and Mukono districts in 2011, 78% of the respondents reported the major constraint of pineapple production as disease.

1.4 Statement of the problem

Pineapple mealybug wilt disease is the recent breakouts of diseases attacking pineapple in many areas of the world (Dey *et al*., 2018). The virus causing the disease in Uganda has not been identified and hence the characteristics of the viruses remain unknown. There is limited or scanty information available on the identity and diversity of pineapple mealybug wilt associated viruses (PMWaVs) causing wilt in Uganda. The mealybug action threshold for transmission of PMWD and sources of resistance to PMWD are not available in Uganda. Therefore, as a starting point to developing effective disease control measure against PMWD, there is urgent need to identify and understand the characteristics of the causal viruses, assessing the mealybug action threshold for transmission and identifying sources of resistance to PMWD. It is against this background that this study was undertaken.

1.5 Justification of the study

The continued spread of pineapple mealybug wilt disease in many parts of the country is a serious threat to livelihoods, food security and pineapple diversity. Additionally, information on the identity of the virus causing pineapple mealybug wilt disease is scanty and limited. Moreover, the

causal viruses strain and the threshold population of mealybugs, the vector for PMWD transmission is not known in Uganda. According to Agrios (2005), the identity of disease causal organisms, their biology, means of spread and host range is imperative to the development of appropriate control measure against any plant disease. The knowledge gap exists in the identity and diversity of the causal viruses of PMWD, mealybug action threshold for the transmission of PMWD and sources resistance to the pineapple mealybug wilt disease. Cognisance of this fact, this study is therefore justified as it seeks to identify and characterise the diversity of the pineapple mealybug wilt disease associated virus in Uganda, establish the mealybug action threshold for wilt disease transmission and identify sources of resistance to PMWD in Uganda. For any accurate assessment and improvement of pineapple crop yield and production the pineapple wilt disease management is imperative, identity and characteristics of the pathogen, the action threshold and sources resistant to the wilt disease should be done.

1.6 Objectives of the study

1.6.1 General objective

To assess the transmission and diversity of pineapple mealybug wilt disease causal viruses to dev elop an effective PMWD management strategy in Uganda.

1.6.2 Specific objectives

1. To identify and characterize viruses causing PMWD in Uganda.
2. To determine the mealybug action threshold for transmission of PMWD in Uganda.
3. To identify sources resistance to PMWD among selected pineapple varieties in Uganda.

1.6.3 Hypothesis

1. Identity and characteristics of the viruses causing PMWD is not known in Uganda.
2. Mealybug action threshold for the transmission of PMWD is not known in Uganda.
3. Sources of resistance to PMWD are not available in Uganda.

1.7 Scope of the study

The study was conducted in Kyambogo University and National Crop Resources Research Institute (NaCRRI) during the period March 2017 and May 2018.

The study focused mainly on the identification and characterization of the viruses causing PMWD in Uganda, mealybug action threshold for the transmission of PMWD and identification of the sources of resistance of pineapple varieties to PMWD in Uganda.

CHAPTER TWO: LITERATURE REVIEW

2.1Etiology and epidemiology of PMWD

Etiology of the PMWD still remains complex and not well resolved. In fact, different researchers expressed and suggested that PMWD has for many years been studied but not properly understood (Gunasinghe and German, 1989; Sether *et al*., 1998). Dey *et al.* (2018) observed that the disease has complex etiology involving association of the virus particles, mealybug vector and their attendant ants. In fact, serological and molecular analysis revealed the presence of at least three distinct pineapple mealybug wilt-associated viruses (PMWaVs) namely, PMWaV-1, PMWaV-2 and PMWaV-3 causing pineapple mealybug wilt disease (Sether *et al.*, 2001,2005; Subere *et al*., 2011). According to Sether (2002), PMWaVs belong to the genus *Ampelovirus* and the family *Closteroviridae*. The PMWaVs are acquired and transmitted by the pink mealybugs *Dysmicoccus brevipes* (Cockerell) and grey mealybug *Dysmicoccus neobrevipes* (Beardsley) (Sether, 1998, 2005). For instance, PMWaV-2 has an important role in the cause of the pineapple mealybug wilt disease. PMWaV-2 in the presence of mealybug feeding leads to significant yield loss (Sether, 2002b; Mau and Kessing, 2007). Meanwhile, the pineapple mealybug wilt associated virus-1 (PMWaV-1) was noted to cause significant yield reduction without the mealybug feeding hence the pineapple fail to develop the symptoms of the disease (Sether, 2002, 2002a, 2005). Also, mealybug feeding on PMWaV-free plants does not induce PMWD symptoms (Sether, 2002). This means that mealybugs are not implicated for transmitting PMWD in pineapple. Pineapple mealybug wilt associated virus two (PMWaV-2) and mealybug feeding led to 100% yield loss (Sether and Hu, 2002). In a survey conducted in Uganda 2013, 17% of the farmers mentioned mealybug wilt disease transmitted by mealybugs as one most important biotic factor affecting pineapple production (Kwikiriza *et al*., 2016). But the presence of PMWaV-2 and mealybug

feeding results in induction of PMWD symptoms (Sether *et al.,* 1998; Sether, 2002; Borroto, 2010) Despite, the numerous viruses reported to be involved in PMWD, pineapple mealybug wilt associated virus two (PMWaV-2) is the major virus associated with disease etiology (Borroto, 2010). In fact, the presence of PMWaV-2 in Hawaii pineapple plants has shown a 99 to 100% correlation with PMWD symptoms (Sether *et al*., 2001). Indeed, there is a strong association between PMWaV and PMWD (Sether and Hu, 1998, 2000), where grey mealybugs (*Dysmicoccus neobrevipes)* and pink mealybug (*Dysmicoccus brevipes*) were identified as vectors of the virus (Sether and Hu, 1998; Ullman *et al*., 1989; Bua *et al*., 2013). The viruliferous mealybugs are transferred from one pineapple plant to the other pineapple within the fields by the tending adult ants, the young nymphs and by wind (Jahn and Beardsley, 2000).

Several species of ants especially the big-headed ant (*Pheidole megacephala*) are associated with mealybugs (Carter, 1959; Petty and Tustin, 1993). In fact, it was reported that mealybugs are in symbiotic relationship with the ants (Philip, 1934; Su, 1979). The mealybug vector when in association with the caretaker ants (*Pheidole megacephala*) protects the mealybugs from the natural enemies and adverse weather condition by building earthen shelter around them, move the mealybugs to protected places, transport the mealybugs from one plant to another plant thereby facilitating mealybug dispersal, ants stimulate increased feeding by the mealybugs and ants remove the honeydew from the mealybugs hence preventing fungi from attacking the mealybugs. According to (Carter 1959), the ants assist in the establishment of mealybug colonies.

2.2 Symptoms of pineapple mealybug wilt disease

Pineapple mealybug wilt disease manifest as wilt of the central leaves due to loss of turgidity in t he leaves and inability of the roots to grow, collapse and rotting. Other symptoms of PMWD is r

eddening of the leaves and leaf margin, curling downward of the leaf tip and dieback (Hernandez *et al.,* 2010).

2.3 Transmission of PMWD

Pineapple mealybug wilt disease is transmitted from one source plant to the other as a result of the mealybug feeding on diseased plant and virus is transmitted to healthy plant. Pineapple mealybug wilt associated virus, the causal organism of PMWD is acquired and transmitted by two species of mealybugs namely the pink pineapple mealybugs (*Dysmicoccus brevipes* (Cockerell) and the grey pineapple mealybug (*Dysmicoccus neobrevipes (*Beardsley) *(*Sether, 1998, 2005). Accordingly, P MWD is only reported from areas of the world where members of the *Dysmicoccus* mealybug sp ecies complex occur and higher incidences of PMWD were associated with high mealybug popul ations (Hernandez *et al.,* 2010). In fact, a strong association was found between PMWaVs and P MWD where both grey and pink pineapple mealybugs were identified as vectors of the virus (Bo se *et al.,* 1990; Bua *et al.,* 2013). PMWaV-1 is correlated with growth reductions of plant crop (B ose *et al.,* 1990). However, PMWaV-2 infection and mealybug feeding are necessary for the deve lopment of PMWD (Carter, 1933b). Although, the yield effects of PMWD are variable, losses am ounting to 100% has been reported (Sether and Hu, 2002). Unless managed, the disease is very d estructive and devastating making commercial growing of pineapple impossible (Bartholomew *et al.,* 2003). However, PMWD only develops in plants infected with a *closterovirus,* designated pin eapple mealybug associated virus (PMWaV-1 & PMWaV-2) that are also exposed to mealybugs f eeding (Sether and Hu., 1999).

2.4 Characteristics of viruses causing PMWD

The *Ampelovirus* is a non-enveloped exceptionally long filamentous particle and flexuous in shape

(https://viralzone.expasy.org/285?outline=all_by_protein). The length is between 1400-2200nm, the virus is (10-13nm) in diameter (https://viralzone.expasy.org/all_by_species/285.html) and coat protein size of 35-37 kilo Dalton (kDa). The virions body is assembled by the major capsid protein (CP) and the tail by the minor capsid protein (CPm) (Melzer *et al*., 2008).

Molecular mechanisms by which the viral suppressor proteins interfere with plant RNA silencing are diverse (Kishore *et al*., 2015). According to Burgy and Havelda (2011), RNA silencing is a powerful defense mechanism that plants employ to defend itself against viral infection. Systemic or long-distance movement of RNA silencing in plants depends upon the amplification of the siRNA silencing signal (Dunoyer *et al*., 2010; Molnar *et al*., 2010) .For characterizing the virus, selected open reading frames (ORFs) of PMWaV-1 and PMWaV-2 were screened for their local suppressor activities in Agrobacterium-mediated transient assays using green fluorescent protein (GFP) in *Nicotiana benthamiana* (Kishore *et al.,* 2015). The systemic results show that PMWaV-2 utilizes a multiple proteins for RNA silencing and suppression mechanism. Two proteins, p20 and coat protein (CP), target local suppression that is, it suppresses plant RNA at the point of infection. Also, the two proteins have systemic silencing, that is, they suppress plant RNA a distance away from the point of infection. However, protein p22 and coat protein duplicate (CPd) target only systemic silencing (Kishore *et al.,* 2015). Of all the proteins tested from the virus PMWaV-2, protein p20 suppressed local silencing while in the PMWaV-1, only one protein p61 had a suppressing activity. Hence, it may be argued that proteins p20 and p22 are the proteins that enhance the ability of PMWaV-2 to cause disease and have an important role in the etiology of wilt disease (Sether, 2002). PMWaVs belong to the genus *Ampelovirus* and the family *Closterovirida e*. (Sether 2002)

2.5 Mealybug action threshold for transmission of PMWD

Carter (1933a, 1939b) demonstrated that mealybug wilt disease was due to toxic saliva of the ve ctor mealybug injected to the pineapple plant leading to symptom development. In fact, when the mealybugs do not feed on the plant recovery occurs ie the symptoms disappear. This is because t he wilt disease occurs only when large numbers of mealybugs are present. According to (Carter a nd Schmidt 1935) one or single mealybug is not capable of transmitting wilt disease but occasion ally as few as five mealybugs per plant could produce typical cases of wilt. However, in similar s tudy, (Carter 1937) demonstrated that single mealybug could occasionally produce wilt in pineap ple. In contrast, (Sether *et al.* 1998) asserted that a higher chance of vector transmission would oc cur when 10, 20 or 40 viruliferous vector mealybugs were used. Indeed, (Jahn *et al.* 2003) observ ed that, the higher the number of mealybugs per pineapple plant, the higher the chances of PMW D manifestation. According to Davily (2002), insect vectors transmit the disease causal pathogen virus after five (5) minutes of feeding on the host plant.

2.6 Resistance to PMWD

Disease management is one of the main challenges affecting production of most crop including the pineapple. The main strategies used in the control of PMWD such as physical, mechanical, biological and chemical means have limitations. For example, mealybugs vectors develops resistance to pesticides used are widely distributed in many agroecological areas (Sether *et al* 2001) and the residual effects of the pesticides to the environment (Thapinta and Hudak, 2000). Accordingly, the use of host resistance is the only economical and sustainable management strategy for both medium and long term basis (Peterson *et al.*, 1989). According to Sether and Hu (2000), Smooth cayenne, the most commonly grown pineapple variety is very susceptible to

PMWD although limited varietal resistance was reported for the control of PMWD (Chan *et al*., 2002). .However, no sources of resistance to PMWD have so far been reported in Uganda although elsewhere in Africa especially in Ghana, MD2 pineapple variety was reported to be less affected by the wilt disease than smooth cayenne and Victoria (sapong *et al*, 2017) . Besides, the polyploidy and vegetatively propagated nature of pineapple makes the identification of sources of resistance in very complicated.

2.7 Literature summary

Pineapple mealybug wilt disease is a serious disease affecting pineapple plant globally. The disease etiology and epidemiology has not been understood in Uganda and therefore there is no documented information on the identity and diversity of the virus; thus complete understanding of the disease remains a challenge. Therefore management of the disease and vector mealybugs either through chemical, biological and cultural means, by identifying and characterizing, assessing mealybug action threshold and screening resistant varieties of pineapple remains a critical issue to deal with within the sector for good productivity in Uganda. However, elsewhere in the world, research has established a close relationship of wilt disease with the mealybug, the vectors that transmit the virus responsible for the cause of the wilt disease (Sether, 2005). Therefore, this study sought to assess the transmission, resistance of different pineapple varieties and molecular identification and diversity of pineapple mealybug associated viruses (PMWaV) in Uganda.

CHAPTER THREE

IDENTIFICATION AND CHARACTERISATION OF DIVERSITY OF VIRUSES CAUSING PMWD IN CENTRAL UGANDA.

3.1. Introduction

Pineapple mealybug wilt associated viruses (PMWaVs) belonging to the genus *Ampelovirus* and family *closteroviridae,* is a complex group of viruses associated with pineapple mealybug wilt disease transmission (Martelli, 2002). The five viruses recognized as PMWaVs 1, 2, 3, 4 and 5, are linked to the etiology of pineapple mealybug wilt disease (Sether *et al.,* 2005b; Gambley, 2008). The virus is transmitted by the two species of mealybugs namely pink mealybug *Dysmicoccus brevipes* (Cockerell) and grey mealybug *Dysmicoccus neobrevipes* (Beardsley) (Sether, 1998, 2002). Therefore, in order to understand the etiology and epidemiology of pineapple mealybug wilt disease, it is imperative that the identity and diversity of the causal viruses is unraveled for screening pineapple for resistance to PMWD. Thus, the objective of this study therefore was to assess the identity and diversity of PMWD casual viruses in central Uganda.

3.2 Materials and methods

3.2.1 Area of study

The study was carried out at Biosciences and Biotechnology laboratory of the National Crop Resources Research Institute (NaCRRI), Namulonge, Wakiso district. Namulonge is 27 km north of Kampala city in central Uganda at an altitude of 1200 meters above sea level and coordinates 0° 31′ 30″ N, 32° 36′ 54″ E.

3.2.2 Sample collection

Both symptomatic and asymptomatic pineapple plant samples were collected from four districts of Masaka, Luwero, Mukono and Kayunga in central Uganda. The districts were identified because of the high intensity of pineapple production in Uganda and the reported widespread occurrences of pineapple mealybug wilt disease (Bua *et al.*, 2013). Whole pineapple plant with symptoms of PMWD was collected from the fields visited and packed in the collection bags. Forty four samples were randomly collected from the districts but more samples were picked from the districts with higher disease prevalence. In total, the number of samples collected was as follows: Luwero (17), Mukono (6), Kayunga (13) and Masaka (8). All the samples were transported to the Department of Agriculture, Kyambogo University for safe storage before laboratory analysis. From Kyambogo University department screen house, about 250g of the fresh pineapple leaf samples from marked plant was cut using sterilized scalpel dipped in 70% ethanol and labeled. The smaller samples were transported in Falcone tubes to the Biosciences and Biotechnology laboratory, (NaCRRI) and immediately stored at -80^{o}C in a freezer in the laboratory for RNA extraction (Carpentier *et al*., 2007).

3.2.3 Preparation of CTAB extraction buffer

The extraction buffer was prepared by weighing one hundred fifty (150) g of Cetyltrimethylamonium bromide (CTAB) in a clean beaker and dissolving in 500mls final volume of sterile distilled water (SDW) step by step. This was followed by the addition of 140ml of 5M Nacl, 20ml of 0.5M Ethylenediaminetetra acetic acid (EDTA) pH 8.0 and 50ml of 1M Tris Hcl pH 8.0, 15g of Polyvinylpyrrolidone (PVP) as described by Winnepenninckxb *et al*. (1993). The content was heated at 50°C while stirring using magnetic stirrer to ensure complete dissolution of PVP. The beaker was covered using aluminum foil during time of heating due to the reason that

aluminum has a relatively high thermal conductivity index, which means it disperses heat evenly around pipette tips and beakers wrapped. After cooling, the CTAB buffer was sterilized in an autoclave at 121°C for 15minutes at 103 kilopascal (kPa) pressure. The buffer was made ready for use after addition of 0.1ml or 100 µL of Beta-mercaptoethanol in a 50ml level Falcone tube (Winnepenninckxb. *et al.,* 1993).

3.2.4 Optimization of PCR protocol

The PCR protocol was optimized by varying and changing the amounts and types of reagent for best result before all the samples kept in stock were extracted. This was done to ensure quantity and quality of total nucleic acid, the cDNA synthesis and then PCR master mix to bring out the best result and gel picture were done (Andrieu and Vessot., 2018).

3.2.5 RNA extraction

Two hundred milligrams (200) mg of pineapple leaf tissue was macerated to fine powder using liquid nitrogen in a sterilized mortar and pestle (Ghosh *et al.,* 2013). Approximately 700µL of extraction buffer was added to the paste and mixed well. The paste was then put in 2ml ependorf tube and vortexed for 2 minutes to disperse the content in the buffer uniformly as described by Keifer *et al.* (2000).

The content of the ependorf tube was incubated in water bath at 65 °C for 30 minutes but mixed by inversion after every 10 minutes. Later the content was left to stand at room temperature for 10 minutes. An equal volume of chloroform: isoamylalcohol in a ratio (24:1) was added and then mixed by inversion for 10 minutes, centrifuged at 8000 revolution per minute (rpm) for 15minutes. The upper aqueous supernatant phase, approximately 450µL was transferred to a sterilized ependorf tube. An equal volume of approximately 500µL of absolute ethanol and 0.03M sodium acetate was then added to the supernatant. The extract was stored at -20 °C for one hour to

precipitate the total nucleic acids. The extract was centrifuged at 13000 rpm for 10 minutes and immediately absolute ethanol decanted off. Five hundred microliters of 70% ethanol was added in the same tube to wash the RNA pellets by tapping the tube, and the extract centrifuged at 13000 rpm for 10 minutes. Ethanol was decanted carefully, and the pellets air dried for about 40 minutes. The dry pellets were then dissolved in 30µl of RNase-free water making the samples ready for RNA quantification (Winnepenninckxb. *et al*., 1993).

3.2.6. RNA quantification

The purity and the concentration of RNA was assessed by determining the absorbance ratio of the samples at 260nm and 280nm using a spectrophotometer as described by John (1992).

3.2.7 Reverse Transcriptase- Polymerase Chain Reaction detection of PMWaV-1 and PMWaV-2 viruses

The viruses causing pineapple mealybug wilt disease was detected using RT-PCR and specific primer pairs. Sense- strand primer and Complementary- strand primer, 223 (5′-TCATTGCACTCACTTATCGTTG-3′) /224 (5′-CATACGAACTAGACTCATACG-3′) and 225(5′-ACAGGAAGGACAACACTCAC-3′) /226 (5′-CGCACAAACTTCAAGCAATC-3′) were used to amplify the viruses PMWaV-2, and PMWaV-1, respectively (Sether, 2001, 2005; Hernández, 2010; Dey, 2014). The heat shock protein 70 homologue (Hsp70h) region of the viral genome was amplified by RT-PCR using the specific primer pairs 223/224 and 225/226.

First strand cDNA was prepared using 5x reaction mix (thermo scientific maxima first strand cDNA synthesis kit) containing 5x reaction buffer, dNTP, oligo (dT) 18, and random hexamer primers (Krug and Berger, 1987). Maxima enzyme mix (contains maxima reverse transcriptase (RT), Ribolock RNase inhibitor), nuclease free water and the sample RNA, the cDNA mix volume

was 20µl. The contents was mixed thoroughly and spanned briefly and incubated at 25 °C for 10minutes followed by 50 °C for 30 minutes, the reaction was terminated by heating at temperature 85 °C for 5 minutes in the Tprofessional thermo cycler Biometra, the cDNA prepared was used for the reverse transcriptase (RT-PCR). PCR master mix contained the following components 5µl of 5× green go Taq reaction buffer (promega-USA), 1µl of 10mM dNTP (thermo scientific-USA), 0.5µl Taq DNA polymerase(thermo scientific-USA), 3µl of 25mM Mgcl (Biolab UK), 1µl Sense strand primer 225 sequence (5′-ACAGGAAGGACAACACTCAC-3′) and 1µl Complementary strand primer 226 sequence (5′-CGCACAAACTTCAAGCAATC-3′), designed for PMWaV1, and complementary strand primer 223 sequence (5′-TCATTGCACTCACTTATCGTTG-3′) and sense strand primer 224 sequence (CATACGAACTAGACTCATACG-3′), designed for PMWaV2 (Horlock, 2003), Two microliters (2µl) of template and eleven point seven microliters (11.7µl) nuclease free water (thermo scientific -USA). The PCR reaction volume of twenty five point two microliters (25.2µl) each PCR tube. The content was spanned briefly to settle at the bottom of the tube. The reaction profile was conducted as follows: Thermo cycling initial (denaturation) was one cycle at 94 °C for 4 minutes, 45 cycles of 94 °C for 1 minute, 54 °C for 1 minute (annealing) and 72 °C for 1 minute (elongation) and finally extension at 72 °C for 10 minutes to extend and join partial genomic clones generated from the random RT-PCR and the Hsp70h-specific PCR (Bettencourt *et al.,* 1999).

Figure 1: Preparation of PCR master mix at Biosciences and Biotechnology laboratory NaCRRI Namulonge, 2017.

3.2.8 Gel electrophoresis

PCR amplicons was separated in 1.2% w/v agarose gel prepared by dissolving 1.2g agarose powder in 100ml of ×1 Tris-acetate-EDTA (TAE) buffer warmed for 2 minutes in a microwave to increase solubility of agarose powder then cooled under water. Five microliters (5µl) ethidium bromide was added to the TAE buffer and mixed gently and uniformly (Joseph and David, 2006). Using appropriate size electrophoresis tank and comb, gel was then casted into the electrophoresis tank and left to solidify for 30 minutes. The PCR products were pipetted separately into each of the wells. One kilobase (1kb) DNA ladder was used to differentiate the band size. The gel was run at 80 volts for 1hour and the images of the separated PCR products was visualized and captured by GBOX syngene gel documentation system (SYNGENE, U: GENIUS 3. UG3/1189 -UK). Thirteen samples were sequenced out of nineteen amplified due to associated cost limitation.

3.2.9 Sequencing

Thirteen PCR products with the base pairs 609 targeting PMWaV-1 using primer set 225/226 were sequenced using Sanger sequencing method at Macrogen, Netherlands. The sequences were trimmed to about 480bp using BIOEDIT software version to get the best quality sequence before blasting the sequences. The trimmed sequences were subjected to basic local alignment tool (BLASTn) for the protein nucleotide identity search, and compare with the sequences of PMWaV-1 available and published in the GenBank database (Zhao and Chu., 2014).

3.2.10 Evolutionary relationship

The evolutionary history of PMWaV was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 241.27734375 (Figure 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Tamura *et al.,* 2004) and are in the units of the number of base differences per sequence. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding (Bofkin and Goldman., 2007). All positions containing gaps and missing data were eliminated. There were a total of 561 positions in the final dataset. Evolutionary analyses were conducted using MEGA6 (Tamura *et al,* 2013).

3.2.11 Evolutionary distances analysis

The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Sharma *et al.,* 2015). The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All

positions containing gaps and missing data were eliminated. There were a total of 338 positions in the final data set. Evolutionary analyses were conducted using MEGA6 (Tamura *et al*., 2013).

3.2.12 Analysis of molecular variance

Analysis of molecular variance (AMOVA) was used to separate the components of variance among the pineapple mealybug wilt-associated virus 1 populations. This was done using GenAlEx v6.5 software (Peakall and Smouse, 2012) with the input data files generated from CREATE v1.37 software (Coombs *et al.,* 2008) and the number of permutations was determined at 999 for significance analysis. AMOVA components were used as estimates of the genetic diversity within and among populations (Meirmans, 2006).

3.3 Results

3.3.1 RNA Quantification

The purity and the concentration of RNA is presented in Table 2. The concentration of RNA ranged from 106 and 522.4 for the samples 19 and 28, respectively. However, the absorbance ratio ranged from 1.56 to 2.08 for the samples 18 and 35 although there was no significant difference in absorbance ratio between samples 22 and 42, respectively.

Table 2: RNA quantification

S/No	Sample ID	Conc Ng/µl	RNA(µg/ml)	Water	Abs Ratio 260/280
1	6	324.5	15.4	-1.4	1.87
2	15	255.2	9.6	4.4	1.58
3	16	114.3	14	0	1.95
4	17	176.8	14	0	1.61
5	18	252.7	14	0	1.56
6	19	106	14	0	1.94
7	22	260.8	19.2	-5.2	2.02
8	25	348.0	14	0	1.75
9	28	522.4	9.6	4.4	1.74
10	33	127.8	39.1	-25.1	2.03
11	35	158.1	31.6	-17.6	2.08
12	39	415.4	12.0	2.0	1.84
13	40	120.3	14	0	2.01
14	42	110.3	45.3	-31.2	2.02

3.3.2 RNA amplification

The presence of pineapple mealybug wilt associated virus one (PMWaV-1) corresponded to the DNA band size of 609bp (Figure 2). The amplicons identity was resolved using basic local align ment search tool (BLAST) for nucleotide similarity (Appendix 2). The isolate MU005 was 94% i dentical to accession number HQ940514.1, Isolates MA012, MU006, LU004 were 99% identical to accessions numbers HQ129930.1, HG940514.1, and HG940514.1 respectively (Appendix 2). . All the isolates were identified as pineapple mealybug wilt associated virus one (PMWaV1) base d on the blast output (Appendix 2) for similarity search and 1kb DNA Gen ladder (Figure 2). However, pineapple mealybug wilt associated virus (PMWaV-2) was not detected (Appendix 4). Out of the forty four total RNA extracted from the pineapple samples nineteen samples amplified while the twenty five samples that were symptomatic of wilt disease did not amplify and thirteen samples were sequenced (Appendix 3).

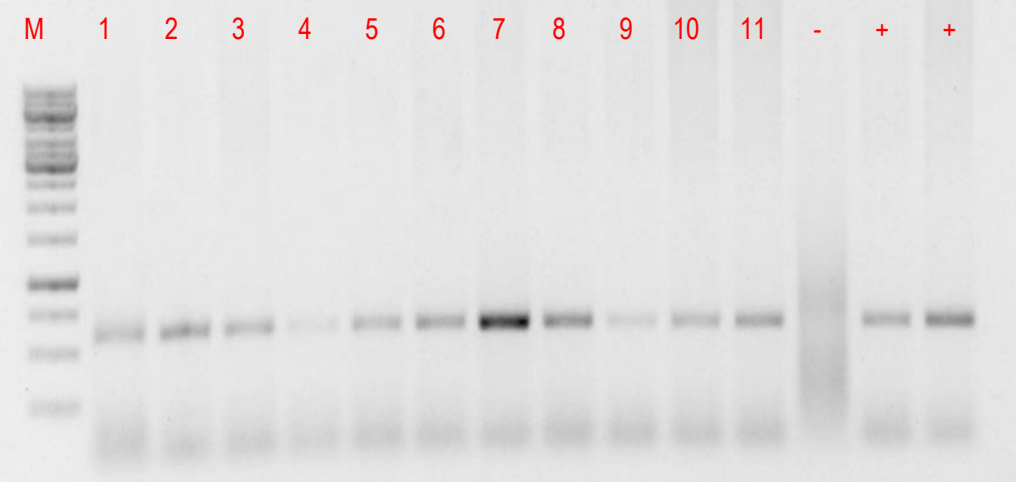


Figure 2: Gel electrophoresis of 11 samples amplified using primer 225/226, Lane M: 1kb gene ladder, lane 1-11 samples and lane marked (-) negative control, (+) positive controls, respectively

3.3.3 Cluster analysis

The results of the analyzed cDNA partial HSP70 gene for heat shock protein 70 sequenced data of 2 samples extracted during the optimization of PCR protocol was preserved and analysed together with 11 samples amplified during PCR amplification. The 13 samples of pineapple mealybug wilt-associated virus 1 revealed close phylogenetic relationships with all the isolates as indicated in the phylogenetic tree (Figure 3). KT322166.1 fell far from the phylogeny because it originate from different taxa it served to root evolutionary relationship. Generally, the evolutionary distances of the isolates appear relatively short, the results affirms that the isolates belong to the same cluster.

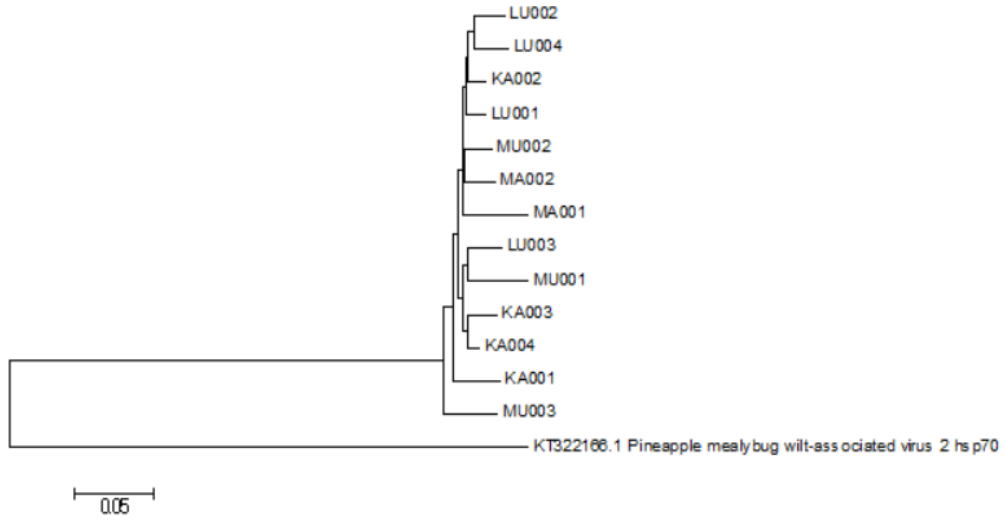


Figure 3: Evolutionary relationships of 13 PMWaV-1

3.3.4 Pair wise population matrix

The genetic distance of each isolates showed that the variation occurs positively with increase in genetic distance of the isolates. Significant differences ($P<0.05$) in genetic differentiation was observed within the sub-population (Table 3). Overall, all pair wise values show a relatively higher variation between and within sub-populations.

Table 3: Pair wise population matrix of Tamura genetic Distance between 13 isolates of Pineapple mealybug wilt associated virus 1

	1	2	3	4	5	6	7	8	9	10	11	12	13
1.LU001	0												
2.LU002	23.00	0											
3.LU003	36.00	35.00	0										
4.LU004	37.00	37.00	43.00	0									
5.MU001	47.00	45.00	44.00	49.00	0								
6.MU002	33.00	27.00	39.00	37.00	42.00	0							
7.MU003	44.00	40.00	43.00	45.00	51.00	38.00	0						
8.KA001	36.00	38.00	42.00	47.00	54.00	45.00	49.00	0					
9.KA002	22.00	26.00	36.00	29.00	45.00	26.00	47.00	42.00	0				
10.KA003	33.00	28.00	36.00	38.00	43.00	35.00	49.00	45.00	29.00	0			
11.KA004	27.00	27.00	39.00	33.00	37.00	30.00	41.00	41.00	22.00	29.00	0		
12.MA001	44.00	43.00	58.00	50.00	61.00	43.00	57.00	62.00	36.00	50.00	43.00	0	
13.MA002	35.00	35.00	36.00	44.00	56.00	40.00	44.00	38.00	35.00	38.00	37.00	54.00	0

3.3.5 Analysis of molecular variance (AMOVA)

The molecular analysis of variance revealed that up to 99% of the diversity was distributed within the population leaving only 1% among the population as indicated (Table 4).

Table 4: Analysis of molecular variance (AMOVA) for 13 Pineapple mealybug wilt-associated virus 1 from central Uganda, 2018

Source of variation	D.f.	SS	MS	Est.Var	Total variance	p-value	F_{ST}
Among population	1	0.417	0.417	0.417	1%	_	_
Within population	22	8.167	0.371	0.374	99%	0.01	0.503
Total	23	8.383	0.788	0.389	100%		

Key d.f. – degree of freedom, SS – sum of squares, MS – mean squares, p – level of significance, F_{ST} – fixation index for genetic differentiation between populations. Probability, P (rand >= data), for F_{ST} is based on standard permutation across the full data set.

F_{ST} = AP/ (AP+WP) = AP/TOT

AP = Est. Var. Among Pops, WP = Est. Var. Within Pops, TOT= Total

3.4 Discussion

Understanding the genetic structure, sequence identity and factors involved in the evolution such as recombination are crucial for the development of durable virus derived disease control strategies (Rubio *et al.,* 2013). The genetic variation and structure of viruses within an infected plant (considered as a virus isolate) also provides important information to understand viral evolution (Alabi *et al.,* 2011). Rapidity and accuracy of identifying the pathogens from the host plant tissues is the most important step to any attempt to control and manage diseases in plants (Khiyami *et al* 2014). According to William *et al.* (1997), good quality RNA is indicated by absorbance ratio 260/280 ranging from 1.8-2.1 on the spectrophotometry or Nano drop-2000 computer program. However, in this study a few samples had absorbance ratio within the acceptable range. The samples with low absorbance ratio (1.56-1.75) than the normal range were isolates 18, 15, 17, 28

and 25, respectively. While those within the acceptable (1.8-2.1) range were isolates 6, 16, 19, 33, 35, 39, 40 and 42. According to (Boesenberg *et al* 2012) the low ratio could have been attributed to factors such as poor extraction procedures, contamination, and low titre of the virus in the samples used, presence of phenolate ion, thiocyanates, organic compounds and others.

The results of this study showed that PMWaV-1 was the only virus found associated with PMWD as opposed to the earlier assertions that PMWD is caused by combination of different pineapple mealybug wilt associated viruses (Sether *et al.,* 2001, 2005; Subere *et al*., 2011). The present study objective was to identify and characterize the viruses that cause PMWD in Uganda, here we discuss the findings of the study. According to Kishore *et al.* (2018), PMWD has a complex etiology involving association of virus particles, mealybug vector and their attendant ants. In fact, serological and molecular analysis revealed the presence of at least three distinct pineapple mealybug wilt-associated viruses) namely, PMWaV-1, PMWaV-2 and PMWaV-3 causing pineapple mealybug wilt disease (Sether *et al.,* 2001,2005; Subere *et al*., 2011). The PMWaVs are acquired and transmitted by the pink and gray mealybugs (Sether, 1998, 2005). In fact, PMWaV-2 has an important role in the cause of the pineapple mealybug wilt disease and in the presence of mealybug feeding leads to significant yield loss (Sether, 2002b; Mau and Kessing, 2007). Meanwhile, the pineapple mealybug wilt associated virus-1 (PMWaV-1) was noted to cause significant yield reduction without the mealybug feeding hence the pineapple fail to develop the symptoms of the disease (Sether, 2002, 2002a, 2005). The results in this study further showed that 99% of the variation in the pineapple mealybug wilt associated virus (PMWaV-1) was within the virus population and only 1% of the variation was between pineapple mealybug wilt associated viruses population. It should therefore be noted that, viruses of the family of *Closteroviridae* are transmitted by insect vectors which favor mixed infections with different viruses or strains of the

same virus. Mixed infections may have resulted to this variation and important evolutionary implications since they can affect the within-isolate population of virus variants and allow interaction or recombination between different virus entities, (Alabi *et al.*, 2011).

Although, a number of pineapple mealybug associated viruses have been reported associated with PMWD, only PMWaV-1 was found in this study using BLAST comparison with the NCBI database. This was consistent with Sether (2001), who found that, only pineapple mealybug wilt associated virus one (PMWaV-1) was the most widely distributed throughout the pineapple growing areas of the world. Analysis of molecular variance (AMOVA) revealed a high value of FST fixation index (0.532, p=0.01) indicating a very strong variability. Actually this confirms a significance differences existing in the pineapple mealybug wilt-associated virus 1. According to (Fumagalli *et al*, 2013), the FST > 0.25 further affirms a very great difference among the sub-populations of pineapple mealybug wilt-associated virus 1 isolated from the central Uganda.

CHAPTER FOUR:

MEALYBUG ACTION THRESHOLD FOR TRANSMISSION OF PINEAPPLE MEALYBUG WILT DISEASE

4.1 Introduction

Several authors have reported the association between the population of mealybugs and the ability to transmit the virus to the host plants. For example, Notte *et al*. (1997) and Tsai *et al.* (2010), indicated that the transmission efficiency of PMWD was high, if high number of the mealybugs that had enough access acquisition time (AAP) on the pineapple plants. However, Kishore *et al.* (2018) demonstrated that exposure to large numbers of mealybugs did not always result in wilt symptoms but may vary depending on breed of the pineapple plant, the origin of planting materials, and growing locations. In fact, PMWD transmission is associated with virus particles, mealybug vectors, and ants which spread the mealybug vectors. Although, two species of mealybug namely, the pink and gray have been associated with PMWD transmission, the information on the action threshold is scanty and limited (Sether *et al.* 1998).Therefore, the objective of this study was to assess the mealybug action threshold for the transmission of PMWD.

4.2 Materials and methods

4.2.1 Experimental site

The experiment was conducted in a screen house at the Department of Agriculture, Kyambogo University. Kyambogo University is located 8km east of Kampala City Centre along the Kampala-Jinja highway by road (https://kyu.ac.ug/index.php/find-us/location) in central Uganda and 0^0 20′54.0″ N, 32^0 37′49.0″ E (latitude 0.348334; longitude 32.630278) 1,240m above sea level (https://en.wikipedia.org/wiki/Kyambogo).

4.2.2 Test plant material collection and detection for latent infection

The test plant materials (Smooth cayenne) used in this study was physically inspected and found to be free from pineapple mealybug wilt disease (PMWD) symptoms. The plant materials suckers were assayed for latent disease infection or status using polymerase chain reaction (PCR) with specific primers targeting the associated viruses PMWaV-1 and PMWaV-2 following the procedure described by (Hu *et al.* 1996; Dey *et al*, 2012).

4.2.3 Experimental design, treatments, potting and management

The experiment was laid out in a completely randomized design (CRD) with eight replications per treatment. The treatments included inoculation of pineapple plants with five levels of viruliferous mealybugs namely, one (1), five (5), ten (10), fifteen (15) and a control (uninoculated) (Jahn *et al.*, 2003;Tsai *et al*., 2010). Forest soil sterilized by direct heating was used for planting the test plants (Figure 4). Six kilograms of sterilized soil was filled in buckets measuring 21.5cm in diameter, 20.cm in height in which material suckers were planted. The test plants were placed in individual cages in the screen house to restrict movement of the mealybugs, and also to reduce effects of wind that disperse the young crawlers or nymph mealybugs especially the grey mealybugs mainly found on the aerial part of the plant (Jahn *et al.,* 2003). Caging would also limit other insect predators like ladybird also called mealybug destroyer (*Cryptolamus montrouzieri*) and Green lacewings (*Chrysoperla sp*), from feeding on the mealybug (Mamoon *et al.,*2016) (Figure 5). Other insects were controlled by maintaining good sanitation in and around the screen house through cleaning washing, and keeping the surrounding grass shorts and good ventilation (Teitel and wenger, 2012). The temperature in the screen house varied between 18 to 22 degrees Celsius. Watering and weed management was done when necessary.

Figure 4: Soil sterilization at Department of Agriculture, Kyambogo University, 2017

Figure 5: Inoculated pineapple test plants in individual cages at the Department of Agriculture, Kyambogo University, 2017.

4.2.4 Inoculation

The pink mealybugs obtained from diseased pineapple plants were given access acquisition period (AAP) of seven (7) days as described by Tanwar *et al.*(2007). A sub population of the mealybug vectors was randomly assayed for their viruliferous potential by PCR (Hu *et al.,* 2005). The viruliferous mealybugs were transferred onto the test plant using a fine paint brush (Figure 6).

Figure 6: Inoculation of pineapple plants with mealybugs at Department of Agriculture, Kyambogo University 2017

4.2.5 Data collection and analysis

Data was collected after 15 days of inoculation access period (IAP) and continued at an interval of 15 days for a period of two months (Sether *et al,* 1998). Two data sets were collected one on disease incidence and other on disease severity (Sseruwagi *et al.,* 2004). Disease incidence was assessed as the number of test plants showing symptoms of disease infection over total number of plants assessed multiplied by hundred. Disease Severity was assessed by calculating average number of chlorotic spots from the middle leaves of infected plant, the number of the spots then scored using

a modified scale of (0-5) where 0 = no symptoms 1 =(1-10%), 2 =(11-25%), 3=26-50%), 4 =51-75%), 5 =(76-100%) (Madden 2007; Masood *et al*., 2010).

Disease incidence and disease severity data was subjected to one-way analysis of variance (ANOVA) using Genstat computer programme (15th edition). Significant differences between the means were separated using the least significant test (LSD) at 5% probability level.

4.3 Results

4.3.1 Disease incidence

The number of mealybugs significantly (P<0.05) influenced the incidence of PMWD in both trials (Table 5). In trial one, 15DAI, the highest and lowest incidence (56.2%) and (3.1%) was recorded from the plants inoculated with fifteen (15) and one (1) mealybugs per plant, respectively. A similar trend was followed at 30DAI, 45 DAI and 60DAI although a higher incidence was recorded at 45DAI and 60DAI, respectively (Figure7a).

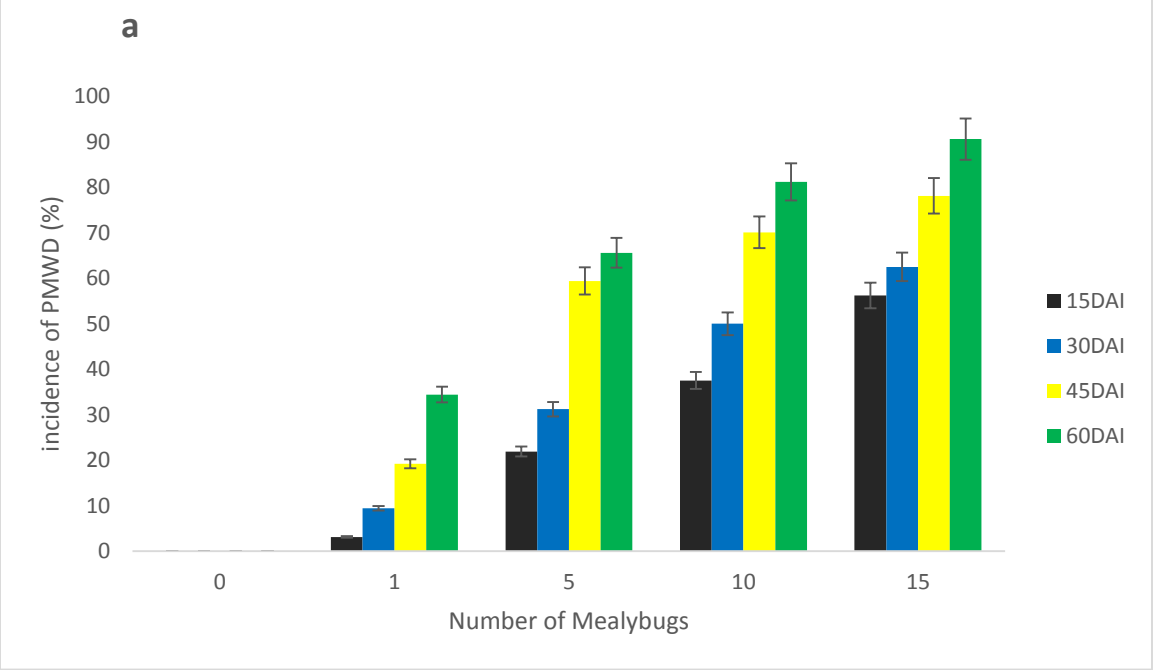
During trial two, a similar trend was followed as in the first trial. The number of mealybugs significantly (P<0.05) influenced the incidence of PMWD (Table 5). At 15DAI, the highest and lowest incidence (52.9%) and (4.3%) was recorded from plants inoculated with 15 and 1 mealybugs per plant respectively. The same trend followed at 30DAI, 45DAI, and 60DAI, respectively. Generally, the highest incidences in the entire trail were recorded in the 45DAI and 60DAI (Figure 7a).

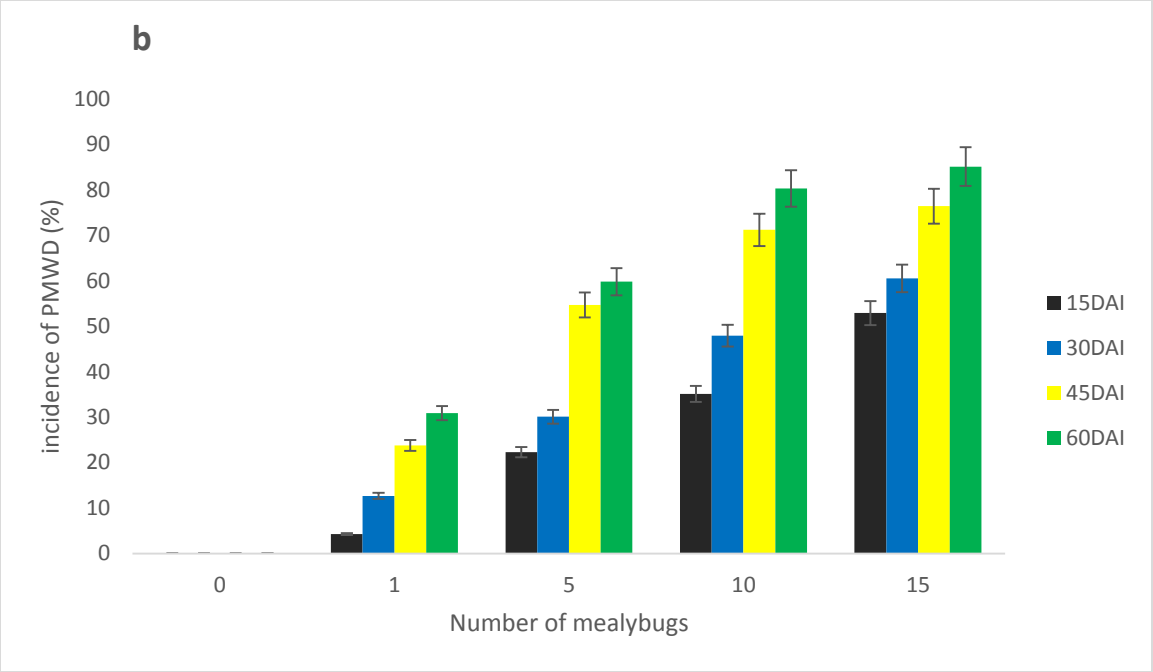
Table 5: Summary of ANOVA for incidence of mealybug action threshold for transmission of PMWD at Kyambogo University, 2017/2018

		Trial one (November - December 2017)			
		15DAI	30DAI	45DAI	60DAI
Source of variation	Degrees of freedom				
Mealybug number	4	2238.28***	2781.25***	5097.66***	5519.53***
Residual	12	35.16	78.12	45.57	55.99
		Trial two	**(March-April 2018)**		
Source of variation	Degrees of freedom				
Mealybug number	4	871.09***	1933.59***	4367.19**	5910.16***
Residual	12	48.18	32.55	85.94	40.36

DAI – Days after inoculation***Significant at <0.001

Figure 7 a and b: Mean disease incidence of mealybug action threshold for transmission of PMWD trial 1 and 2 at Kyambogo University, 2017/ 2018





The number of the mealybugs significant (P<0.05) influenced the severity of PMWD in both trials (Table 6). In the first trail, 15DAI the highest and the lowest severity (2.8%) and (0.5%) was recorded from plants inoculated with 15 and 1 mealybugs per plant, respectively. A similar trend was followed at 30DAI, 45DAI, and 60DAI. Although a high severity was recorded 45DAI and 60DAI respectively (Figure 8a). During trial two, similar trend was followed, as in the first trial. The number of mealybugs significantly (P<0.05) influenced the severity of PMWD (Table 6). At 15DAI the highest and the lowest severity (2.6%) and (0.4%) was recorded from plants inoculated with 15 and 1mealybugs per plant respectively. The same trend followed at 30DAI, 45DAI, and 60DAI, respectively. Generally, the highest severity in the entire trial was recorded in 45DAI and 60DAI (Figure 8a).

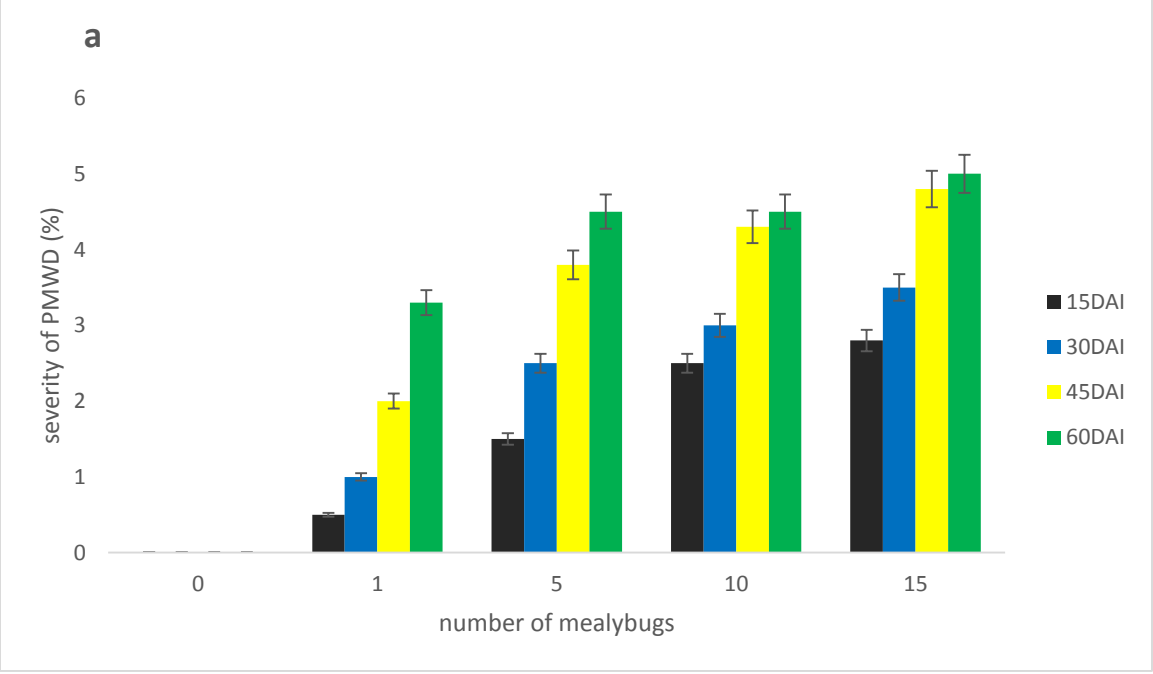
Table 6: Summary of ANOVA for severity of mealybug action threshold for transmission of PMWD at Kyambogo University, 2017/2018

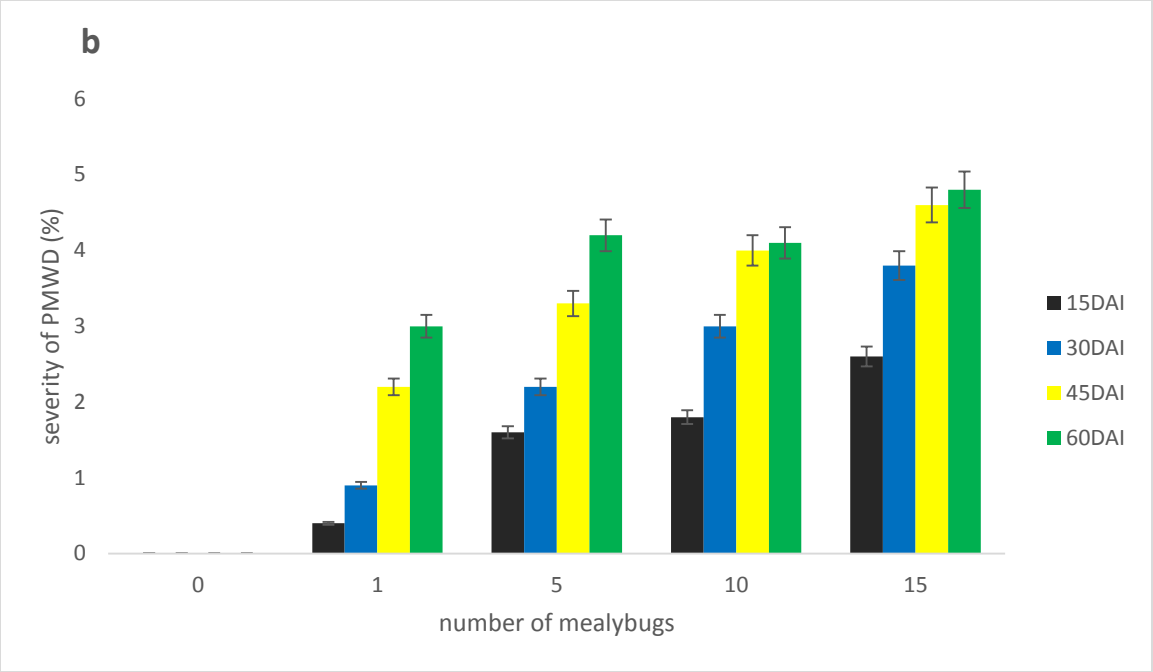
		Trial one (November – December 2017)			
		15DAI	30DAI	45DAI	60DAI
Source of variation	Degrees of freedom				
Mealybug number	4	5.8000***	8.5000***	15.1750***	17.1250***
Residual	12	0.3667	0.6333	0.5750	0.1250
		Trial two(March – April 2018			
Source of Variation	Degrees of freedom				
Mealybug number	4	7.6750***	13.0750***	16.5000***	17.0500***
Residual	12	0.2083	0.5417	0.1333	0.2167

DAI – Days after inoculation

******* Significant at <0.001

Figure 8: a and b mean disease severity for mealybug action threshold for transmission of PMWD trial 1 and 2 at Kyambogo University 2017/2018





4.4 Discussion

Exposure to groups of 10 and 15 viruliferous mealybugs resulted in significantly higher transmission and infections of pineapple plants than either single or 5 viruliferous mealybugs, respectively. This is because inoculation with a high number of mealybugs resulted into transmission of high viral load capable of causing PMWD. The present study objective was to determine mealybug action threshold for the transmission of PMWD and here we discuss the findings. The transmission efficiency of the virus increased with increase in the number of mealybugs reflecting high virus retention (VR) after sufficient virus acquisition access period (AAP) (Roivainen, 1976) This therefore means that the inoculation acquisition period (IAP) was shortened since more vectors (15 mealybugs) threshold transmitted sufficient virus. The virus introduced was able to move to the vascular tissues and later symptoms of infection manifested more in plants inoculated with higher number of mealybugs. This is consistent with Sether *et al.* (1998) who reported that a higher (10-20) mealybug threshold transmitted more virus than a lower

threshold. Similarly, Notte *et al*. (1997) had demonstrated that the transmission efficiency of PMWD was high where a high number of the mealybugs that had longer AAP were used.

Additionally, the variation in mealybug action threshold for transmission was probably as result of differences in feeding rate and virus retention. This therefore has a pronounced effect on the amount of virus acquired by the mealybug and subsequently transmitted to the host plant. Transmission efficiency increases, where high number of mealybugs feeds on the host thereby introducing more virus pathogen. This finding is consistent with Notte *et al* (1997) who reported that if sufficient virus is ingested by the vectors, the chance of transmission would be high provided adequate vectors population was used. In contrast, Kishore *et al*. (2018) observed that exposure to large numbers of mealybugs did not always result in wilt symptoms. But similar study revealed that the transmission and levels of all PMWaVs in pineapple were also found to differ depending on other factors including host, origin of plant material, feeding behavior, the mouth part stylets, viral load and complexes of the viruses as well as virus retention time in the mealybug (Stafford *et al.,* 2012). This therefore could account for the result of this study since it was conducted in different geographical location.

CHAPTER FIVE:

IDENTIFICATION OF SOURCES RESISTANCE TO PINEAPPLE MEALYBUG WILT DISEASE IN UGANDA

5.1 Introduction

Use of host plant resistance involves the use of cultivars that able to tolerate the pathogen and provides the cheapest and most economical option available to plant disease management in changing climate (Juroszak and Tiedemann, 2011). However, breeding for resistance is always hampered by a number of factors including lack of resistant genes, limited knowledge on the genes for resistance and breakdown of resistant genotypes due to the emergence of virulent pathogens strain among others (Palloix *et al.,* 2009). Therefore, it is imperative that the search for resistant genotypes is done routinely so as to increase the availability of the resistant varieties to increase yield. Accordingly, resistance to PMWD can be achieved through screening and identification of the genotypes for resistance to the vector and pathogen (Soler *et al*., 2003). The other disease management strategies like the use of chemicals, biological and physical methods may have negative impacts on the ecosystem. Therefore, due to the limitations in, the effectiveness of the biological control, physical and chemical control (Furness 1976; Smith &Gardner, 1951), and economically effective no cash involvement by the resource poor farmers (Sharma and Ortiz., 2002) effort should be concentrated to words identifying cultivars with resistance to major pests like mealybugs in pineapples and diseases such as pineapple wilt. Therefore, the objective of this study was to identify sources of resistance to PMWD in central Uganda.

5.2 Materials and methods

5.2.1 Germplasm and inoculum collection

The pineapple germplasm used in this study was collected from National crop resources research institute (NaCRRI), Namulonge. The five cultivars of pineapple namely Smooth cayenne, Red Spanish, *Sassilimu*, MD2 and Victoria were first assayed for latent infection by (PMWaV-1) and (PMWaV-2) strains using RT-PCR (Mo, 2012) and varieties were chosen because they are the most commonly grown and available in Uganda. Smooth Cayenne; has large fruits (1.5-2.5kg), yellow flesh soft and juicy with sugar (13-19 °Brix) suitable for processing (https://www.samvalleyfarms.com/?page_id=6). Red Spanish; weigh up to 1-2kgs, pale yellow in color with soothing fragrance, square shape, hard spiky leaves (https://www.doityourself.com/stry/4-different-varieties-of-pineapples). Victoria; has small fruits (0.5-1kg), full yellow shell when ripe, pulp is crispy and sweet (14-18 Brix), excellent flavor and shelf life (https://www.samvalleyfarms.com/?page_id=6). MD2 hybrid; has medium large (1.3-2.5kg) cylindrical fruits with intense yellow color is sweet with low fibre and acidity, contains as much as four times vitamin C than regular varieties, it is high in sugar (13-17°Brix) (https://www.samvalleyfarms.com/?page_id=6) and *Sassilimu* a local cultivar grown in most districts in central Uganda (Anonymous,2017;Oculi *et al.,* 2020)

5.2.2Experimental setup

This study was conducted in the screen house at Department of agriculture, Kyambogo University the experiment layout as described in chapter three.

5.2.3 Preparation of CTAB extraction buffer

One hundred fifty grams (150g) of Cetyltrimethylammoniumbromide (CTAB) was weighed in a clean beaker and dissolved in 200mls of sterile distilled water (SDW). The content was warmed at 50^0C and stirred using magnetic stirrer to ensure complete dissolution. The following components were added, 140mls of 5M Nacl, 20mls of 0.5M ethylenediaminetetra acetic acid (EDTA) pH 8.0, 50mls of 1M Tris Hcl pH 8.0, 15g of polyvinylpyrrolidone (PVP), and sterile distilled water (SDW) to make the final volume 500mls. The beaker was covered with aluminum foil during warming. After the contents was clearly dissolved and cooled the buffer was sterilized in an autoclave adjusted at 121^0C for 15mins at 103 kPa pressure. The buffer was made ready for use by adding 0.1ml or 100µL of beta-mercaptoethanol to each 50ml level Falcone tube**.**

5.2.4 RNA extraction

Total nucleic acid extraction from asymptomatic test plants to detect latent infection of the planting materials, the complementary DNA synthesis, and the reverse transcriptase polymerase chain reaction detection of pineapple mealybug wilt associated viruses (PMWaV-1 and PMWaV-2) gel electrophoresis protocols are as described in chapter three.

5.2.5 Source of inoculum

The mealybugs used in this study were collected from pineapple plants infected with pineapple mealybug wilt disease source plant. Viruliferous mealybugs used in this study were fed on the source diseased plants collected from farmer fields in Kayunga district following a seven days access acquisition period (AAP) as described by (Jane and Capobianco 2013). A sample of the mealybug populations was assayed to confirm their viruliferous status using PCR

5.2.6 Extraction of total nucleic acid from mealybugs

Ten (10) mealybugs were crushed in a sterilized (mortar and pestle. Pre warmed 600µL of 10% CTAB extraction buffer and 3µL of proteinase k was added in each tube 2mls ependorf tube and vortexed for 5 minutes. The mixture was incubated overnight in water bath at 65^0C, and then cooled at room temperature before spinning at 14000rpm for 10 minutes. An equal volume of chloroform: isoamylalcohol 24:1 was added and mixed by inversion for 5 minutes and Centrifuged at 14000rpm for 10 minutes. The supernatant was collected in fresh microfuge tubes and the pellets discarded. 400µLchilled isopropanol was added and mixed gently until white flakes appeared, the content was stored at -35^0C for an hour. The samples were later thawed to room temperature and spanned at 10000 rpm for 10 minutes the supernatant decanted. 400µL 70% ethanol, plus 100µL ammonium acetate was added to the pellet and spanned at 10000 rpm for 10 minutes. The supernatant was carefully decanted. 400µLof absolute alcohol was added and spanned at 10000rpm for 10 minutes, the supernatant was decanted and pellets dried at room temperature for 40 minutes (Ivanova and Kuzmina, 2013).

The pellet was again resuspended in 20µl of RNase-free water. The purity and the concentration of RNA was assessed by determining the absorbance ratio of the sample at 260nm and 280nm using a spectrophotometer (Desjardins and Conklin., 2010).

5.2.7 Complimentary deoxyribonucleic acid (cDNA) synthesis

First strand cDNA was prepared using maxima first strand cDNA synthesis kit (thermo scientific USA) which contains two main components as 5x reaction mix (containing 5x reaction buffer, dNTP, oligo (dT) 18, random hexamer primers) and maxima enzyme mix (contains maxima reverse transcriptase (RT), Ribolock RNase inhibitor), Nuclease free water, and RNA template , the cDNA mix volume was 20µl. (Kaihara and Agresti., 2018). Contents was mixed gently and

spanned briefly, then incubated at 25^0C for 10 minutes followed by 50^0C for 30 minutes, the reaction was terminated by heating at temperature of 85^0C for 5 minutes in the Tprofessional thermo cycler Biometra (Darissa *et al*., 2010).

5.2.8 Detection of PMWaV-1 and PMWaV-2

PCR master mix contained the following components, 5µl of 5× green go Taq reaction buffer (promega-USA), 1µl of 10mM dNTP, 0.5µl Taq DNA polymerase (thermo scientific-USA), 3µl of 25mM Mgcl (Biolab UK), primer set 225/226 1µl sense and 1µl complementary strand primers targeting PMWaV-1 and primer set 223/224 sense and complimentary strand targeting PMWaV-2 (Sether, 2001). 2 µl of the template and 11.7µl Nuclease free water (NFW) the PCR reaction volume of 25.2µl was added to each PCR tube and the content spanned briefly. The reaction profile was. Thermo cycling initial (denaturation) was one cycle at 94^0C for 4 minutes, 45 cycles of 94^0C for 1 minute, 54^0C for 1 minute (annealing) and 72^0C for 1 minute (elongation) and finally extension at 72^0C for 10 minutes (Nitsche *et al*., 2006).

5.2.9 Inoculation of test plants

The test plants were inoculated by mechanical methods which involved the use of paint brush for removal and transfer of the mealybugs from source plant to the test plants (Figure 7). The viruliferous mealybug fed on diseased pineapple plants for access acquisition period (AAP) of seven days were transferred to the test plants. Each test plant was inoculated with 15 mealybugs (Jahn *et al,* 2003; Tsai *et al,* 2010). This was to quickly and rapidly cause the transmission of the virus and infection to the plants as observed in chapter four. Twelve test plants in first trial were screened and inoculated per variety. The experiment was repeated twice.

Figure 9 Inoculation of test plants with viruliferous mealybugs at Department of Agriculture, Kyambogo University, 2017

5.2.10 Data collection and analysis

Two sets of data was collected, on disease incidence and disease severity, this was started 15days after inoculation time. Disease incidence was expressed as the number of treated test plants or experimental units showing symptoms of disease infection over the total number of plants assessed multiplied by hundred. The basis for assessing disease incidence was that, middle leaves of treated plants showing symptoms over the total number of treated plants assessed. The top leaves were not selected because the top new leaves may recover and not show symptoms of disease yet they could be infected, the bottom leaves may also show deficiency of nutrient nitrogen which in most cases starts from lower and bottom then spreads to other leaves secondly the plants may be self-adjusting as a result of new indoor environment. The first trial data was recorded from November-December 2017 and the second trial data recorded from March- April 2018. Disease severity was assessed by calculating average number of chlorotic spots on the middle infected leaves. The number of spots then rated using a modified scale of (0-5) where 0=no symptoms1=(1-10%),2=(11-25%),3=(26-50%),4=(51-75%.and 5=75-100% (Madden.2007;Masood *et al*., 2010).

The basis for assessment was that, middle leaves of infected test plant were selected, average number of spots on the leaves determined to assess the severity of damage.

Incidence and severity data was analyzed by one way analysis, the variance, mean disease incidence and mean severity were generated using Genstat computer program (15th edition). The significance level for the means were separated by the least significance test (LSD) AT 5% probability level. Area under disease severity index progress curve (AUSiPC) was calculated by addition of the first two severity values recorded, the result divided by two to find the average or mid-value of the two then multiply the average or mid-value by the time interval, which is the number of days from the first recording to the second recording. The severity figures are calculated using the formula described by Koros *et al.* (2018).

$$\text{AUSiPC} = \sum_{1}^{n-1}\left(\frac{SS_1 + SS_2}{2}\right)x\left(t_2 - t_1\right)$$

Where; SS1 is disease severity score at time t_1 and SS2 disease severity score at t_2

Data on disease incidence and severity was collected on a fortnight interval for two months for both trial one and two. The first and second trial ran from November to December 2017 and March to April 2018, respectively. A total of one hundred twenty (120) experimental units were screened in the two separate trials, twelve (12) plants were assigned treatments per variety in the first trial and in the second trial hence the experiment was repeated two times.

5.3 Results

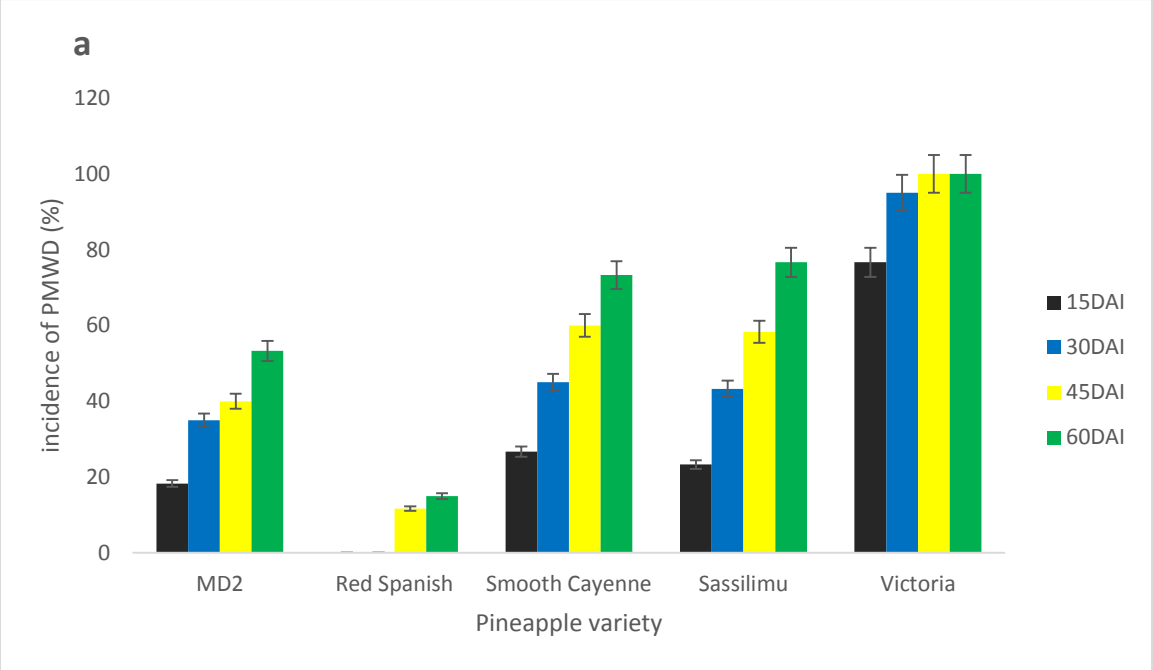
There was significant difference ($P<0.05$) in the incidence of PMWD among the pineapple variety during both first and second trials (Table 7). In the first trial, at 15DAI, the highest and lowest disease incidence of 76.7% and 0% was recorded from Victoria and Red Spanish, respectively (Figure 10a). A similar trend was followed at 30DAI, 45DAI and 60DAI although higher incidences were recorded at 45DAI and 60DAI, respectively (figure 10a and b). During the second trial, at 15 DAI, the highest and lowest disease incidence of 81.7% and 1.7% was recorded from Victoria and Red Spanish, respectively (Figure 10b). A similar trend was followed at 30DAI, 45DAI, and 60DAI although similarly as above higher incidences were recorded at 45DAI and 60DAI, respectively

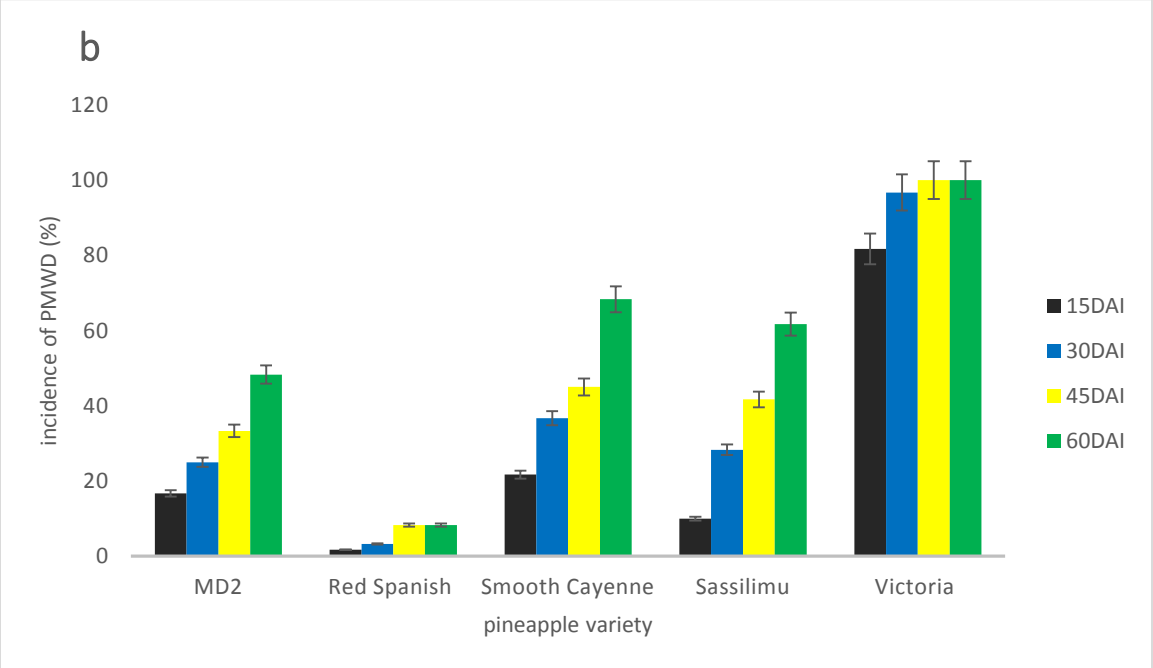
Table 7: Summary of ANOVA for incidence of PMWD on 5 pineapple varieties grown at Kyambogo University, 2017/2018

		Trial one (November – December 2017)			
		15DAI	30DAI	45DAI	60DAI
Source of variation	Degrees of freedom				
Variety	4	4080.4***	5773.4***	5199.2***	5072.92***
Residual	16	275.0	126.0	125.7	77.43
		Trial two(March – April 2018)			
Source of variation	Degrees of freedom				
Variety	4	5062.2***	6134.85***	5643.50**	5553.09***
Residual	16	127.8	56.60	37.14	42.37

DAI – Days after inoculation***Significant at <0.001

Figure 10 a and b: Mean disease incidence of PMWD trial 1 and 2 on 5 pineapple varieties grown at Kyambogo University, 2017/2018





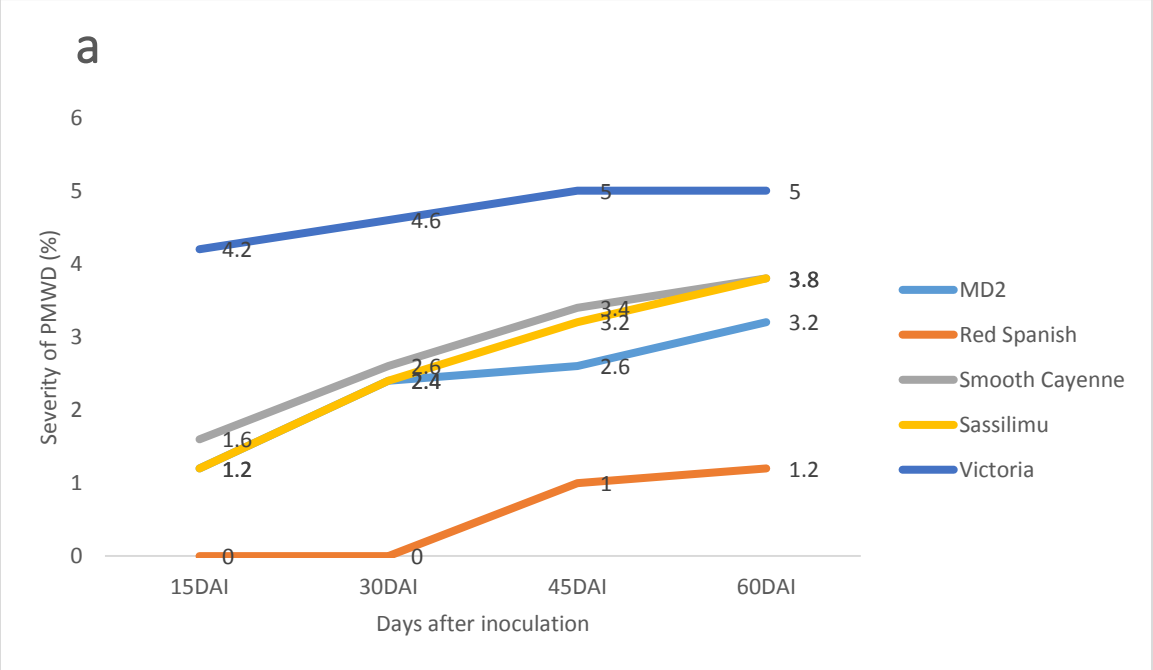
There was significant (P<0.05) difference in severity of PMWD among the pineapple varieties during the first and second two trials (Table 8). In the first trial, 15DAI, the highest and the lowest (4.2%) and (0%) disease severity was recorded from Victoria and red Spanish respectively (Figure 11a). the same trend was followed at 30DAI, 45DAI, and 60DAI respectively. although high severity was recorded 45DAI and 60DAI. during the second trials, similar trend was followed as in the first trial. There was significant (P<0.05) difference in the severity of PMWD among the variety (Table 8). At 15DAI, the highest and the lowest (4.4%) and (0.2%) disease severity was recorded from Victoria and Red Spanish, respectively (Figure 11b). The same trend was followed at 30DAI 45DAI and 60DAI although higher severities were recorded 45DAI and 60DAI, respectively.

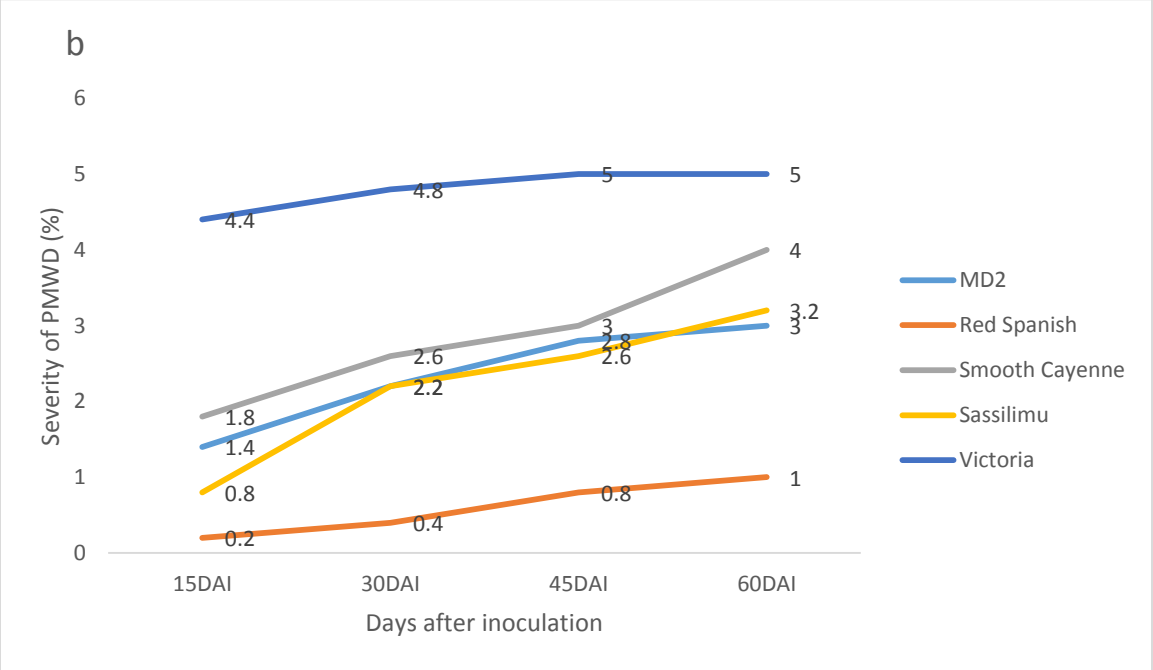
Generally, in terms of resistance classification, lower AUSiPC was observed in Red Spanish, mid curves in MD2, Sassilimu and Smooth Cayenne while the highest curve was observed in Victoria in trial one (Figure 8a) and in second trial , similar trend for severity was observed (Figure 8b).

Table 8: Summary of ANOVA for severity of PMWD on 5 pineapple variety grown at Kyambogo University, 2017/2018

		Trial one(November – December 2017)			
		15DAI	30DAI	45DAI	60DAI
Source of Variation	Degrees of freedom				
Pineapple variety	4	12.0400***	13.3000***	10.4400***	9.7000***
Residual	16	0.8400	0.2500	0.3650	0.2750
		Trial two (March-April 2018)			
Source of Variation	Degrees of freedom				
Pineapple variety	4	13.0600***	12.3400***	11.1400**	10.9400***
Residual	16	0.3850	0.3400	0.1900	0.1400

Figure 11 a and b: Mean disease severity of PMWD trial 1 and 2 on 5 pineapple variety grown at Kyambogo University, 2017/ 2018





5.4 Discussion

Pineapple varieties show variation in the levels of resistance to PMWD, the lower area under severity index progress curve (AUSiPC) observed in Red Spanish represented low disease progress and greater resistance to PMWD the higher area under severity index progress curve (AUSiPC) observed in Victoria indicated rapid disease progress and hence higher susceptibility. However, MD2, Sassilimu and Smooth Cayenne curves appeared in between Red Spanish and Victoria probably indicating mild resistance to PMWD. The objective of the present study was to identify sources of resistance to PMWD in selected pineapple varieties in Uganda. Here the study revealed that, Victoria variety of pineapple was more susceptible to PMWD red Spanish was more resistant to PMWD. The reason for the variation in the resistance of varieties to PMWD was not clear but it is suggested to be as a result of genetic difference between the varieties. However, the reason would needs clarification in another field study for pineapple variety resistance. Xiaodan and Ray (2018) in a study of genome of pineapple disease resistance gene observed that, susceptibility of the cultivated pineapple variety was due to the less genetic diversity. In fact, reports have shown that cultivated pineapple varieties tend to be vulnerable compared to their wild counterpart varieties that had greater gene diversity. Earlier, Rohrbach and Donald (2003) reported that some varieties of pineapple such as Smooth cayenne had mild resistance to pineapple mealybug wilt disease and other pineapple diseases such as fusariosis and fruit core rot. Accordingly, Sapong *et al.* (2018) in a survey conducted in Ghana reported that 96.6% of the respondents reported PMWD had less effect on MD2 but the disease had more effect on Smooth cayenne and Victoria.

Generally, Ocwa *et al.* (2016) reported that resistance to pineapple disease by pineapple varieties can be explained by a number of factors including genetic constitution, age of the plant, and nature

of pathogen and predisposing environmental conditions. Additionally, Kus *et al.* (2002) observed that as plants mature and the leaf ages, the plants become increasingly resistant in symptom development to normally virulent pathogens. The degree of host plant susceptibility or resistance to pathogen infection dependent partially on a number of factors including the insect vector feeding mouth parts, it's probing behavior and in some cases on the presence of the phenolic compounds which are associated with the defense of the plant (Marie and Eric.; 2007 Amita *et al.* 2010). It should be mentioned here that by the time of this study was undertaken, there was no study done to show the pineapple varieties resistant to wilt in Uganda and elsewhere hence this study provides basis for future study in screening more pineapple varieties for resistance to wilt disease. It was again important to mention here that, this study did not concisely specify the factors(s) justifying the variation in resistance to PMWD. Despite this gap, this study has provided insight that resistance to PMWD is available in central Uganda though there is need to screen more germplasm and confirm other cultivars that are resistant besides red Spanish. Additionally, Red Spanish can be used as donor material for further breeding works.

CHAPTER SIX:

GENERAL DISCUSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General discussion

Pineapple mealybug wilt associated virus 1 (PMWaV-1) was identified as the causal virus of PMWD in central Uganda although the other PMWaVs have been reported elsewhere. Although, the study covered only four districts in Uganda, it represents the first comprehensive study on the identification and characterization of casual viruses of PMWD. According to Sether (2002, 2005), the pineapple mealybug wilt associated virus-1 (PMWaV-1) was noted to cause significant yield reduction without the mealybug feeding hence the pineapple fail to develop the symptoms of the disease. However, in this study, it was not possible to assess the effects of PMWaV-1 on growth and yield performance of pineapple. The mealybug action threshold for transmission of PMWD have been studied and 10 and 15 mealybugs have been identified as the threshold to the transmission of PMWD, due to the high incidence and severity. Elsewhere it was reported that as low as one mealybug was capable to cause infection. This study only covered few groups of mealybugs, however it provided information about the mealybug population that may cause serious transmission to the pineapple plant. According to (Roivainen, 1976) transmission of PMWaVs was efficiency and higher as the number of mealybugs increases and high virus retention (VR) and longer virus acquisition access period (AAP). Similarly (Sether *et al.* 1998) reported that a higher (10-20) mealybug threshold transmitted more virus than lower action threshold of the mealybugs.

Five pineapple varieties have been screened for their resistance to PMWD in Uganda and the different varieties showed varying levels of severity. Although selected varieties were used, the

study provided an understanding and insight of or knowledge on the different varieties of pineapple susceptibility to wilt disease. Red Spanish variety showed low susceptibility to PMWD while Victoria was more susceptible to wilt MD2 smooth cayenne and *sassirimu* had moderate susceptibility. Accordingly to (Sapong *et al.* 2018) MD2 variety was more tolerant to PMWD than Smooth cayenne and Victoria. The disease had less effect on MD2 than the varieties mentioned above. Rohrbach and Donald (2003) reported that Smooth cayenne had mild resistance to pineapple mealybug wilt disease and other pineapple diseases such as fusariosis and fruit core rot. The control of viral diseases according to (Leal, 2003) was possible through the use of resistant varieties and (John *et al.* 2003) reported that, a high diversity within the genotypes of the crops provides better management of viral disease through resistance.

6.2 Conclusions

i. Pineapple mealybug wilt associated virus one (PMWaV-1) was identified as the causal virus causing PMWD. In fact, 99% of the variation in the pineapple mealybug wilt associated virus was within the virus population and only 1% of the variation was between the populations.

ii. The mealybug action threshold for the transmission of PMWD ranged between 10 and 15. In fact, the transmission of PMWD was associated with higher mealybug number/population as opposed to low number.

iii. Varying degree of resistance to PMWD was observed among the pineapple varieties with Red Spanish being resistant, MD2, Sassilimu and Smooth Cayenne moderately resistant and Victoria being susceptible

6.3 Recommendations

From the findings of the study and the conclusions, the followings recommendations were advanced.

i. Another diversity study with large samples collected far and wide in the different geographical regions of Uganda is recommended to adequately conclude on the diversity of viruses causing PMWD in Uganda
ii. Mealybug action threshold of 15 can be used for screening PMWD resistance.
iii. Molecular studies may be necessary to provide additional insights and understanding the mechanism of resistance to PMWD.
iv. The variety Red Spanish should be grown by farmers as it showed high resistance to PMWD.

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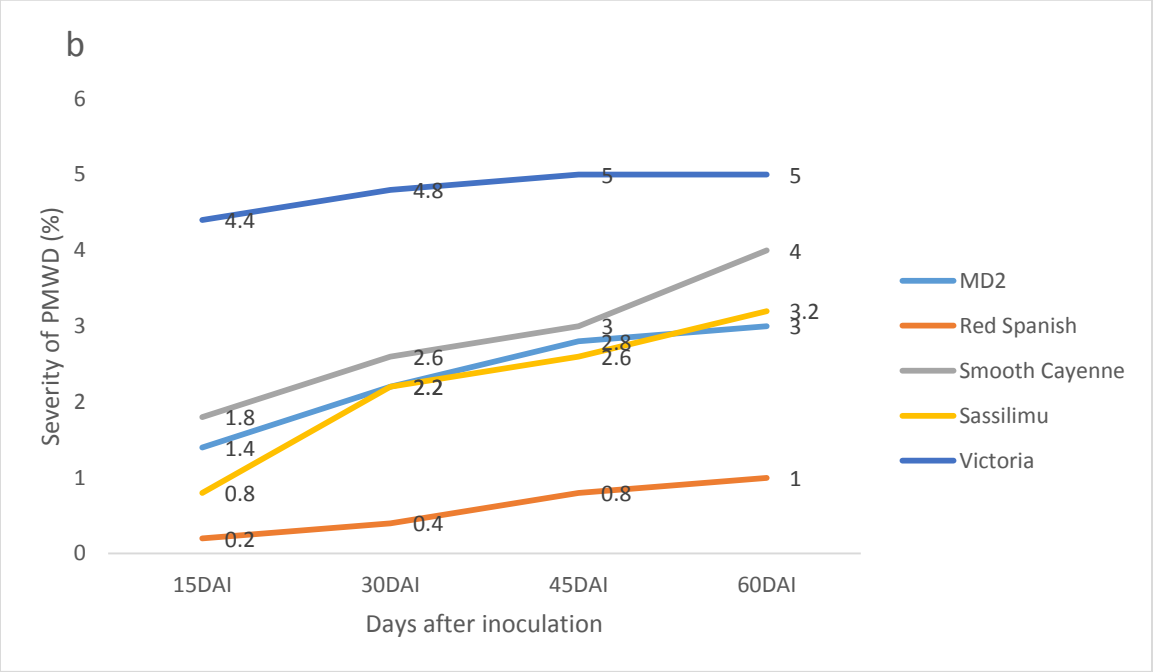
APPENDICES.

Appendix: 1 **AUSiPC of PMWD for five Pineapple varieties in second trial.**



Appendix: 2 BLAST result.

Genetic identity of pineapple mealybug wilt associated virus from central Uganda.

S/no	Isolate	% Query Coverage	E-value	% Identity	GI-Accessions	Identification
1	LU001	19	0.0	97	HG940514.1	Pineapple mealybug wilt-associated virus 1
2	LU002	19	0.0	98	HG940514.1	Pineapple mealybug wilt-associated virus 1
3	LU003	34	0.0	98	HG940514.1	Pineapple mealybug wilt-associated virus 1
4	LU004	80	0.0	99	HG940514.1	Pineapple mealybug wilt-associated virus 1
5	MU005	34	0.0	94	HG940514.1	Pineapple mealybug wilt-associated virus 1
6	MU006	22	0.0	99	HG940514.1	Pineapple mealybug wilt-associated virus 1
7	MU007	88	0.0	98	HG940514.1	Pineapple mealybug wilt-associated virus 1
8	KA008	19	0.0	95	HG940514.1	Pineapple mealybug wilt-associated virus 1
9	KA009	35	0.0	98	HG940514.1	Pineapple mealybug wilt-associated virus 1
10	KA010	21	0.0	97	HG940514.1	Pineapple mealybug wilt-associated virus 1
11	KA011	30	0.0	96	HG940514.1	Pineapple mealybug wilt-associated virus 1
12	MA012	32	0.0	99	HQ129930.1	Pineapple mealybug wilt-associated virus 1
13	MA013	16	0.0	97	HG940514.1	Pineapple mealybug wilt-associated virus 1

Appendix 3: Aligned sequences.

Appendix 4; RNA EXTRACTION PCR RESULT SHEET (AMPLICONES +VE AND –VE)

SAMPLE CODES	DISTRICTS	PMWaV1	PMWaV2	BOTH 1&2
LWR 1	LUWERO	-	-	-
LWR 2		+	-	-
LWR 3		+	-	-
LWR 4		-	-	-
LWR 5		-	-	-
LWR 6		+	-	-
LWR 7		-	-	-
LWR 8		+	-	-
LWR 9		+	-	-
LWR 10		-	-	-
LWR 11		-	-	-
LWR 12		+	-	-
LWR 13		-	-	-
LWR 14		-	-	-
LWR 15		+	-	-
LWR 16		+	-	-
LWR 17		+	-	-
MKN 18	MUKONO	+	-	-
MKN 19		+	-	-
MKN 20		-	-	-
MKN 21		-	-	-
MKN 22		+	-	-
MKN 23		-	-	-
KYG 24	KAYUNGA	-	-	-
KYG 25		+	-	-
KYG 26		-	-	-
KYG 27		-	-	-
KYG 28		+	-	-
KYG 29		-	-	-
KYG 30		-	-	-
KYG 31		-	-	-
KYG 32		-	-	-
KYG 33		+	-	-
KYG 34		-	-	-
KYG 35		+	-	-
KYG 36		-	-	-
MSK 37	MASAKA	-	-	-
MSK 38		-	-	-
MSK 39		+	-	-
MSK 40		+	-	-
MSK 41		-	-	-
MSK 42		+	-	-
MSK 43		-	-	-
MSK 44		-	-	-

+ Means sample tested positive for the virus one.

- Means sample tested negative for the virus one