Research Article

Interaction between Cowpea Aphid-Borne Mosaic Virus Isolates and Its Effect on Passion Fruit Woodiness Disease on *Passiflora edulis* Sims and *Passiflora ligularis* Juss

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In East Africa, passion fruit woodiness disease is caused by potyviruses, among which are Cowpea Aphid-Borne Mosaic Virus (CABMV) and Uganda Passiflora Virus (UPV). Previous studies suggest that synergistic interaction of viruses causes mild or severe outcomes of the disease. However, mixed infections of these viruses have not been documented. The aim of this study was to determine the effects of the interaction between two *CABMV* isolates causing passion fruit woodiness disease in Kenya. Healthy plants were mechanically inoculated at six-leaf stage. Four treatments were used: *CABMV* isolate 1, *CABMV* isolate 2, mixture of the two isolates, and noninoculated control. The test plants were maintained in a screen house, and data on symptom severity and rate of disease development were recorded. ELISA test was conducted to confirm virus presence and distribution. The results indicate that the *CABMV* isolates used are systemic. There were various differences in disease progression, which was faster in the sweet passion variety than in the purple variety. However, the latter had a higher disease severity. Coinfected plants expressed severe symptoms compared to the singly infected ones, indicating synergistic interaction between the viral isolates. This implies that coinfection has adverse impact resulting in higher economic losses.

1. Introduction

Passion fruit is an economical viable crop, grown in Kenya, to improve livelihoods of small holder farmers. The crop is among the top three exported fruits. It earns Kenya about Sh1.64 billion annually in foreign exchange [1]. *Passiflora ligularis* (sweet passion) and *Passiflora edulis* (purple passion) are the two major species grown in Kenya. These varieties are susceptible to CABMV [2]. The key species commonly grown for export in Kenya is *Passiflora edulis*. Viral diseases in passion fruit production are of great concern because they affect the fruit quality and quantity and the orchard age wherever the fruit is grown [3]. In Kenya, woodiness disease is prevalent in passion fruit orchards and can cause losses of up to 90% or even as high as 100% in the presence of other fungal diseases such as fusarium wilt [4]. Virus pathogens responsible for woodiness disease include Cowpea Aphid-Borne Mosaic Virus (CABMV), Passion Fruit Woodiness Virus (PWV), and Uganda Passiflora Virus (UPV) [5, 6]. However, country-wide surveys carried out in 2008–2010 in Kenya showed that Passion Fruit Woodiness Virus (PWV) was not present and instead indicated a high prevalence and incidence of CABMV in passion fruit orchards in Kenya [3]. It had earlier been assumed that the variation of disease symptoms observed was due to a different novel potyvirus, like UPV, but sequencing work done on woodiness virus isolates in Kenya by Kilalo (2012, unpublished) indicated the presence of CABMV only in the passion plants showing different symptoms. Thus, there is an assumption that several CABMV isolates or strains could exist in the country.

It is common to find multiple viruses infecting a single host plant [7], and in such cases, the effects of the disease are compounded [8]. Viral coinfection may lead to symptoms exacerbation or plant protection depending on whether the virus interaction is synergistic or antagonistic [9]. The effects of synergistic interaction have huge economic and epidemiological implications because they aggravate virus symptoms consequently causing significant production losses [10].

While viruses occurring in the same host interact with each other to influence plant host interaction [8], most research has focused on their individual effects. However, considering that mixed infections have a greater impact on productivity, they deserve attention too [11]. In several other commercial crops such as maize, pepper, and watermelons, synergy between potyviruses in mixed infections has been widely reported [12]. However, information on mixed-virus infections and their effects on passion fruits is limited.

When in a mixture, viruses may recombine and evolve, producing more virulent strains which differ from known populations. This necessitates the need for frequent identification of emergent viruses and strains [8]. Therefore, the development of effective disease control strategies and diagnostic tools requires an informed knowledge on disease development. The interest of this research was to test the effects of interaction between two CABMV isolates that cause woodiness disease in Kenya on type of symptoms induced and symptom severity.

2. Materials and Methods

2.1. Infected Plant Materials (Virus Inocula). Plant leaves showing passion fruit woodiness disease symptoms were obtained from diseased plants maintained in a greenhouse at the University of Nairobi, College of Agriculture and Veterinary Sciences and farmers' field in Embu where previous collections had been made. The plants were tested for the presence of CABMV using ELISA. Only the farmers' field samples with CABMV were used in this study. Deep sequencing of the samples maintained in the green house at the University of Nairobi and in the farmers' field had revealed the presence of CABMV and no other potyvirus [13]. Virus obtained from the farmers' field was named virus isolate 1, while that obtained from the greenhouse was named virus isolate 2. These two woodiness disease causing viruses induced distinct symptoms and severity to passion fruit plants.

2.2. Passion Fruit Seedlings. The seeds of *P. ligularis* and *P. edulis* were obtained from Nakuru market and sown in nursery bed protected by screen nets and maintained underrequisite agricultural practices such as constant watering and weed control. After two months, the seedlings were transplanted into pots containing a mixture of sterilized gravel, farmyard manure, and soil in ratio of $2 : 1 : 1$, and 70 g diammonium phosphate fertilizer. Thereafter, the plants were maintained in a glass house. At four-leaf stage after emergence, the plants were tested for CABMV presence

using RT-PCR, following the manufacturer's protocol (Invitrogen, California).

Briefly, RNA was extracted from the leaves using ZR plant RNA miniprep kit™, following the manufacturer's instructions (Zymo Research, USA). Thereafter, cDNA was synthesized using the RT-PCR SuperScript™ III Reverse Transcriptase 1st-Strand cDNA kit (Invitrogen, California), following the manufacturer's protocol. The primer used in this synthesis is Reverse 5′-CTGCTGAGGAATTTAAGA-GACAAG-3′. The cDNA obtained was ready for use in a PCR downstream. A total of $20 \mu L$ of the PCR reaction mixture was used for each sample to amplify CABMV and UPV using the cDNA synthesized strands. The primer sequences for Cowpea Aphid-Borne Mosaic Virus were Forward   5′-GATGCAGGCAAGGATAAAGAAAAG-3′ and Reverse 5′ -CTGCTGAGGAATTTAAGAGACAAG- 3′ position 131/739 amplifying a 628 bp product, a partial coat protein gene for CABMV. The Uganda Passiflora Virus primer sequences used were Forward 5′-GCACGAAATT-CAAGAATACCTTAG-3′ and Reverse 5′-GACTTCA-TAAAATCAAATGAGTA-3′ position 558/1039 amplifying a 772 bp product, a partial coat protein gene of UPV. This was done to ensure that the seedlings were free from all viruses causing woodiness disease in East Africa. The mixture was made of $7.75 \mu L$ sterile distilled water, $0.25 \mu L$ Taq polymerase enzyme, $0.5 \mu L$ F primer, $0.5 \mu L$ R primer, $5 \mu L$ PCR buffer, $1 \mu L$ DNTP, and $2 \mu L$ cDNA. The PCR profile consisted of an initial denaturing step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30-second denaturation, 58°C for 30-second annealing, and 72°C for 45-second extension, followed by a 72°C final extension for 7 minutes, and a final hold step at 4°C. The UPV primers were annealed at 48°C for 1 minute. The PCR products were then separated on a 1.5% agarose gel. Virus-free test plants were then used in the virus transmission test.

2.3. Virus Transmission Test. The experiment was carried out in a completely randomized design using 10 replicates per treatment. Four treatments (CABMV isolate 1, CABMV isolate 2, a mixture of the two isolates, and a buffer only for negative control) were used. Transmission test was carried out based on the procedure by Freitas and Rezende [2] as follows. Single-virus extracts from infected plants were prepared by grinding infected leaves in phosphate buffer at a ratio of $1 : 10$ (tissue weight : buffer volume). Mixed-virus inocula were then prepared by grinding equal weights of the leaves infected with woodiness virus in phosphate buffer. Carborundum powder was dusted onto two leaves per plant and the virus sap rubbed to it using the forefinger. The inoculated leaves were washed gently with distilled water after 5 minutes. Data on disease symptoms was collected once weekly for eight weeks after inoculation. The disease severity was measured based on a 1 to 5 scale, which was slightly modified from Novaes and Rezende [14] as follows: plants without mosaic symptoms and leaf deformations and with good development of leaves (1), those with slight symptoms (~25% of the leaf area with small mosaic) (2), those with moderate symptoms with mild mosaic and no leaf

deformation (∼50% of the leaf area with mosaic) (3), those with strong symptoms (∼75% of the leaf area with mosaic) (4), and those with leaves with severe mosaic, blisters, and leaf deformations and later systemic necrosis to death (5).

2.4. Determination of the Presence and Distribution of CABMV Isolates.

ELISA test was done twice in order to elucidate whether two different viruses causing woodiness disease occupied the same niche in commonly grown Kenyan variety. The presence of potyvirus in plant parts (stems, leaves, and roots) was first confirmed with general potyvirus antisera using Antigen-Coated-Plate ELISA (ACP-ELISA) as per the procedure of Clark and Adams [15]. This was to confirm whether there were other potyviruses other than CABMV. Thereafter, specific CABMV antisera (DAS-ELISA) was used to determine/confirm the presence of the virus within the test samples and especially those that tested positive for the general potyvirus test. The ELISA kits (DSMZ Institution for Microbiology, Germany) were used as per the manufacturers' instructions. In total, the roots, stem, and leaves for four plant samples showing positive virus symptoms were used for each treatment.

2.5. Test for General Potyvirus Presence in the Samples.

The presence of potyviruses was conducted according to the procedure of Clark and Adams [15]. The roots, stem, and leaves of each test plant were crushed in extraction buffer. Thereafter, 200 ul aliquots of test samples were added to the wells in duplicate. The plates were then incubated overnight at 4°C. The plates were washed in PBS-Tween. Blocking was done to the wells by adding 200 ul of PBS-Tween containing 2% skimmed milk to each well and incubating for 30 minutes at 37°C. The blocking solution was removed, and the plates were tapped dry. Monoclonal antibody was added to the wells after diluting it in a conjugate buffer at a ratio of 1 : 1000. Incubation was then done at 37°C for 30 minutes followed by washing in PBS-Tween three times. RAM-AP was diluted in a conjugate buffer at a ratio of 1 : 1000; then aliquots of 200 ul were added to each well before incubation for an hour at 37°C. The plates were washed with washing buffer 3 times after the incubation period. After drying the plates, P-nitrophenyl phosphate (1 mg/ml) was added to the wells, and the plates were incubated at 37°C for 30 to 60 minutes.

2.6. Test for CABMV in the Samples.

The presence of CABMV was tested following the procedure conducted by Clark and Adams [15]. The wells were coated with CABMV antibody diluted in coating buffer at a ratio of 1 : 1000 and incubated at 37°C for 4 hours. Thereafter, the plates were washed using PBS-Tween and blotted dry. This washing step was repeated thrice. The plant parts (roots, stem, and leaves) were ground in extraction buffer (0.02 M phosphate buffer, pH 7.4) separately at a ratio of 1 : 20 and then sieved through cheesecloth to extract the virus [16]. An aliquot (200 ml) of the test samples was added to duplicate wells. Since the test was done in duplicate, samples with mean absorbance values

more than two times the average of the negative control were considered positive.

2.7. Data Analysis.

A completely randomized design was used in this study. Data collected was analyzed by one-way analysis of variance (ANOVA) PROC GLM, using SAS (9.7) to determine the difference in the average values of disease symptoms severity among the treatments. The most susceptible passion fruit variety was determined by comparing their mean severity score. The means were compared using LSD test at $P = 0.05$ significance.

3. Results

As a preliminary experiment, 5-month-old test plants were used and only 2 plants per treatment developed the disease symptoms. This rendered the trial unsuccessful. For this reason, this study was conducted using younger plants at six-leaf stage.

All the test plants used for the study were negative for CABMV on RT-PCR test prior to inoculation (Figure 1). Plants treated with CABMV isolate 1 only were the first to show initial disease symptoms after 7 days of inoculation, while those inoculated with virus isolate 2 only showed symptoms 10 days after inoculation and those inoculated with a mixture of the two viruses showed symptoms 14 days after inoculation.

Virus infected plants exhibited typical symptom characteristics of passion fruit woodiness disease which are leaf mosaic and yellowing, leaf puckering, leaf blisters, stunted growth, and hardening of the leaves and stem (from the tip). However, the intensity of the symptoms varied for each virus treatment and plant variety used.

Coinfected plants presented more severe symptoms compared to singly infected ones. In *P. ligularis* variety, coinfection resulted in increased intensity of mosaic symptoms, leaf puckering, followed by intense chlorosis and leaf curling, whereas in *P. edulis,* coinfection resulted in increased leaf rolling, puckering, and blisters formation. Mosaic, reduced leaf size, and severely deformed apical leaves and vines were also observed. In addition, buds formed at the tip were crinkled, and infected leaves dropped after rapid yellowing (Figure 2).

Those infected with singly virus isolate 1 showed vein clearing, deformed leaves, blisters, and golden mosaic. Single inoculation with virus isolate 2 induced rugose leaves with diffused mosaic and leaf blisters (Figure 2). Coinfection resulted in the highest severity score of 3.2679 which was significantly different ($P < 0.05$) from the singly infected viruses (2.9107 and 2.8393) (Table 1). For each virus treatment, disease severity increased with time (Figure 3).

There was a significant difference ($P < 0.05$) in varietal susceptibility to the viruses. The purple (*P. edulis*) variety had a mean severity score of 2.65; thus it was more susceptible than the sweet passion (*P. ligularis*) variety whose score was 2.14. Woodiness disease severity on *P. edulis* variety was significantly higher relative to the *P. ligularis* variety (Table 2).
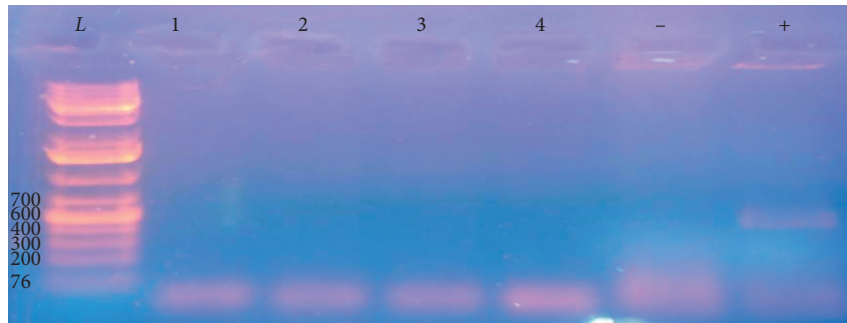
FIGURE 1: PCR amplification products of CABMV; 1, 2, 3, and 4 are the first four seedlings testing negative for CABMV. Negative control (−) is a sample from disease-free seedlings. Band size was compared with a 1 kb ladder (L). The positive control (+) formed a band at 650 bp, which is the expected size for CABMV. It was obtained from diseased plant maintained in a screen house. All the seedlings tested showed similar results.
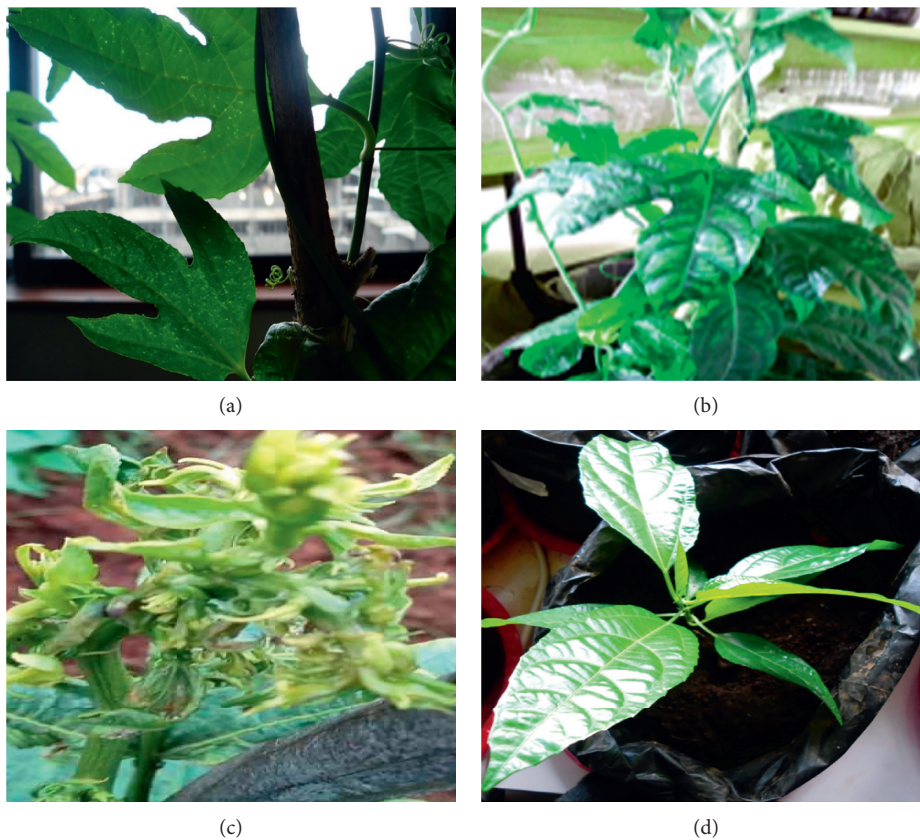

FIGURE 2: Foliar symptoms at eight weeks after inoculation. Diffused leaf mosaic by CABMV isolate 2 (a), concentrated mosaic, rugose leaf, vein clearing, and deformed leaves by CABMV isolate 1 (b), leaf curl, whole leaf mosaic, leaf deformation, blisters formation, and leaf roll by dual infected virus isolates (c), and healthy plant (d).

3.1. Determination of Distribution of CABMV in Different Passion Fruit Plant Parts. There were variations in the distribution of the viruses in the plant parts (Table 3); all the plants which tested positive for general potyvirus antibody were also positive for CABMV antibody. In coinfected plants, 50% of the leaves and the stems were positive for CABMV, while only 20% of the roots were positive. In plants inoculated with CABMV isolate 1, 50% of the roots and leaves tested negative for CABMV and all the stem part tested positive for CABMV. The CABMV isolate 2 had all the leaves and stem testing positive for CABMV, but only 20% of the roots tested positive (Table 4).

4. Discussion

The current findings confirm the susceptibility of commercially grown passion fruit species to Cowpea Aphid-Borne Mosaic Virus (CABMV) and also preliminarily report the presence of two distinct CABMV isolates in Kenya whose disease severity is higher with incidences of coinfection. It is

TABLE 1: Effects of CABMV isolates 1 and 2 and the mixture on *P. edulis* based on disease symptom severity.

Treatment	Mean severity score	*t*-grouping
Mixture of CABMV isolate 1 and isolate 2	3.2679	a
CABMV isolate 1	2.9107	b
CABMV isolate 2	2.8393	b
Control	1.0000	c

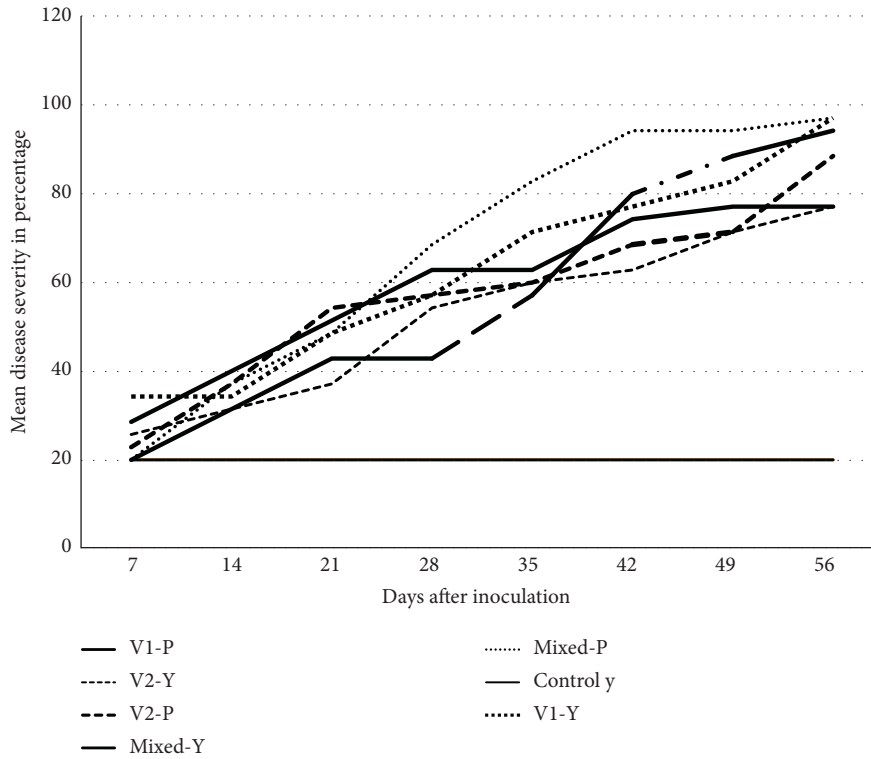Means with the same letter along the column are not significantly different using LSD test at $P = 0.05$.

FIGURE 3: Disease progression in *P. ligularis* and *P. edulis* 28 days after inoculation. y is the sweet passion variety (*P. ligularis*), while p represents the *P. edulis* variety. V1 represents virus isolate 1, V2 is virus isolate 2, and mixed represents a combination of CABMV isolate 1 and CABMV isolate 2.

TABLE 2: Comparison of *P. ligularis* and *P. edulis* passion fruit reaction to a mixture of the two virus isolates causing woodiness disease.

Variety	Mean symptom severity	*t*-grouping
P. edulis	2.65	a
P. ligularis	2.14	b

Means with the same letter along the column are not significantly different.

possible that the observed phenomenon can be attributed to alteration of genes which are not affected in a single-virus infection [10] and possible strain differences that need to be studied further.

In the preliminary studies of this experiment where five-month-old plants were used as test plants, an average of two plants per treatment showed the viruses symptoms 21 days after inoculation implying that disease severity may be influenced by the age of the plants making older plants less susceptible [17].

The findings of this study show that disease severity dramatically increased due to infection with two CABMV isolates pointing to virus isolate synergy. Changes leading to synergy in plants include an increase of both virus titres, an asymmetric increase of one virus titre with no change in the other, and reduction of one virus titre with the other remaining constant [8]. The results corroborate those of Gil-Salas et al. [18] which reported synergistic mixed-virus infection among potyviruses. Other similar synergistic diseases include cassava mosaic disease [19] and the maize lethal necrosis disease [20]. The phenomenon of two or more viruses coexisting in the same plant is not rare [21]. The mixed virus can cause either synergistic or antagonistic interaction [8]. The mixture creates unpredictable consequences on disease epidemiology and biology [21]. However, not all cases of coinfection are detrimental to the plant. For instance, a dual infection involving tomato spotted wilt virus and peanut mottle virus does not result in intensification of the symptom severity [22].

TABLE 3: Determination of potyvirus presence in *P. edulis* plants parts by ACP-ELISA.

Inoculum	Mean absorbance value of plants at 405 nm wavelength		
	Leaves	Stems	Roots
CABMV isolate 1	0.134 positive	0.119 positive	0.1605 positive
	0.286 positive	0.177 positive	0.1155 negative
	0.267 positive	0.234 positive	0.225 positive
	0.124 positive	0.1275 positive	0.1585 positive
CABMV isolate 2	0.135 positive	0.124 positive	0.1025 negative
	0.125 positive	0.135 positive	0.1045 negative
	0.103 negative	0.198 positive	0.4215 positive
	0.160 positive	0.164 positive	0.176 positive
CABMV mix isolates	0.1145 negative	0.109 negative	0.105 negative
	0.9675 positive	0.234 positive	1.9605 positive
	0.1715 positive	0.0905 negative	0.1345 positive
	1.266 positive	0.161 positive	0.156 positive
Control-ve	Mean = 0.0585		

Note. Absorbance value at 450 nm. Samples with mean absorbance values higher than two times the negative control average (healthy plant parts), which is 0.117, were considered positive.

TABLE 4: Determination of CABMV isolate distribution in *P. edulis* by DAS-ELISA, absorbance value for CABMV assay indicates the presence or absence of the virus in different organs.

Plant part	Virus type	Mean absorbance value	Reaction to antisera
Leaves	CABMV 1	0.229	Positive
		0.14	Negative
		0.141	Negative
		0.2145	Positive
	CABMV 2	0.3015	Positive
		0.4895	Positive
		0.239	Positive
		0.2545	Positive
	Mixed	0.1505	Negative
		1.165	Positive
		0.1925	Positive
	Control	0.683	Positive
		0.21275	
Stem	CABMV 1	0.3015	Positive
		0.4895	Positive
		0.239	Positive
		0.2545	Positive
	CABMV2	0.1325	Positive
		0.373	Positive
		0.159	Negative
		0.1995	Positive
	Mixed	0.2405	Positive
		0.164	Negative
		0.283	Positive
	Control	0.1565	Negative
		0.186	
Roots	CABMV 1	0.164	Negative
		0.1335	Positive
		0.2255	Positive
		0.1425	Negative
	CABMV 2	0.288	Positive
		0.3965	Positive
		0.3315	Positive
		0.1485	Negative
	Mixed	0.146	Negative
		0.157	Negative
		0.149	Negative
	Control	0.411	Positive
		0.225	

Note. Absorbance value at 450 nm. Samples with mean absorbance values higher than two times the negative control average (healthy plant parts) were considered positive.

In a mixed-virus infection, some plants maintain the symptoms of the viruses in question, while in others the symptoms are altered. For instance, a combination of pepper golden mosaic virus (PepGMV) and pepper huasteco virus (PHV) elicits different symptoms after interaction [8]. The results of the current study show symptom alteration. The increase of symptoms severity for coinfected plants suggests the presence of cumulative RNA suppressing mechanism from the two virus isolates that facilitate synergism. This phenomenon of symptoms development aggravation in coinfected plants has been reported by Lamichhane and Venturi [9].

In general, the disease severity increased with time in all treatments. However, there was varietal difference in the sweet passion and purple variety in the rate of disease progression. The plants inoculated with the single isolates were the first to be infected compared to the coinfected ones. In fact, coinfected plants first showed symptoms after 14 days of inoculation, while singly infected ones showed symptoms after 7 and 10 days. The plants treated with CABMV isolate 1 were the first to show symptoms followed by CABMV isolate 2 and finally the mixture. This difference in latency period suggests that virus isolate 1 initiates an efficient systemic infection relative to virus isolate 2. When the two virus isolates were mixed, systemic infection was established after two weeks suggesting that virus isolate 2 interfered with virus isolate 1 kinetics by making it slower. However, since the severity of the disease increased faster in

mixed infection after the initial infection, it implies that virus isolate 2 has a negative influence on isolate 1 during the early phase of infection, but later virus isolate 2 facilitates infection systematically. The difference in disease progression among these virus isolates implies that plant resistance to these viruses is dependent on the mechanism that facilitates movement of the pathogen between cells or those that inhibit the process of a specific virus replication in the tissues [8].

The movement of virus from the inoculated leaves to new leaves is slowed down after a while in singly infected plants due to resistance against the virus [18]. However, in mixed-virus infection, virus movement within the plants increased

tremendously after the first symptoms. This phenomenon has been attributed to the suppression of the host plant RNA silencing mechanism [23]. Interaction of viruses occurs as a result of complex processes that include negative interference between involved viruses at certain development stages and synergism in other phases [11]. Alves-Júnior [24] reported similar findings to those of a study of an interaction between tomato yellow spot virus and tomato rugose mosaic virus.

All plants inoculated with the virus isolates tested positive in ELISA test. This indicates that the virus isolates used were strains of CABMV or possibly a very close variant, which could also be a causative agent of woodiness disease in Kenya. In addition, it was observed that the isolates induced different symptoms in the plant. CABMV isolate 1 was more severe than virus isolate 2. The two CABMV isolates 1 and 2 showed unique symptom development in the passion fruit although they belonged to the same virus species. Similar findings have been reported in strains of cassava mosaic disease where different strains were observed to show diverse infection dynamics and contrasting phenotypes [25].

The distribution of CABMV isolate 1 within the plant showed some differences with the other treatments, and this has an implication in detection and sampling. In this case, the plants with leaves testing negative for potyvirus had the stem and roots testing positive. Conventionally, the leaves have been the gold standard for virus detection [26]. However, these results show that the absence of virus in the leaves does not necessarily mean that the plant is virus free. Therefore, a negative leaf test should not be interpreted as a definite diagnosis of this virus isolate. This information helps in the selection of plant parts with high viral load for sampling.

When testing for virus isolate 2, the virus was widely distributed in the plant; thus all the tissues can be used in detection. However, the virus titre (absorbance) value of the leaves was relatively higher than in the roots and the stem, thus making the leaves the best part for diagnostic purposes. Epidemic outbreaks of woodiness disease causing virus can be attributed to the thorough distribution in the stem, roots, and leaves. This is because the viruses can be acquired by the vectors in any part of the infected plant as they feed.

5. Conclusion

The current results have provided a framework for understanding the epidemic of woodiness disease which is currently ongoing in all passion fruit growing areas globally. The disease rapid spread can be attributed to readily available inoculum in all plant parts and increased virus concentration in the plant due to synergistic interaction. This information on the distribution of CABMV in different tissues aids in providing sensitive diagnosis during the early phase of disease development. Therefore, this study outcome is important in the management of passion fruit woodiness disease.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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