Transmission of a New *Cowpea Aphid Borne Mosaic Virus* Isolate in Passion Fruits

Kiptui Lucy[1*], Kilalo Dora[2] and Toroitich Faith[1]
[1]*Department of Biological Sciences, Egerton University, P.O Box 536 Egerton, Kenya*
[2]*Department of Plant Science and Crop Protection, University of Nairobi, P.O Box 29053, Kangemi, Kenya*
**Corresponding author:lujekip@gmail.com*

Received	Reviewed	Accepted	Published
27th August 2019	18th October 2019	21st November 2019	6th January, 2020

Abstract

Passion fruit woodiness is an important potyviral disease affecting passion fruits in the world. The causative agent for this disease in Kenya is *Cowpea aphid borne mosaic virus*. However, this disease manifestation in some fields at the present is different in regards to disease severity and symptomatology from the commonly known variant. The newly identified isolate was named CABMV virus isolate1, while the commonly known one was named virus isolate 2. Currently, information on CABMV isolate 1 is scarce relative to that of CABMV isolate 2, which has been studied widely. This study was carried out to determine whether virus isolate 1 is transmitted by seed and select aphid species. The select aphid species are pest of crops grown near or alongside passion fruits in most Kenyan farms. For seed transmission test, mature seeds were isolated from fruits harvested from infected passion fruits plants maintained in a greenhouse. Thereafter, the seeds were planted in a nursery bed which was screened against aphids. The resultant seedlings had no symptoms associated with virus isolate 1. Furthermore, using reverse transcription–polymerase chain reaction, seed transmission rate of 0% was observed, showing that this isolate 1 is not transmitted by seed. Aphid transmission test was carried out in passion fruit plants using aphids from farmers' fields in Njoro. Four aphid species which are *Acyrthosiphon pisum, Aphis fabae, Schizaphis graminum* and *Myzus persicae* were selected for the study. The aphids were allowed 30 minutes virus acquisition period on virus isolate 1 infected passion fruits plants. Thereafter, the aphids were left to feed on healthy test plant for inoculation to take place. Symptoms development on healthy plants signified successful transmission by the aphids. Aphid transmission test results indicated that *Myzus persicae, Acyrthosiphon pisum,* and *Aphis fabae* successfully transmitted the virus.

Key words: Acquisition, Aphids, Passiflora, Passion fruit woodiness, Seed Transmission.

Introduction

Passion fruit is a high market value horticultural crop in Kenya and its production is of high economic importance (Wangungu *et al*., 2014; Match Maker Associates (2017). The fruit is mostly grown for wealth generation and livelihoods improvement since it has a fast period of maturity, which is only 9 months and it requires minimum labour and land space (Wangungu *et al*., 2014). However, there has been a decline in passion fruit production due to pests and diseases, of importance is passion fruit woodiness disease which accounts for 80- 100 % yield loss (Otipa *et al*., 2008). This pathogen is therefore of great economic importance to farmers.

Passion fruit woodiness disease is widely spread in passion fruit growing areas in Kenya and it is the most important viral disease affecting passion fruit orchard in the world. In East Africa, Passion fruit woodiness virus (PWV), Cowpea aphid-borne mosaic Virus and a more recently identified Ugandan passiflora virus (UPV) (Ochwo-Ssemakula *et al*., 2012; Cerqueira-Silva *et al.*, 2014) are the known causal agents. Passion fruit woodiness disease greatly reduces the quantity and quality of fruit, therefore, causing significant loss to farmers. The disease generally causes woodiness of the fruit, which becomes dwarfed, and loses much of the pericarp, therefore becoming unmarketable. It also reduces leaf area thereby decreasing productivity and shortens the economic lifespan of the orchard (Trevisan *et al*., 2006). The most common symptoms in the field include; stunted growth, leaf mottling, leaf mosaic, vein clearing, and leaf chlorosis (Novaes and Rezende, 2003; Cerqueira-Silva *et al*., 2014).

Generally, all viruses exhibiting similar symptom in passion fruit belong to the family *potyviridae* including *East Asian Passiflora virus* (EAPV), CABMV and PWV. These viruses are transmitted by aphid vectors among other means such as mechanical transmission during grafting and pruning. Limited information on CABMV strains hinders the development of an effective control strategy for this economically important virus. In addition, lack of understanding of this disease attributes to its high prevalence in Kenya at the present, thus there is a need to verify its mode of transmission. This study was carried out to determine the specific aphid vectors for the new virus isolate observed to cause unique and more severe symptoms in the field (Kiptui *et al*, In press), and whether seeds transmit this virus. Since aphid vectors do not colonize passion fruit plant, this research will also

provide an insight into the possible alternate host of the vectors that transmit the virus.

Materials and Methods

Seed Transmission Test

Raising of Seedlings

Passion fruit seeds were isolated from woodiness disease infected plants maintained in a screen house in the College of Agriculture and Veterinary Sciences, University of Nairobi and infected farms around Njoro farms in Nakuru County. The seeds were sown in separate nursery beds protected by screens nets against aphids in the garden. After two months of constant watering and weed control, one hundred and twenty seedlings were transplanted into pots and taken to a glass house at Biological Sciences Department, Egerton University. At four leaf stage after emergence (Novaes and Rezende, 2003), a leaf from each seedling was plucked and tested for the presence of each virus using reverse transcriptase polymerase chain reaction coupled with gel electrophoresis. Virus presence in the seeds was also confirmed using RT-PCR.

Extraction of RNA

The RNA was extracted using "ZR plant RNA miniprep kit ^{tm}" (Zymo Research, USA) according to the manufacturer's protocol. Thereafter, complementary deoxyribose nucleic acid was extracted from the RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit (California), and the cDNA obtained was amplified using Thermo Scientific PCR kit (California) as per the manufacturer instructions. A total of 20µL of the PCR reaction was made for each sample of CABMV isolate 1 cDNA synthesized strand (7.75 µL sterile distilled water, 0.25 µL Taq polymerase enzyme, 0.5 µL F primer, 0.5 µL R primer, 5 µL PCR buffer, 1 µL DNTP and 2 µL cDNA). Coat protein specific CABMV virus primers used were:

Forward: ACTGAACTCAAAAAGCTTTATTATACC;
Reverse: GTAATCACCTAGGCATGTAGA.

The PCR profile used was an initial denaturing step of 94° C for 5 minutes, followed by 35 cycles of 94° C for 30 seconds denaturation, 58° C for 30 seconds annealing and 72° C for 45 seconds extension, followed by a 72° C final extension for 7 minutes, and a final hold step at 4° C. PCR products was separated on a 1.5% TBE agarose gel.

Gel Electrophoresis

Gel electrophoresis was done to separate DNA molecules present in order to determine the presence of CABMV virus. Agarose gel was prepared by dissolving 1.5g of agar in 100 ml of TBE buffer then liquefied by heating in a microwave and cooled; thereafter 5 µL ethidium bromide was added and swirled. Agarose was then dispensed in a combed casting tray and left for 30 min to solidify, thereafter the gel was transferred into the tank and comb removed. In the first well, a standard 1kb gene ruler ladder was placed. One microlitre loading dye was then added to 5 µL samples then loaded to the rest of the well; the voltage was adjusted to 100v then switched on for 1 hour. The gel was transferred to UV transilluminator for viewing. Scoring was done visually based on the presence or absence of bands.

Aphids' Transmissibility Test

Transmission of CABMV Isolate 1

To determine the potential aphid vectors of virus isolate 1, experiments were carried out under glasshouse condition at Egerton University. Complete randomized design was used in setting out the experiments. *Schizaphis graminum* (wheat aphids), *Acyrthosiphon pisum* (pea aphids), *Myzus persicae* (green peach aphid) and *Aphis fabae* (bean aphid) were collected and reared in their respective host plants, these aphids were chosen for this experiment because they are abundant in most passion fruit producing areas in Kenya. Ten healthy plants were used for each treatment (aphids' species) and replicated 3 times. The aphids were gently removed from the host plant using camel brush and placed on petri-dishes with moist Whatman filter paper for 60 minutes starvation period. Freshly cut leaf discs from CABMV isolate 1 infected passion fruit were then given to the aphids in the same petri-dishes to allow virus acquisition for 30 minutes. Thereafter, the aphids were collected and transferred onto healthy passion fruit plants. Five aphids per plant were used (Green, 1991; Chang, 1992; Noveas and Rezende, 2003).

The aphids were allowed 4-5 hours inoculation period then killed with an insecticide (cypermethrin). The plants were then placed in an isolated area in the glass house and watered regularly. Non-viruliferous aphids fed on healthy passion fruit plants served as negative control, whereas plants mechanically inoculated with virus isolate 1 were the positive controls. Symptoms development like leaf mosaic, lesions, leaf deformations, and tissue necrosis after inoculation indicated successful transmission by aphid vector. Symptoms development were observed over 4 weeks.

DAS- ELISA Confirmatory Test for CABMV Isolate 1 Presence in *Passiflora edulis* plants showing symptoms after aphid inoculation

This test was conducted according to Clark and Adams (1977) procedure. The wells were coated with Poty virus monoclonal antibody diluted in coating buffer at a ratio of 1:1000. The plates were incubated at 37 °C for 4 hours. Thereafter, the plates were washed using PBS-Tween and blotted dry using a tissue paper. This washing step was repeated 3 times. The plant parts (roots, stem, and leaves) were grounded in extraction buffer (0.02 M phosphate buffer, pH 7.4) in a ratio of 1:20 then sieved through cheesecloth to extract the virus. To the wells, 200 ml aliquots of the test samples were added into the plates. Thereafter, the plates were incubated overnight at 4 °C. The plates were then washed using PBS-Tween three times. To the well 200 ml aliquots of enzyme conjugate was added to the wells. Then the plates were incubated at 37 °C for 2 hours. Washing was done three times before addition of 200 µl aliquots of freshly prepared substrate (1 mg /ml para-nitrophenyl- phosphate in substrate buffer) to each well. The plates were then covered and incubated at 37°C for 60 minutes. The results were read using an automated photometer (ELISA-reader).

Statistical Analyses

The difference in the rate of transmission between the aphids' species was determined by one way analysis of variance using SAS statistical package. This was done in order to determine the efficacy of *Schizaphis graminum* (wheat aphids) and *Acyrthosiphon pisum* (pea aphids), *Myzus persicae* (green peach aphid) and *Aphis fabae* (bean aphids) in virus isolate 1 transmission. Least Significance Differences was used in the separation of means.

Results and Discussion

In the glasshouse the seeds germination was good and the seedlings appeared healthy as they grew vigorously. Out of 120 seedlings, four seedlings had leaves with yellowish green colour but no woodiness disease was evident. Reverse transcriptase carried out had all plants testing negative for *Cowpea Aphid Borne Mosaic Virus*. On running the samples through gel electrophoresis, no bands were formed on the wells placed with the samples (figure 1). However, the positive control which was a leaf infected with CABMV alongside the samples showed a band of around 650 base pairs as shown in figure 1 below.

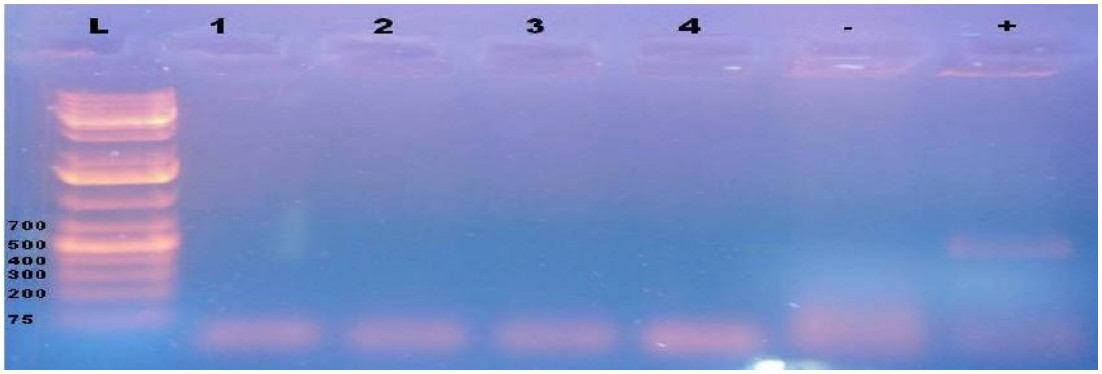

Figure 1: PCR amplification products of CABMV; 1, 2, 3, 4 are the first four seedlings testing negative for CABMV. Negative control (-) is a sample of disease free seedlings. Band size was compared with a 1kb ladder (L). The positive control (+) formed a band at 650 bp, which is the expected size for CABMV virus. All the seedlings tested showed similar results.

Seeds play an important role in the dissemination of some plant viruses. Nevertheless, not all infected seeds lead to seedlings infection (Konate, Sarra and Traore, 2001). These results indicated that the *CABMV* isolate1 is not transmitted through infected seeds. Other virus pathogens have also been reported to be seed -borne but not seed transmissible (Zhou, Chen, Wang, Li, Tang, and Zhou, 2015). For instance, Zucchini yellow mosaic virus (ZYMV) (Desbiez, and Lecoq, 1997), rice yellow mottle virus (RYMV) (Konate *et al.*, 2001).

Evidence from this study suggests that virus isolate 1 that causes woodiness disease is not vertically transmitted by seeds. Therefore, certification of nurseries rather than seeds in a bid to control the virus is necessary. In the management of other seed-borne viruses, standardized methods for testing virus methods should not be restricted to serological and immunological test only, because they detect the presence or absence of a virus antigen regardless of the viability. Hence, the efficacy of seed assays test should be ascertained by plant out experiments before adoption and standardization are done. Seeds are mostly the primary source of inoculum which may result in rapid virus spread by the aphids' vector to cause epidemics (Maule and Wang, 1996; Simmons and Munkvold, 2014). Infection of plants at a younger stage causes more loss that when the plants are older (Konate *et al*., 200l).

The aphid species which transmitted CABMV isolate 1 from this study are *M. persicae, A. fabae* and *A. pisum.* The degree of transmission varied from one aphid species to another with *M. persicae* being the most efficient (Table 1).

Table 1: Efficacy of select aphids in the transmission of CABMV isolate 1 in passion fruits

Species	**t grouping**	Mean of test plants showing woodiness symptoms
Positive control	a	7.3333
Myzus persicae	a	7.0000
Aphis fabae	ab	6.3333
Acyrthosiphon pisum	b	5.3333
Schizaphis graminum	c	1.0000
Negative control	c	0.0000
Lsd (p<0.05)	1.5579	
F value	42.14	

Mechanically inoculated plants served as a positive control, while the negative control was healthy plants. Significance at $p<0.05$.

Based on this research *M. persicae* has the highest ability to transmit CABMV isolate 1 followed by *A. fabae and A. pisum* consecutively. The transmission ability of *M. persicae* and *A. fabae* was not statistically different. However, there was a significant difference in the rate of transmission between *A. pisum* and *M. persicae. Schizaphis graminum* has a minor importance in virus isolate 1 transmission since it has no significant difference with the control used. Based on ELISA test, all the symptomatic plants were positive for potyviruses confirming transmission ability by *M. persicae*, *A. pisum* and *A. fabae* (Table 2).

Table 2: Absorbance values for transmissibility test samples of CABMV by selected aphid species

Sample number	***Myzus persicae***	***S. graminum***	***Aphis fabae***	***Acyrthosiphon pisum***	**control**
1	1.4565	0.6875	2.4135	0.9245	0.385
2	0.6405	0.491	1.4525	0.771	0.353
3	1.2465	0.385	1.5045	0.7485	0.3325
4	1.259	0.353	1.425	1.338	0.301
5	1.4565	0.3325	1.626	1.7475	0.4035
6	0.626	0.301	1.7375	1.8425	0.37
7	0.58	0.3685	1.569	0.606	0.367
8	2.461	0.5615	0.8135	0.636	0.491
8	1.555	0.606	2.116	0.3875	0.54
10	1.572	0.6465	1.678	0.938	0.583

Note. Absorbance value above 0.7325, which is twice the average of negative control mean absorbance were considered positive while below were considered negative.

These experimental results concur with previous studies which attribute the spread of woodiness virus to aphids and mechanical inoculation through grafting. For instance, CABMV, PWV, *Cucumber mosaic virus* (CMV) and EAPV are not seed transmissible (Nascimento *et al*., 2006; Bashir *et al*., 2002; Ochwo-Ssemakula *et al*., 2012; Kilalo *et al.,* 2013). Transmission of this *Virus* isolate by *M. persicae, A. fabae,* and *A. pisum* confirms that they are among the main species responsible for woodiness disease as affirmed by Kilalo *et al*., (2013) and Garcêz *et al.*, (2015).

Several aphid species such as *Toxoptera citricidus, M. persicae, Aphis solanella, A. fabae* Scopoli, and *Aphis gossypii* have been shown to transmit different strains of woodiness disease viruses (Cerqueira-Silva *et al*., 2014; Garcez *et al.,* 2015). These vectors are not colonizers of passion fruit plants. According to Rodrigues *et al* (2016) seasonal abundance affects the transmission efficiencies of the aphid vector. Analyzed dominance and occurrence indices show that *A. fabae, A. gossypii* and *M. persicae* are the most frequent species that fly over the passion fruit orchards (Nantale *et al.,* 2014). In addition, *M. persicae* and *A. fabae* are efficient virus transmitters because of its polyphagous nature (Rodrigues *et al*., 2016). In the same way, this study results underscore the fact that *M. persicae* and *A. fabae* are important in virus transmission.The absence of transmission by *S. graminum* species can be attributed to lack of structural helper protein that mediates virus retention in the stylets of the aphid vector (Revers and García, 2015).

Conclusion

This study underscores the fact that passion fruit seeds do not transmit CABMV virus isolate 1, and this implies that seed certification may not be necessary in the management of this disease. Additionally, it sufficed the fact that aphids transmit virus isolate 1 and the most efficient aphid vectors are *M. persicae, A. fabae,* and *A. pisum*. Hence, aphid control is important for the management of the disease. Since the aphids, which transmit virus isolate 1 are found in plants commonly intercropped with passion fruits in Kenya, management of passion fruit woodiness disease should include monitoring of aphids in beans, maize, potatoes, and peas grown near passion fruit orchards. Furthermore, plants showing vectors symptoms should be sprayed using insecticides, and aphid resistant plants should be selected in areas where passion fruits are grown.

Acknowledgments

The authors are grateful to the Department of Biochemistry where viral infection studies were conducted and BecA-ILRI through the Swedish International Development Cooperation Agency (SIDA) that partly funded the study. Additionally, they are highly indebted to Ms. Sicily Muchira of University of Nairobi and Kenya Plant Health Inspectorate Services virology staff for guidance accorded when undertaking laboratory work.

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