DISEASE ASSESSMENT, INFERENCES ON VIRUS MOVEMENT, AND SEEDLING DEVELOPMENT IN TROPICAL PUMPKIN (*CUCURBITA MOSCHATA*) INFECTED WITH *PAPAYA RINGSPOT VIRUS* AND *ZUCCHINI YELLOW MOSAIC VIRUS*

by

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ABSTRACT

Papaya ringspot virus (PRSV) and *Zucchini yellow mosaic virus* (ZYMV) frequently infect tropical pumpkin (*Cucurbita moschata*) in Puerto Rico. Breeding programs depend on the development of efficient and reliable methods of assessing resistance. The goal of this research was to determine the best method to differentiate the level of resistance among genotypes of tropical pumpkin for both PRSV and ZYMV, determine if there are differences in seedling development of *C. moschata* infected with PRSV, ZYMV, or both PRSV+ZYMV, and to evaluate whether 'Menina' can serve as a source of resistance. The cotyledons of seven genotypes were inoculated with PRSV, ZYMV or PRSV+ZYMV. Analysis of the viral serological titer in the first five leaves was performed using ELISA tests. An ELISA reading <0.400 (A_{405nm}) was considered to be negative for presence of virus. Symptoms and fresh and dry weight were taken. Small leaves, intervenal chlorosis, leaf deformation, curled leaves, and mosaic were some of the symptoms observed in susceptible genotypes inoculated with PRSV and ZYMV. Smaller leaves and chlorosis were observed in 'Menina' inoculated with PRSV and ZYMV and no symptoms were observed in 'Nigerian Local'. For PRSV, sampling of the fourth leaf was required to differentiate between resistant versus susceptible genotypes. For ZYMV, leaves 2, 3, and 4 can be used to differentiate resistant from susceptible genotypes. No differences were found in ELISA readings from plants with single versus double inoculation. No differences in fresh and dry weight were found between uninoculated versus inoculated plants. Negative ELISA readings were obtained in the inoculation of susceptible genotypes with sap of inoculated resistant genotypes. 'Menina' was observed to be a useful source of resistance to ZYMV and PRSV. The results of this study can be used when developing a breeding program for resistance to these two viruses.

RESUMEN

Papaya ringspot virus (PRSV) y *Zucchini yellow mosaic virus* (ZYMV) frecuentemente infecta la calabaza tropical (*Cucurbita moschata*) en Puerto Rico. Los programas de mejoramiento dependen del desarrollo de métodos eficientes y confiables para evaluar la resistencia. El objetivo de esta investigación fue determinar el mejor método para diferenciar el nivel de resistencia para PRSV y ZMYV entre los genotipos de calabaza tropical, determinar si existen diferencias en el desarrollo de plántulas de *C. moschata* infectadas con PRSV, ZYMV, o PRSV+ZYMV, y evaluar si 'Menina' puede ser utilizada como una fuente de resistencia. Los cotiledones de siete genotipos fueron inoculados con PRSV, ZYMV o PRSV+ZYMV. Análisis de título serologíco viral en las primeras cinco hojas se realizó mediante pruebas ELISA. Una lectura de ELISA <0.400 (A_{405nm}) se consideró que era negativa para presencia de virus. Se recopiló datos de síntomas, peso fresco y peso seco. Hojas pequeñas, clorosis intervencionista, deformación de la hoja, hojas rizadas y mosaico fueron algunos de los síntomas observados en los genotipos susceptibles inoculados con PRSV y ZYMV. Se observaron hojas pequeñas y clorosis en 'Menina' inoculada con PRSV y ZYMV y no se observaron síntomas en 'Nigerian Local'. Para PRSV, se requirió el muestreo de la cuarta hoja para diferenciar entre genotipos resistentes con susceptibles. Para ZYMV, las hojas 2, 3 y 4 puede ser utilizada para diferenciar genotipos resistentes de los susceptibles. No se encontraron diferencias en las lecturas de ELISA de plantas con la inoculación simple y doble. No se encontraron diferencias en peso fresco y peso seco entre plantas inoculadas y no inoculadas. Se obtuvieron lecturas de ELISA negativas en la inoculación de genotipos susceptibles con savia de genotipos resistentes previamente inoculados. Se observó que 'Menina' fue una fuente útil de resistencia a PRSV y ZYMV. Los resultados de este estudio pueden ser utilizados en el desarrollar en un programa de mejoramiento para la resistencia a estos dos virus.

The sky is the limit. Only we can decide our path and our future.

I want to dedicate this work to my family, especially my mother Linette Vélez-Rodriguez and my father Felix Miranda-Berrios for having always supported me and inspired me to follow my dreams. To my grandmother Sonia Rodríguez because I know that she would be happy to see that one of her grandchildren followed in her footsteps. Finally, to my beautiful dog Mei, for having accompanied me during these years, and having given me so much happiness.

ACKNOWLEDGEMENTS

The greatest changes and contributions to the world are done by hard working people with the capacity to successfully employ teamwork. Working in unison with a team gives space to maximize the work done and to look at things in different perspectives, leading to a much greater result. It is important to always remember that we alone cannot do much, but working together with other people can result in something much greater than expected.

Because of this I would like to give thanks to all the people that made this research happen. First, I want to thank Dr. Linda Wessel-Beaver, for all the guidance and patience, for the knowledge and opportunities that she has provided me during these past few years. To Dr. Jose C. Verle Rodríguez for having taught me to always work hard and always learn and update my knowledge. Also, to show my appreciation to Dr. Feiko Fewerda for all his advice and conversations about how to grow as an individual in a professional field. To my laboratory partner Wilfredo R. Seda-Martínez for having assisted me during the experiments and for all the laughter and wonderful memories that we had in the laboratory and in our Cornell University internship. To Dr. Lydia Rivera Vargas for having allowed me to use her laboratory and equipment. Thanks to the project USDA-NIFA Award No. 2015-51181-24285 (Subaward No. RC105573UPRM (CucCAP, Z295) and USDA-NIFA Hatch Accession No. 1000526 (H443) for funding my research. Finally, I would like to thank all the people at the Department of Agro-Environmental Sciences that helped with this research.

Most importantly, I want to thank my parents for having always been there for me, for having always helped me overcome the obstacles of life, and for all their love. Without them, I would not be the person I am today.

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1 INTRODUCTION

In Puerto Rico, tropical pumpkin *(Cucurbita moschata)* is the second most important vegetable crop in terms of revenue with a production of 300,636 hundredweight, generating a gross income of $6,039,000 (Department of Agriculture of Puerto Rico, 2015). Some of the cultivars of pumpkin produced in Puerto Rico are 'Soler', 'Taina Dorada' and 'Verde Luz'. However, 'Soler' is the cultivar that predominates in plantings in Puerto Rico because of its agronomical characteristics and consumer acceptance. These three pumpkin cultivars have been developed by Dr. Linda Wessel-Beaver at the Agricultural Experiment Station (AES) of the University of Puerto Rico, Mayagüez Campus (UPRM).

A survey conducted in 2001 and 2002 concluded that there was a high incidence of *Papaya ringspot virus* (PRSV) and *Zucchini yellow mosaic virus* (ZYMV) in cucurbits in Puerto Rico (Paz-Carrasco and Wessel-Beaver, 2002). This information was confirmed in research conducted in Puerto Rico during 2006 to 2011 again showing that PRSV and ZYMV are the most frequent virus species infecting various cucurbit species (Rodrigues et al., 2012). Virus and severe virus vector outbreaks are a frequent and major cause associated with low yields and limitations to growing cucurbits in Puerto Rico. Overlapping of susceptible cucurbit crops and continuous growing of cucurbit crops throughout the year makes Puerto Rico, an ecologically diverse island with an abundance of alternative host, an excellent and dynamic environment for plant viruses to evolve (Rodrigues et al., 2012). For this reason, it is very difficult to develop strategies of control that do not consider the use of genetic resistance.

Because of the high incidence of viral disease in tropical pumpkin in Puerto Rico, there is a necessity to study and understand the pathogen-plant interaction which influences how disease resistance is assessed in a breeding program. Breeding programs depend on the development of

efficient and reliable methods of assessing resistance to develop new cultivars with resistance to both PRSV and ZYMV, the most common viruses in tropical pumpkin in the island.

A mayor challenge that breeding studies confront is with the method of classification used to determine which plants are resistant and which plants are susceptible or what is the degree of resistance or susceptibility in a cultivar or line. Another problem is that there are differences in the type and number of resistance genes in different genotypes making the determination of a resistance gene model more complicated. Further research must be done to determine and explain the type of resistance genes in *Cucurbita*. The most efficient way of reducing yield losses cause by viral diseases is genetic resistance (Brown et al., 2003).

The goal in this research was to study the serological detection of ZYMV and PRSV during seedling development of *C. moschata*, to determine the best method to differentiate the level of resistance among genotypes of tropical pumpkin for both PRSV and ZYMV, to determine if there are differences in seedling development of *C. moschata* infected with PRSV, ZYMV, and both PRSV+ZYMV, and to evaluate the usefulness of 'Menina' as a source of resistance to PRSV and ZYMV. This information should aid in the development of an efficient and reliable method to determine the degree of resistance or susceptibility of ZYMV and PRSV in genotypes of *C. moschata*.

2 LITERATURE REVIEW

Cucurbita is an important genus in the *Cucurbitaceae* family. There are five domesticated species: *C. pepo, C. moschata, C. maxima, C. argyrosperma,* and *C. ficifolia* (Bisognin, 2002). "Pumpkin", "squash", and "gourd" are common names for these species. Gourds are exclusively *C. pepo,* but the term pumpkin and squash do not refer to a particular *Cucurbita* species. This research focuses on tropical pumpkin, *C. moschata*, or "calabaza" as it is known in Puerto Rico. In other parts of the American tropics other common names are used in Spanish for *C. moschata*.

2.1 PUMPKIN VIRAL DISEASE

Viral diseases are a major problem in the *Cucurbitaceae*. *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV, also known as WMV-2), and *Cucumber mosaic virus* (CMV) are four viruses that affect cucurbit production worldwide (Zitter et al., 1996). Zitter et al. (1966) established a serological difference between two types of WMV (WMV-1 and WMV-2) determining that WMV-1 was PRSV. All of these viruses are transmitted in a non-persistent manner by several species of aphids and mixed infections are common (Lecoq et al., 1998). It has been estimated that ZYMV can cause up to 48% in total yield loss and up to 84% loss in marketable yield in *Cucurbita maxima,* affecting the marketable fruit weight and incidence of fruit blistering (Fletcher et al., 2000). However, little is known about the direct percent of yield loss of all four viruses in the different species of the *Cucurbita* genus and even less about the yield losses that can result when more than one virus infects a plant.

Since the 1930s, virus-like symptoms among cucurbits have been recognized in Puerto Rico. Since then, many surveys have been done to report new viral diseases found in cucurbits and establish viral incidence. Cook (1936) reported viral disease symptoms in cucumber sampled

between 1935 and 1936. Adsuar and Cruz Miret (1950) reported and characterized a "Virus A" and a "Virus B" affecting summer squash, tropical pumpkin, melon, and watermelon. Pérez (1963) establish that "Virus A" and "Virus B" were WMV and CMV, respectively. The same researcher also reported *Squash mosaic virus* (SqMV) was affecting melon (Perez, 1958). Escudero (1992) reported that between 1982 and 1991 ZYMV, PRSV and WMV-2 (now usually known simply as WMV) were commonly found affecting cucurbits in Puerto Rico.

A survey conducted between 2001 and 2002 established the incidence of ZYMV, PRSV, WMV-2, CMV, SqMV, *Tomato spotted wilt virus* (TSWM) and germiniviruses in tropical pumpkin, summer squash, cucumber, melon and watermelon in Puerto Rico (Paz-Carrasco and Wessel-Beaver, 2002). A total of 320 samples were taken during this period. About 60% to 70% of samples were infected with ZYMV and PRSV, 20% to 30% of samples were infected with WMV-2 and TSWV, and less than 5% of the samples were infected with germinivirus. CMV was not founded in this survey. They also reported that up to 5 viruses were presence in the same tissue and that PRSV and ZYMV were present in most of these mixed infections.

Recent research conducted at the AES-UPRM between 2006 and 2011 demonstrated that there was a high incidence of PRSV and ZYMV infecting various cucurbit species in Puerto Rico (Rodrigues et al., 2012). These two viruses are both transmitted by many species of aphids in a non-persistent manner (Pinto et al., 2008). *Squash vein yellow virus*, a *Potyviridae* transmitted by whiteflies, was reported in 2013 infecting cucurbits (Acevedo et al. 2013). They belong to the *Potyviridae* family, one of the largest plant virus families worldwide with 30% of known plant viruses, and many of its members represent a major threat in agricultural crops (Dunham, et al., 2014; Riechmann et al., 1992). So far, the *Potyviridae* family is the most common viral family infecting *C. moschata* in Puerto Rico (Paz-Carrasco and Wessel-Beaver, 2002).

2.2 *PAPAYA RINGSPOT VIRUS*

In 1949, Jensen used the term papaya ringspot to describe a papaya disease in Hawaii. (Gonsalves, 2010). PRSV causes a major disease in cucurbits. This virus is grouped into the papaya-infecting type, PRSV-P, which affects papaya and cucurbits, and the cucurbit infecting type, PRSV-W, which affects only cucurbits but not papaya (Tripathi, et al., 2008). PRSV is transmitted in a non-persistent manner by many species of aphids to many species of the *Cucurbitaceae* family (Lecoq et al., 1998). This means that the virus is acquired and transmitted by its vector in short periods of time that are measured in seconds to a minute and the virus does not have the capacity to replicate in his vector (Tripathi et al., 2008). Mottling, mosaic and leaf deformity are some characteristic symptoms of the PRSV in cucurbits (Paz-Carrasco and Wessel-Beaver, 2002.).

PRSV has a single-stranded positive RNA with an elongated and flexuous particle of 760-800 x 12 nm (Zhao, et al., 2015). Viral particles contain 94.5% protein and 5.5% nucleic acid and the capsid does not have an outer membrane. The PRSV genome consists of ~10,324 nucleotides (Azad, et al., 2014). The genome encodes a single large protein that contains ~3,344 amino acids which is cleaved into smaller proteins that have different functions. The suggested locations of the cleavage sites predict various proteins consisting of pinwheel type 1 (PI), helper component (HC-Pro), third protein (P3), cylindrical inclusion protein (CI), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), and coat protein (CP, 35K).

The P1 protein is encoded, cleaved and moved systemically in infected plants (Azad et al., 2014). The aphid transmission, symptom expression, long distance movement, genome

amplification, and suppression of post transcriptional gene silencing (PTGS) is mediated by the multifunctional protein HC-Pro. Because HC- Pro is a suppressor of RNA silencing, it can affect development pathways in plants and help in the establishment of the heterologous virus. Another multifunctional protein is the C1 that has NTP binding site, NTPase, RNA binding, and RNA helicase activity. The NIa has two domains defined as the N-terminal genome-linked protein (VPg) and C-teraminal domain. For priming RNA synthesis, the VPg is required. NIb is a codependent RNA polymerase that has been shown to have replicase activity. The coat protein (CP) is involved in aphid transmission, systemic movement and the encapsidation of the viral RNA.

2.3 *ZUCCHINNI YELLOW MOSAIC VIRUS*

Zucchini yellow mosaic virus (ZYMV) was first described in Italy by Lisa et al. (1981) and in France by Lecoq et al. (1981). ZYMV is the most serious threat to *Cucurbita* (pumpkins and squash) production in many regions (Pachner et al., 2011). For this reason, it is important to find a source of resistance that can avoid or reduce viral disease such as ZYMV in *Cucurbita ssp*. 'Nigerian Local' from Nigeria, 'Menina' from Portugal, and 'Soler' from Puerto Rico have been identify as sources of resistance to ZYMV in *C. moschata* (Pachner et al., 2015). Severe mosaic, deformation, blistering, reduced size of the leaf, and stunted plants are some of the symptoms in the aerial part of the plant caused by ZYMV (Department of Agriculture and Food - Australia, 2016). Foliar yellow mosaic, distortion, and necrosis in the aerial part of the plant and small, deformed, and green mottled fruit formation have also been reported (Humaydan, 1983).

ZYMV has a single-stranded positive RNA with an elongated and flexuous particle of 680-730 nm (Gal-On, 2007). The RNA genome size is 9.6 kb. The genome encodes a single polyprotein of 3080 amino acids cleaved by three viral proteases and processed into ten putative functional

and mature proteins. These proteins are pinwheel type 1 (PI), helper component (HC), third protein (P3), 6K1, cylindrical inclusion protein (Cl), 6K2, viral encoded protein (VPg), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), and coat protein (CP). The CP and HC-Pro have an important role in the virus plant to plant movement by the vector.

2.4 APHID VECTOR

Aphids have the capacity of transmitting viruses from one plant to another one, because of the biological and molecular interactions with viruses. They can transport virus in persistent and non-persistent manners (Brault et al., 2010). CMV, PRSV, ZMYV, and WMV are some of the viruses that aphids can transmit from plant to plant. (Lecoq et al., 1998). These viruses can cause a yield lost in many crops worldwide and they can be found in cucurbit crops in Puerto Rico. Previous research has shown that a large number of aphid species are capable of viral transmission (Simmons et al., 2013). *Aphis nerii*, *Myzus persicae*, and *A. gossypii* are some examples of viral vectors and many reports have shown that they are capable of transmission of ZYMV and PRSV and other viruses from the *Potyviridae* family. The CP and the HC are two proteins essential for potyvirus transmission from plant to plant by aphids.

2.5 PLANT BREEDING REASEARCH

Plant breeding research has focused on determining the gene model that explains resistance against PRSV and ZYMV. Brown et al. (2003) reported the inheritance of resistance to four major cucurbit viruses found in North America, including ZYMV and PRSV. The cultivar used in their study was ‘Waltham’ as the highly susceptible parent and ‘Nigerian Local’ as the source of

resistance. F1, F2 populations, and backcross populations were generated for inheritance studies of resistance to each virus. They concluded that resistance to ZYMV is attributed to a single dominant gene, *Zym*, previously reported by Paris et al. (1988). Brown et al. (2003) also reported that inheritance of resistance to PRSV can be attributed to a single recessive gene and it was proposed to designate this gene as *prv*.

In contrast to what was proposed by Brown et al. (2003), a study conducted in Puerto Rico proposed that resistance for PRSV is controlled by at least two genes: a dominant gene for resistance together with an epistatic dominant suppressor gene (McPhail Medina et al., 2012). In this study several F2 populations were generated using 'Nigerian Local' as the resistant parent and several tropical pumpkin cultivars as susceptible parents. Plants in the F2 populations were rated on a scale from 0 to 3 where 0 corresponded to no visible symptoms and 3 corresponded to severe symptoms. Plants with severity of 0 were classified as resistant and plants with severity of 1, 2, or 3 were classified as susceptible. The F2 population segregated 13:3 (susceptible:resistant). A two gene model of dominant suppression epistasis provided the best overall fit of the data.

The one gene model proposed by Brown et al. (2003) for resistance to ZYMV has also now been discarded. Pachner et al. (2011) made an extensive study of various *C. moschata* genotypes carrying resistance to ZYMV. They concluded that a number of genes explain resistance to ZYMV, depending on the source. 'Nigerian Local' carries two dominant genes for resistance (*Zym-0* and *Zym-4*) 'Menina' carries a single dominant resistant gene (*Zym-1*) and the Puerto Rican cultivar 'Soler' carries a recessive gene for resistance (*zym-6*).

The literature contains little information about virus symptomatology caused by the interaction between PRSV and ZYMV. Nor is there much information about how PRSV and

ZYMV and a double infection with PRSV+ZYMV might affect the development of resistant genotypes.

3. MATERIALS AND METHODS

3.1 PREPARATION OF INOCULUM

A continuous source of virus inoculum was needed for this research. Experiments were conducted using infected fresh leaves of 'Waltham'. Fresh inoculum was produced from a stock of dried or lyophilized infected tissue stored at -20°C. Fresh inoculum was produced in the following manner: 'Waltham' seeds were planted in the laboratory under artificial lighting with 12 hours of light and 12 hours of darkness. Five to seven days post-germination, cotyledons were mechanically inoculated with a virus solution. The virus solution was prepared with dried or lyophilized pumpkin (*C. moschata*) tissue that had the virus of interest (PRSV or ZYMV), using 0.12 g lyophilized tissue with 10 ml of 0.02 M phosphate buffer, pH 7.0. The phosphate buffer was prepared mixing 3.48 g of potassium hydrogen phosphate (K_2HPO_4) and 2.72 g of potassium dihydrogen phosphate (KH_2PO_4) in 1 liter of distilled water. Seedlings were individually inoculated with PRSV or ZYMV. The third or fourth leaf of plants to be used as a source of inoculum were tested with ELISA (enzyme-linked immunosorbent assay) (Clark and Adams, 1977) (described below) to ensure that plants used as the source of fresh inoculum carried the virus of interest. All plants with a negative ELISA absorbency reading (<0.400 at A_{405nm}) or contaminated with another virus were eliminated.

3.2 ELISA PROCEDURE

The Double Antibody Sandwich (DAS) Enzyme-linked immunosorbent assays (ELISA) used during thesis research were done using commercial kits from Agdia (Elkhart, Indiana) for PRSV and ZYMV. Positive and negative controls were also obtained from Agdia. The Agdia commercial buffer solution was used as an additional negative control. For ELISA tests, leaf

samples were repeated in two wells, one on the left and one on the right side of each 96-well plate. Readings (light absorbencies) were taken with the plate reader Multiskan® FC Microplate Photometer (Fisher Scientific, Vantaa, Finland) at a wave-length of 405 nm ($A_{405\ nm}$). Preliminary tests inficated that negative controls from Agdia typically gives ELISA readings of 0.150 to 0.200. Therefore, ELISA readings less than 0.400 were considered as negative for presence of either PRSV and ZYMV.

3.3 EXPERIMENT 1: VIRAL SEROLOGICAL TITER IN THE FIRST FOUR LEAVES SAMPLED AT 20 DAYS POST-INOCULATION

The genotypes 'Waltham', 'Moschata 166', 'Taina Dorada', and 'Soler' (known to be susceptible to PRSV and ZYMV), and the genotypes 'Menina', and 'Nigerian Local' (known to be resistant to PRSV and ZYMV) were planted in 9.8 cm plastic pots filled with ProMix® BX (Premier Tech Horticulture, Quakertown, Pennsylvania) in the greenhouse during March to April 2016. Five to seven days post-germination, cotyledons were mechanically inoculated with PRSV, ZYMV or with a phosphate buffer treatment was applied (control). The proportion of the viral solution was 1.0 g of fresh leaf pumpkin tissue from the inoculum and 10 ml of phosphate buffer. Five replicates (plants) of each combination of six genotypes and three virus treatments (PRSV, ZYMV, and the control) were used (a total of 18 treatment combinations). Plants were fertilized with 20-20-20 (N-P-K) soluble fertilizer. Approximately 30 ml of the fertilizer solution (15 g of 20-20-20 fertilizer in 3.79 L of water) was added to each pot once a week. Photographs of each plant were taken approximately 20 days post-inoculation. Tissues of the first four leaves were sampled 20 days post-inoculation for PRSV and 21 days post-inoculation for ZYMV and tested with ELISA for the appropriate virus (PRSV or ZYMV). Control plants were tested for both PRSV

and ZYMV. An ELISA reading (A_{405nm}) of < 0.400 was considered as negative for virus. For each virus (PRSV and ZYMV), and for each leaf, data was analyzed as a one-way analysis of variance to determine if there were significant differences among genotype means. When the F test was significant at the 0.05 probability level, Fisher's Least Significant Difference (LSD) was used to compare genotype means. Pearson's correlation was used to test the association between ELISA readings taken from each of the four leaves.

3.4 EXPERIMENT 2: VIRAL SEROLOGICAL TITER IN THE FIRST FOUR LEAVES SAMPLED AS EACH LEAF FULLY EXPANDS

The same protocols described for Experiment 1 were used in Experiment 2 except 'Verde Luz' was substituted for 'Taina Dorada', and tissue of the first four leaves were sampled every three to four days as each leaf fully expanded. Three individual trials were conducted from June to August 2016 with each experiment consisting of three to four replicates (plants) of each genotype x inoculum treatment combination. Photographs of the plants were taken each time an ELISA was performed. Data was analyzed as in Experiment 1, combining the data from the three trials.

3.5 EXPERIMENT 3: EFFECT OF SINGLE AND DOUBLE INOCULATION WITH PRSV AND ZYMV ON SEROLOGICAL VIRUS TITER IN LEAVES 1, 4, AND 5, AND ON SEEDLING DEVELOPMENT

Seeds of genotypes 'Waltham', 'Moschata 166', 'Menina', and 'Nigerian Local' were planted in the greenhouse during November to December 2016 and inoculated with PRSV, ZYMV

or PRSV+ZYMV or phosphate buffer (control) as previously described. Plants were fertilized with 20-20-20 soluble fertilizer once a week as in previous experiments. As each leaf fully expanded, tissue of the first, fourth, and fifth leaf were sample and tested with ELISA. At 18 days post-inoculation, plants were photographed. Plants were then harvested, and fresh weight measured. Each plant was put inside a paper bag and dried in an oven at 60 °C for approximated 72 hours. ELISA data was analyzed individually for each viruses, as a factorial arrangement (4 genotypes x 2 inoculation treatments) in a one-way analysis of variance. Fresh and dry weight data was analyzed as a factorial arrangement (4genotypes x 4 inoculation treatments) in a one-way analysis of variance. Means were compared with LSD at 0.05 probability.

3.6 EXPERIMENT 4: INOCULATION OF SUSCEPTIBLE GENOTYPES WITH SAP OF INOCULATED RESISTANT GENOTYPES

Cotyledons of 5 to 7 days old plants of resistant genotypes ‘Menina’ and ‘Nigerian Local’ were mechanically inoculated with either PRSV or ZYMV. 18 days post-inoculation, tissue from these plants (‘Menina’ inoculated with PRSV, ‘Menina’ inoculated with ZYMV, ‘Nigerian Local’ inoculated with PRSV, and ‘Nigerian Local’ inoculated with ZYMV) were used as inoculum to mechanically inoculate cotyledons of four to five plants of susceptible genotypes ‘Waltham’ and ‘Moschata 166’ at approximately 6 days post-seeding. At 20 day-post-inoculation, the fourth leaf of each inoculated plant was tested with ELISA for the virus used in the inoculation. Readings of <0.400 were consider negative for virus. Data was analyzed as a factorial arrangement (2 genotypes x 2 inoculation treatments) in a one-way analysis of variance. Means were compared with Fisher’s Least Significant Difference test at the 0.05 level of probability.

4. RESULTS

Control (uninoculated) plants of both the susceptible and resistant genotypes of tropical pumpkin did not show any virus symptoms (Figure 1). Development of small leaves, intervenal chlorosis, and leaf deformation were observed in ‘Waltham’ inoculated with PRSV (Figure 2) and ZYMV (Figure 3). Also, curled leaves were observed in ‘Waltham’ inoculated with ZYMV. Small, deformed leaves were observed in ‘Moschata 166’ inoculated with PRSV and ZYMV. Intervenal chlorosis and curled leaves were observed in ‘Moschata 166’ inoculated with ZYMV. Intervenal chlorosis and mosaic were observed in ‘Taina Dorada’ and ‘Soler’ inoculated with PRSV and ZYMV. Also, small leaves and leaf deformation were observed in ‘Taina Dorada’ and ‘Soler’ inoculated with ZYMV. Smaller leaves and general leaf chlorosis were observed in ‘Menina’ inoculated with PRSV and ZYMV compared to uninoculated plants of ‘Menina’ (Figure 4). No symptoms were observed in ‘Nigerian Local’ inoculated with virus solution.

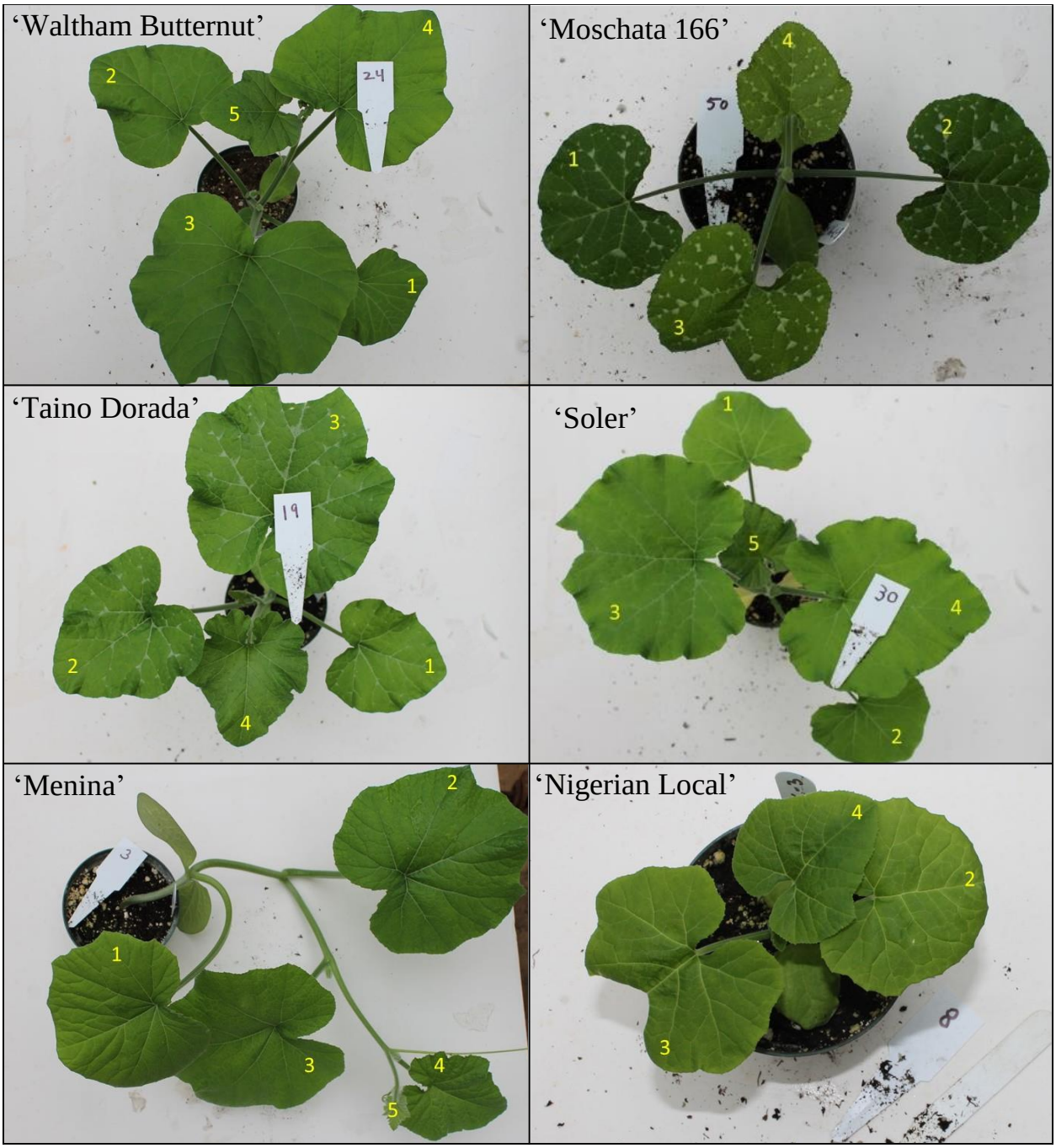

Figure 1: Twenty-two-day-old control (uninoculated) plants of six genotypes of *Cucurbita moschata*. ‘Menina’ and ‘Nigerian Local’ are resistant to *Papaya ringspot virus* and *Zucchini yellow mosaic virus*. The other genotypes are susceptible. Numbers indicate leaf position 1 (oldest leaf) to 4 (youngest leaf). Scale reference: pot size is 9.8 cm.

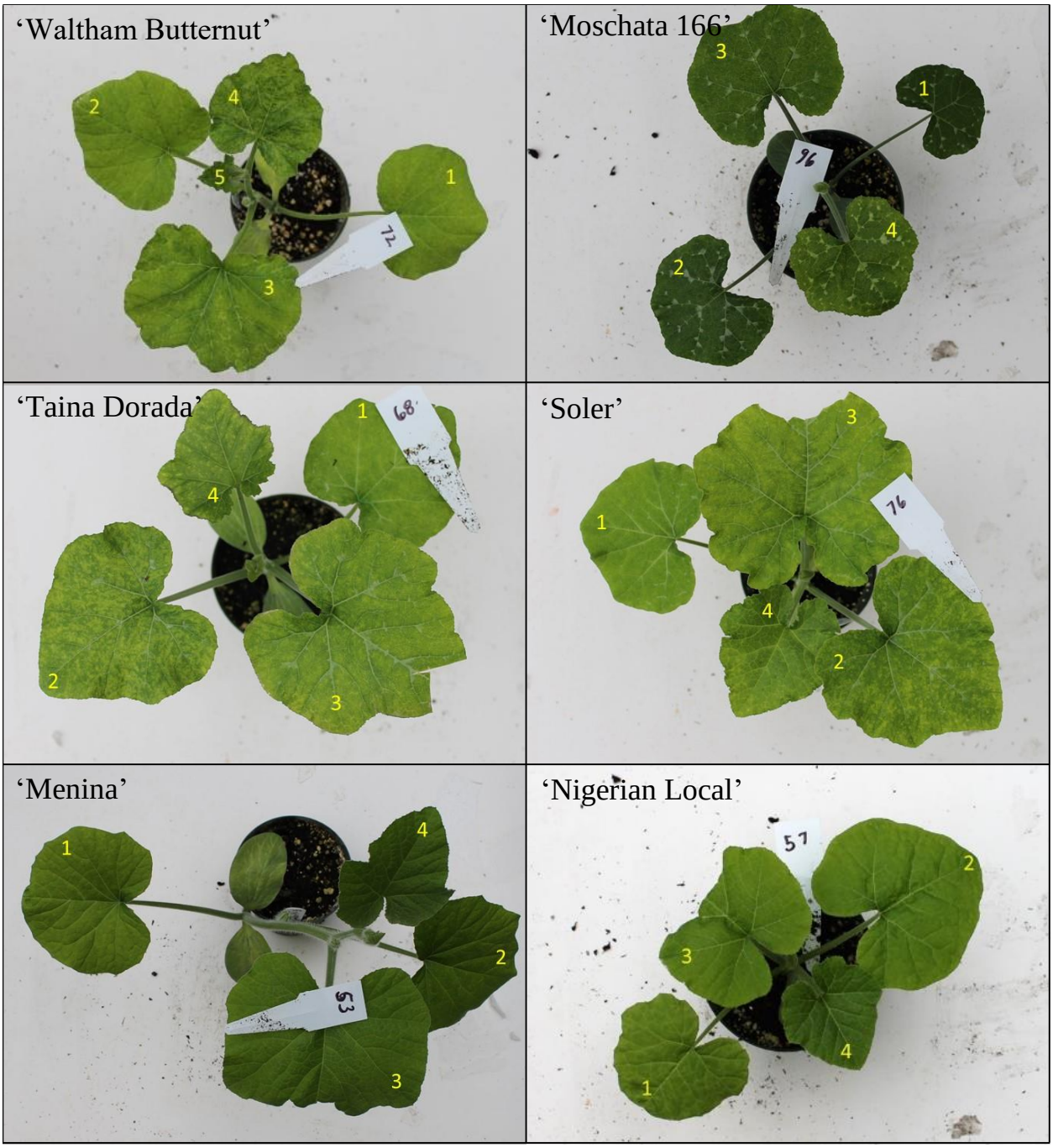

Figure 2: Twenty-two-day-old plants of six genotypes of *Cucurbita moschata*. Cotyledons were inoculated with *Papaya ringspot virus* 6 days after planting. ‘Menina’ and ‘Nigerian Local’ are resistant to *Papaya ringspot virus*. The other genotypes are susceptible. Numbers indicate leaf position 1 (oldest leaf) to 4 (youngest leaf). Scale reference: pot size is 9.8 cm.

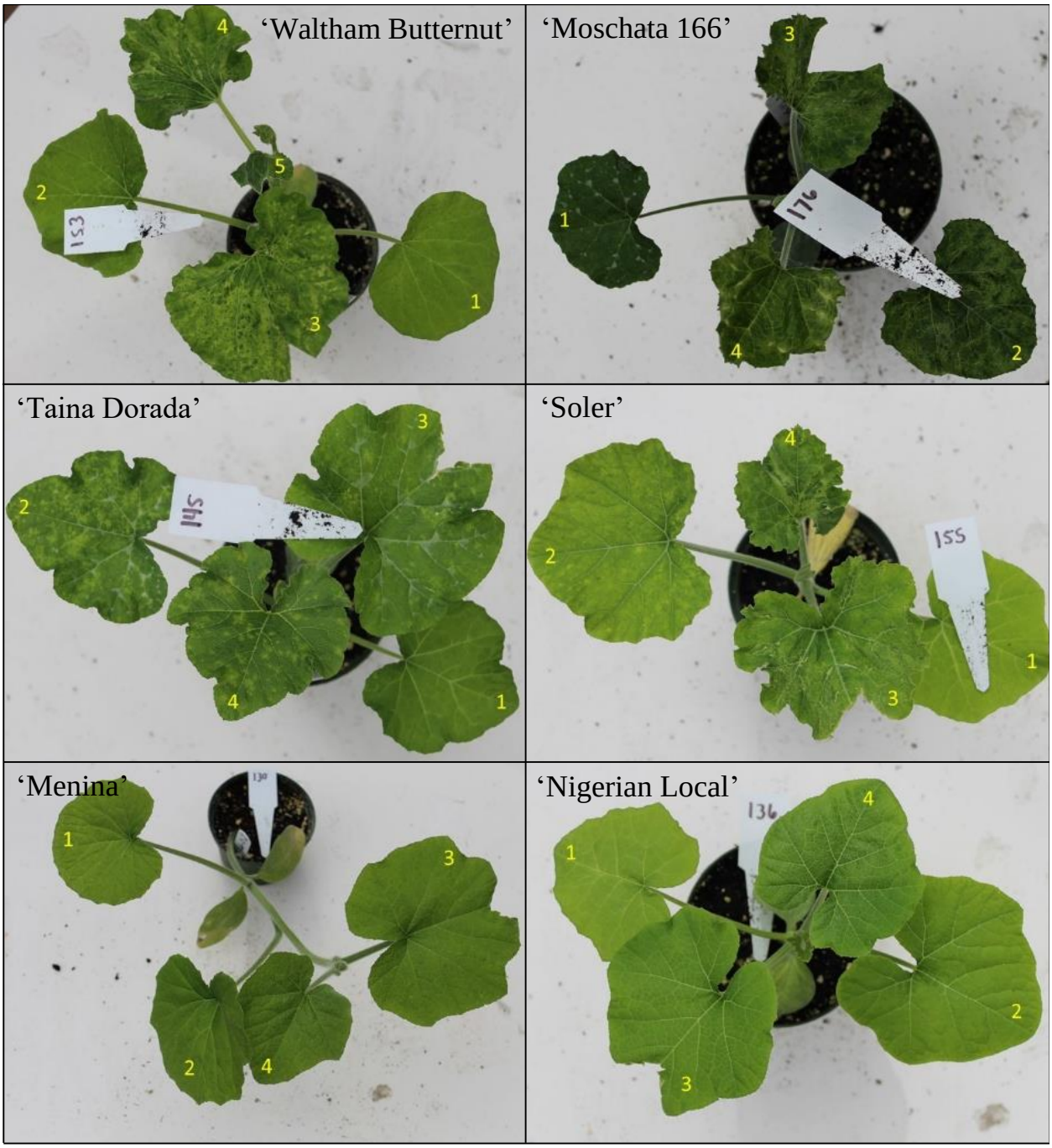

Figure 3: Twenty-two-day-old plants of six genotypes of *Cucurbita moschata*. Cotyledons were inoculated with *Zucchini yellow mosaic virus* 6 days after planting. ‘Menina’ and ‘Nigerian Local’ are resistant to *Zucchini yellow mosaic virus.* The other genotypes are susceptible. Numbers indicate leaf position 1 (oldest leaf) to 4 (youngest leaf). Scale reference: pot size is 9.8 cm.

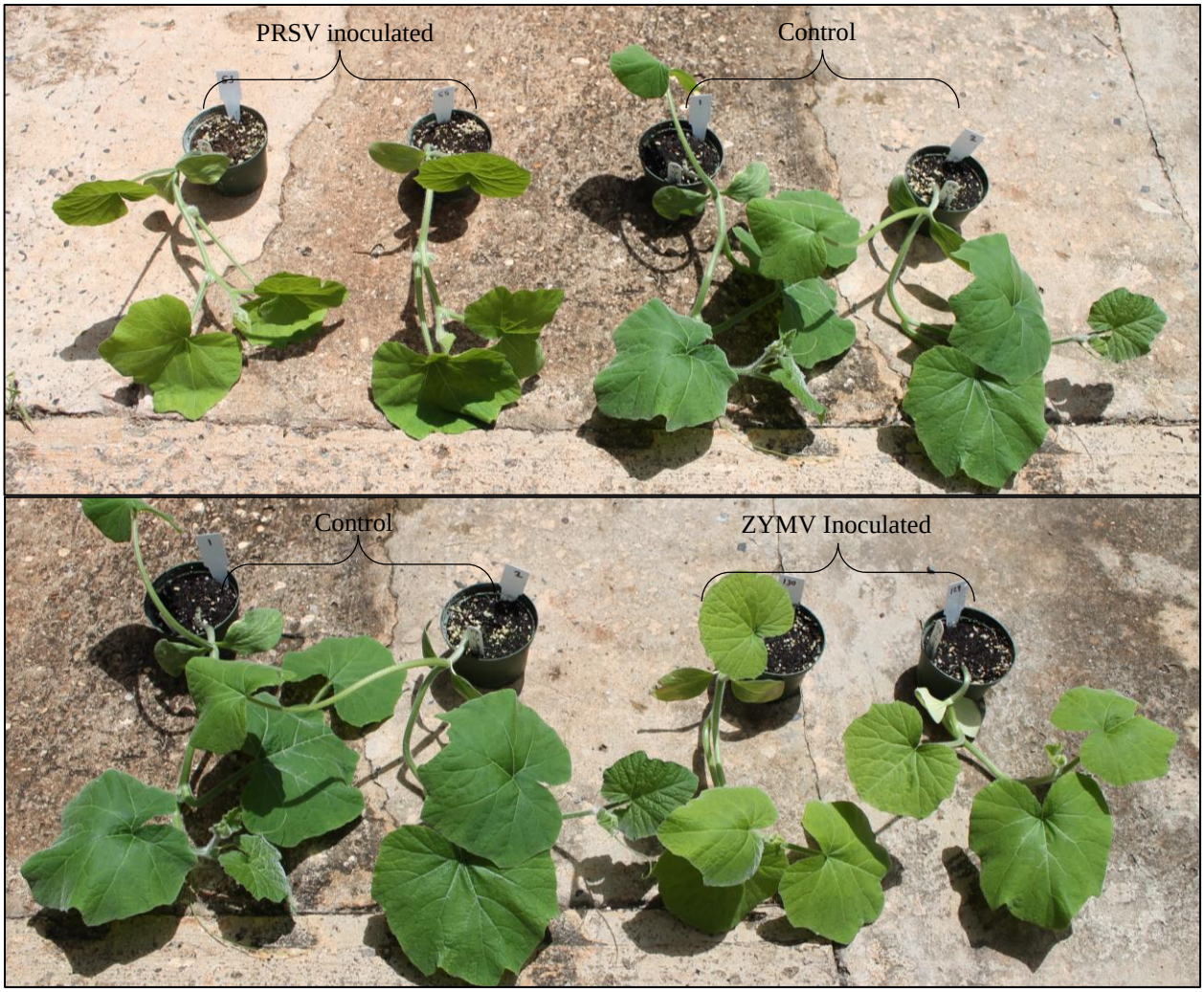

Figure 4: Twenty-three-days-old plants of *Cucurbita moschata* cv. Menina inoculated with *Papaya ringspot virus* (above) or *Zucchini yellow mosaic virus* (below) versus control (uninoculated) plants of the same age. Scale reference: pot size is 9.8 cm.

4.1 EXPERIMENT 1: VIRAL SEROLOGICAL TITER IN THE FIRST FOUR LEAVES SAMPLED AT 20 TO 21 DAYS POST-INOCULATION

In this experiment, all leaves were sampled 20 days after inoculation. In leaf 1, there was no significant difference between PRSV ELISA readings of resistant versus susceptible genotypes and all genotypes had positive readings (high serological titer, A_{405nm}>0.400) (Table 1). PRSV ELISA readings of susceptible 'Soler' and resistant 'Nigerian Local' were different from all other genotypes, but all six genotypes continued to have positive readings. PRSV ELISA readings in leaf 3 did not allow differentiation between resistant and susceptible genotypes. In leaf 4, resistant versus susceptible genotypes were clearly separated. 'Menina' and 'Nigerian Local' had negative PRSV ELISA readings. 'Soler' was weakly positive and 'Waltham', 'Moschata 166', and 'Taína Dorada' were positive for PRSV. In general, PRSV ELISA readings in susceptible genotypes remained high in leaves 1 to 4, while readings in the resistant genotypes, especially in 'Menina', were lower in later-appearing leaves.

PRSV ELISA readings in leaf 1 were not significantly correlated with readings in leaf 2, 3 or 4 (p=0.09 to 0.13) (Table 2). This continued to be true for leaf 2 versus leaf 3 and leaf 4 (p=0.07, 0.14). Readings in leaves 3 and 4 were moderately correlated ($r = 0.51$, $p<0.01$).

For ZYMV in Experiment 1, all leaves were sample at 21 days after inoculation. Resistant genotypes 'Menina' and 'Nigerian Local' had negative ZYMV ELISA readings in all four leaves and readings were significantly different from the susceptible genotypes (Table 3). 'Waltham', 'Moschata 166', 'Taína Dorada', and 'Soler' had positive ZYMV readings in all leaves. ZYMV ELISA readings among all four leaves positions were moderately to highly correlated (r =0.58 to 0.82, $p<0.01$) (Table 4).

Table 1: Mean *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with PRSV. ELISA readings were taken in leaves sampled 20 days post-inoculation.

		Leaf position[2]							
Genotype	**Phenotype[1]**	**1**		**2**		**3**		**4**	
Waltham	S	1.168	a	1.410	a	1.323	a	1.551	a
Moschata 166	S	1.170	a	1.094	a	1.199	a	1.354	ab
Taína Dorada	S	1.106	a	1.344	a	0.992	a	0.991	bc
Soler	S	0.824	a	0.866	ab	0.862	a	0.571	cd
Menina	R	1.158	a	1.275	a	0.787	a	0.382	d
Nigerian Local	R	0.606	a	0.507	b	0.488	a	0.347	d
Mean		1.005		1.083		0.942		0.866	
F-test		0.4182		0.0221		0.1189		0.0002	
F-LSD (0.05)		N/A		0.5563		N/A		0.5330	

[1]Phenotype (S=susceptible and R=resistant to PRSV) based on previous studies.

[2] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.

F-LSD= Fisher's Least Significant Difference at α=0.05.

Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

N/A – F-LSD values were not included when the probability of the F-test was $p>0.05$.

Table 2: Pearson's correlations between *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with PRSV.

	Leaf position [1, 2]		
Leaf Position	**1**	**2**	**3**
2	0.28 (p=0.13)		
3	0.33 (p=0.08)	0.33 (p=0.07)	
4	0.31 (p=0.09)	0.27 (p=0.14)	0.51 (p<0.01)

[1] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.

[2] ELISA readings from susceptible ('Waltham Butternut', 'Moschata 166', 'Taína Dorada', and 'Soler') and resistant genotypes ('Menina', and 'Nigerian Local') were used for calculating Pearson's correlation.

Table 3: Mean *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with ZYMV. ELISA readings were taken in leaves sampled 21 days post-inoculation.

		Leaf position[2]							
Genotype	**Phenotype[1]**	**1**		**2**		**3**		**4**	
Waltham	S	1.833	a	1.933	a	1.753	ab	0.923	b
Moschata 166	S	2.061	a	1.620	a	1.391	ab	1.203	ab
Taína Dorada	S	1.125	b	1.905	a	1.873	a	1.537	a
Soler	S	2.263	a	1.984	a	1.356	ab	1.016	b
Menina	R	0.242	c	0.185	b	0.217	c	0.162	c
Nigerian Local	R	0.209	c	0.292	b	0.145	c	0.177	c
Mean		1.289		1.320		1.123		0.836	
F-test		<0.0001		<0.0001		<0.0001		<0.0001	
F-LSD (0.05)		0.4741		0.5785		0.4986		0.3972	

[1]Phenotype (S=susceptible and R=resistant to ZYMV) based on previous studies.

[2] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.

F-LSD= Fisher's Least Significant Difference at α=0.05.

Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

Table 4: Pearson's correlations between *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of Cucurbita moschata inoculated with ZYMV.

	Leaf position [1, 2]		
Leaf Position	**1**	**2**	**3**
2	0.74 (p<0.01)		
3	0.70 (p<0.01)	0.82 (p <0.01)	
4	0.58 (p<0.01)	0.77 (p <0.01)	0.79 (p <0.01)

[1] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.

[2] ELISA readings from susceptible ('Waltham Butternut', 'Moschata 166', 'Taína Dorada', and 'Soler') and resistant genotypes ('Menina', and 'Nigerian Local') were used for calculating Pearson's correlation.

4.2 EXPERIMENT 2: VIRAL SEROLOGICAL TITER IN THE FIRST FOUR LEAVES SAMPLED AS EACH LEAF FULLY EXPANDED

In Experiment 2, leaves were sampled every three to four days as each of the first four leaves fully expanded, starting with leaf 1 three to four days after inoculation. No difference in PRSV ELISA readings were observed in resistant versus susceptible genotypes as the first and second leaves emerged and expanded (Table 5). 'Menina' and 'Nigerian Local' had negative PRSV ELISA readings in all four leaves. By leaf 3, PRSV ELISA readings in susceptible genotypes 'Waltham' and 'Moschata 166' were significantly different from those in resistant genotypes 'Menina' and 'Nigerian Local'. In leaf 4, 'Waltham', 'Moschata 166', and 'Verde Luz' were susceptible, 'Soler' was intermediately susceptible, and 'Menina' and 'Nigerian Local' remained resistant. In strong contrast to Experiment 1, PRSV ELISA readings were negative in both susceptible and resistant genotypes when leaf 1 was sampled about 4 days post-inoculation. As leaves continued to be sampled, PRSV ELISA readings increased in susceptible genotypes and remained low in resistant genotypes.

Correlations between PRSV ELISA readings in leaf 1 and leaves 2, 3, and 4 were negative, but not always significant ($p=0.01$ to 0.08) (Table 6). Correlations between leaf 2 versus leaves 3 and 4 were moderately positive. Readings in leaves 3 and 4 were strongly correlated (0.70, $p<0.01$).

Table 5: Mean *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with PRSV. ELISA readings were taken as each leaf expanded.

		Leaf position[2]			
Genotype	**Phenotype[1]**	**1**	**2**	**3**	**4**
Waltham	S	0.287 a	0.542 a	0.841 a	0.975 a
Moschata 166	S	0.279 a	0.566 a	0.822 a	1.014 a
Verde Luz	S	0.332 a	0.473 a	0.732 ab	1.024 a
Soler	S	0.307 a	0.306 a	0.393 bc	0.586 b
Menina	R	0.348 a	0.333 a	0.392 bc	0.348 c
Nigerian Local	R	0.303 a	0.202 a	0.259 c	0.402 c
Mean		0.309	0.404	0.573	0.725
F-test		0.5794	0.2518	0.0013	<0.0001
F-LSD (0.05)		N/A	N/A	0.3492	0.2013

[1]Phenotype (S=susceptible and R=resistant to PRSV) based on previous studies.
[2] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.
F-LSD= Fisher's Least Significant Difference at α=0.05.
Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).
N/A – F-LSD values were not included when the probability of the F-test was p>0.05.

Table 6: Pearson's correlations between *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of the first four leaves, sampled as each leaf fully expanded, of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with PRSV.

	Leaf position [1, 2]		
Leaf Position	**1**	**2**	**3**
2	-0.26 (p=0.08)		
3	-0.37 (p=0.01)	0.58 (p<0.01)	
4	-0.36 (p=0.02)	0.50 (p<0.01)	0.70 (p<0.01)

[1] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.
[2] ELISA readings from susceptible ('Waltham Butternut', 'Moschata 166', 'Verde Luz', and 'Soler') and resistant genotypes ('Menina', and 'Nigerian Local') were used for calculating Pearson's correlation.

In Experiment 2, leaves were sampled every three to four days as each of the first four leaves fully expanded, starting with leaf 1 three to four days after inoculation. There were no

significant differences among genotypes in ZYMV ELISA readings in leaf 1 (Table 7). In the second, third, and fourth leaves, ELISA readings for resistant 'Menina' were different from readings of the susceptible genotypes. 'Nigerian Local' had negative ELISA readings in all four leaves and can be distinguished from at least some, but not all, of the susceptible genotypes from leaf 1. 'Waltham', 'Moschata 166', 'Verde luz', and 'Soler' had positive ELISA readings in all four leaves.

Table 7: Mean *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of the first four leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with ZYMV. ELISA readings were taken as each leaf expanded.

		Leaf position[2]							
Genotype	**Phenotype[1]**	**1**		**2**		**3**		**4**	
Waltham	S	0.806	a	0.472	bc	1.240	ab	2.348	a
Moschata 166	S	1.248	a	0.672	b	1.394	a	1.902	b
Verde Luz	S	0.854	a	1.068	a	0.752	bc	1.529	bc
Soler	S	0.965	a	0.688	b	0.920	ab	1.288	c
Menina	R	0.432	a	0.273	c	0.247	c	0.331	d
Nigerian Local	R	0.267	a	0.229	c	0.222	c	0.311	d
Mean		0.762		0.567		0.796		1.285	
F-test		0.0869		0.0008		0.0001		<0.0001	
F-LSD (0.05)		N/A		0.3614		0.5418		0.3922	

[1]Phenotypes (S=susceptible and R=resistant to PRSV) based on previous studies.
[2] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.
F-LSD=Fisher's Least Significant Difference at α=0.05.
Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).
N/A – F-LSD values were not included when the probability of the F-test was $p>0.05$.

Correlations between ZYMV ELISA readings leaf 1 and leaves 2, 3, and 4 were either not significant ($p>0.05$) or only moderated correlated ($r=0.51$, $p<0.01$) (Table 8). Correlations were

lower between leaf 2 and leaf 3 (r=0.37) and not significant between leaf 2 and leaf 4 (p=0.14). The correlation between readings in leaf 3 versus leaf 4 were moderate (r = 0.48).

Table 8: Pearson's correlations between *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of the first four leaves, sampled as each leaf fully expanded, of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with ZYMV.

	Leaf position [1, 2]		
Leaf Position	**1**	**2**	**3**
2	0.14 (p=0.34)		
3	0.51 (p<0.01)	0.37 (p=0.01)	
4	0.23 (p=0.14)	0.23 (p=0.14)	0.48 (p<0.01)

[1] Leaf position 1 = oldest leaf, leaf position 4 = youngest leaf.
[2] ELISA readings from susceptible ('Waltham Butternut', 'Moschata 166', 'Verde Luz', and 'Soler') and resistant genotypes ('Menina', and 'Nigerian Local') were used for calculating Pearson's correlation.

4.3 EXPERIMENT 3: EFFECT OF SINGLE AND DOUBLE INOCULATION WITH PRSV AND ZYMV ON SEROLOGICAL VIRAL TITER IN LEAVES 1, 4, AND 5, AND ON SEEDLING DEVELOPMENT

In Experiment 3, as in Experiment 2, leaves were sampled every three to four days as each of the first four leaves fully expanded, starting with leaf 1 three to four days after inoculation. For plants inoculated with PRSV, there were significant differences in PRSV ELISA readings among genotypes ($p < 0.0001$) in all three leaves positions (Table 9). However, in leaf 1, these differences depended on the type of inoculation (interaction F test: $p < 0.0001$). In leaf 1 and leaf 4 of susceptible 'Waltham' inoculated with PRSV+ZYMV resulted in lower PRSV ELISA readings compared to 'Waltham' inoculated solely with PRSV. This difference was no longer evident in leaf 5. PRSV-inoculated versus PRSV+ZYMV-inoculated plants of the other three genotypes showed no difference in PRSV ELISA readings. Genotypes susceptible to PRSV could be

distinguish from resistant genotypes beginning in leaf 1 (as it expanded), although the separation of these two groups was stronger in leaves 4 and 5. ‘Menina’ and ‘Nigerian Local’ gave negative readings (<0.400) in the first and fourth leaf, for both treatments (inoculation with PRSV and PRSV+ZYMV). In the fifth leaf ‘Menina’ and ‘Nigerian Local’ inoculated with PRSV and ‘Nigerian Local’ inoculated with PRSV+ZYMV gave weakly positive readings for PRSV (readings from 0.414 to 0.459). ‘Menina’ inoculated with PRSV+ZYMV gave a negative reading for PRSV. ‘Waltham’ and ‘Moschata 166’ had positive ELISA readings in all leaves in both treatments.

Mean ZYMV ELISA readings of the first, fourth, and fifth leaves of susceptible and resistant genotypes inoculated with ZYMV and double-inoculated with PRSV+ZYMV are show in Table 10. Leaves were sample every three to four days as each leaf fully expanded, starting with leaf one three to four days after inoculation. There was no difference in ZYMV ELISA readings between susceptible ‘Waltham’ and the resistant genotypes. This was true no matter which leaf was tested. In contrast, ‘Moschata 166’ exhibited significantly lower ZYMV ELISA readings when inoculated with ZYMV+PRSV compared to ‘Moschata 166’ inoculated only with ZYMV. Genotypes susceptible to ZYMV could be distinguished from resistant genotypes based on ELISA readings taken in leaf 1 and these differences continued through leaf 5.

Table 9: Mean *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of the first, fourth, and fifth leaves of susceptible and resistant genotypes of *Cucurbita moschata* inoculated with PRSV and double inoculation with PRSV and *Zucchini yellow mosaic virus* (ZYMV). Readings were taken as each leaf expanded.

			Leaf position[2]					
Genotype	**Phenotype[1]**	**Inoculum**	**1**		**4**		**5**	
Waltham	S	PRSV	0.882	a	0.873	a	1.145	a
		PRSV/ZYMV	0.416	c	0.678	b	1.099	a
Moschata 166	S	PRSV	0.602	b	0.771	ab	1.187	a
		PRSV/ZYMV	0.461	bc	0.811	ab	0.741	b
Menina	R	PRSV	0.397	cd	0.392	c	0.414	b
		PRSV/ZYMV	0.339	cde	0.392	c	0.379	b
Nigerian Local	R	PRSV	0.234	e	0.343	c	0.454	b
		PRSV/ZYMV	0.237	de	0.332	c	0.459	b
Mean			0.446		0.574		0.735	
Genotype F-test			<0.0001		<0.0001		<0.0001	
Inoculum F-test			0.0001		0.4226		0.2036	
Interaction F-test			0.0001		0.3207		0.2979	
LSD (0.05)			0.1559		0.2064		0.40762	

[1]Phenotypes (S=susceptible and R=resistant to PRSV) based on previous studies.
[2] Leaves were sampled every three to four days as each leaf fully expand, starting with leaf 1 three to four days after inoculation. Leaf position 1 = oldest leaf, leaf position 5 = youngest leaf.
LSD= Least Significant Difference at α=0.05 for the genotype x inoculum combinations of treatment.
Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

'Nigerian Local' inoculated with ZYMV and 'Menina' and 'Nigerian Local' inoculated with both viruses gave negative readings for the first, fourth, and fifth leaves. 'Menina' inoculated with ZYMV gave positive readings for the first leaf and negative readings for the fourth and fifth leaves. Resistant genotypes 'Menina' and 'Nigerian Local' could be distinguish from the susceptible genotypes in the fourth and fifth leaves. These two genotypes gave negative ELISA

readings that were significantly different from the rest of the genotypes for both inoculation treatments. 'Waltham' and 'Moschata 166' had positive ELISA readings in all leaves in both treatments.

Table 10: Mean *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of the first, fourth, and fifth leaves of four genotypes of *Cucurbita moschata* inoculated with ZYMV and both ZYMV and *Papaya ringspot virus* (PRSV).

			Leaf position [2]		
Genotype	**Phenotype[1]**	**Inoculum**	**1**	**4**	**5**
Waltham	S	ZYMV	1.271 a	1.130 ab	1.087 b
		ZYMV/PRSV	1.475 a	1.306 a	0.792 bc
Moschata 166	S	ZYMV	1.249 a	1.292 a	1.640 a
		ZYMV/PRSV	0.422 b	0.758 b	0.673 c
Menina	R	ZYMV	0.518 b	0.211 c	0.179 d
		ZYMV/PRSV	0.279 b	0.195 c	0.172 d
Nigerian Local	R	ZYMV	0.183 b	0.180 c	0.225 d
		ZYMV/PRSV	0.203 b	0.196 c	0.230 d
Mean			0.700	0.659	0.625
Genotype F-test			<0.0001	<0.0001	<0.0001
Inoculum F-test			0.0518	0.3961	0.0027
Interaction F-test			0.0058	0.0950	0.0026
LSD (0.05)			0.4247	0.4194	0.4017

[1]Phenotypes (S=susceptible and R=resistant to PRSV) based on previous studies.
[2] Leaves were sampled every three to four days as each leaf fully expand, starting with leaf 1 three to four days after inoculation. Leaf position 1 = oldest leaf, leaf position 5 = youngest leaf.
LSD= Least Significant Difference at α=0.05 for the genotype x inoculum combination of treatments.
Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

Mean weight of fresh and dry tissue of susceptible and resistant genotypes uninoculated (control) or inoculated with PRSV, ZYMV, or PRSV+ZYMV are shown in Table 11. The fresh weight of 'Waltham' inoculated with PRSV+ZYMV was significantly less than the mean fresh

weight of the control. However, the mean weight of ‘Waltham’ inoculated with PRSV or with ZYMV was not significantly different from that of the control. The dry weights of ‘Waltham’ inoculated with ZYMV or inoculated with both viruses were significantly less than the dry weight of the control. However, the dry weight of ‘Waltham’ inoculated with PRSV was not significantly different from the control and ZYMV inoculation. No significant differences were found among inoculation treatments, including the control, for either fresh or dry weight of ‘Moschata 166’. Fresh weight of ‘Menina’ inoculated with ZYMV was significantly greater than that of the control. Control plants of ‘Menina’ also tended to have a lower dry weight compared to ‘Menina’ inoculated with virus. No significant differences were found for fresh and dry weight among inoculated treatments. For ‘Nigerian Local’, no significant differences were found among uninoculated and inoculated treatments for both fresh and dry tissue weight.

Table 11: Mean of fresh and dry tissue of twenty-two-day-old plants of four different genotypes of *Cucurbita moschata* uninoculated (control) and inoculated with *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), and both PRSV and ZYMV.

			Weight (g)[2]			
Genotype	**Phenotype[1]**	**Inoculum**	**Fresh Tissue**		**Dry Tissue**	
Waltham	S	Control	27.52	cde	3.18	a
		PRSV	25.57	ef	2.86	abc
		ZYMV	27.20	def	2.41	cd
		PRSV/ZYMV	21.22	g	2.19	d
Moschata 166	S	Control	15.02	h	1.72	e
		PRSV	16.59	h	1.93	de
		ZYMV	15.03	h	1.61	e
		PRSV/ZYMV	13.89	h	1.57	e
Menina	R	Control	31.03	bcd	2.34	cd
		PRSV	31.40	abc	2.96	ab
		ZYMV	35.13	a	2.94	ab
		PRSV/ZYMV	33.54	ab	2.75	abc
Nigerian Local	R	Control	22.17	fg	2.41	bcd
		PRSV	19.19	gh	2.35	cd
		ZYMV	24.06	efg	2.76	abc
		PRSV/ZYMV	18.56	gh	2.32	cd
Mean			23.56		2.39	
Genotype F-test			<0.0001		<0.0001	
Inoculum F-test			0.0233		0.1133	
Interaction F-test			0.1040		0.0009	
LSD (0.05)			4.614		0.514	

[1]Phenotypes (S=susceptible and R=resistant to PRSV) based on previous studies.

[2] Fresh weight was taken 18 days post inoculation. Plants were cut and placed inside a paper bag and dried in an oven at 60 °C for approximated 72 hours for dry measures.

LSD=Least Significant Difference at α=0.05 for the genotype x inoculum combination of treatments.

Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

4.4 EXPERIMENT 4: INOCULATION OF SUSCEPTIBLE GENOTYPES WITH SAP FROM PREVIOUSLY INOCULATED RESISTANT GENOTYPES

Susceptible genotypes 'Waltham' and 'Moschata 166' had negative ELISA readings when inoculated with fresh inoculum from resistant genotypes 'Menina' and 'Nigerian Local' that had been previously inoculated with either PRSV (Table 12) or ZYMV (Table 13). No virus symptoms were observed on either the source plants ('Menina' and 'Nigerian Local' inoculated with virus) nor the test plants ('Waltham' and 'Moschata 166'). However, source plants had weakly positive ELISA readings in some cases. 'Menina' source plants inoculated with PRSV or ZYMV had ELISA readings of 0.374 and 0.671, respectively. 'Nigerian Local' source plants inoculated with PRSV or ZYMV had ELISA readings of 0.462 and 0.360, respectively.

Table 12: Mean *Papaya ringspot virus* (PRSV) ELISA readings (A_{405nm}) of susceptible genotypes 'Waltham' and 'Moschata 166' inoculated with sap from plants of 'Menina' and 'Nigerian Local' inoculated with PRSV

Inoculum Source	**Tested Genotype**	**PRSV ELISA Reading**
Menina	Waltham	0.310 a
	Moschata 166	0.275 a
Nigerian Local	Waltham	0.339 a
	Moschata 166	0.259 a
Mean		0.296
Genotype F-test		0.0723
Source of inoculum F-test		0.8182
Interaction F-test		0.4461
LSD (0.05)		0.090

The fourth leaf was sample at 20 days after inoculation.
LSD= Least Significant Difference at α=0.05 for the inoculum source x tested genotype combination of treatments.
Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

Table 13: Mean *Zucchini yellow mosaic virus* (ZYMV) ELISA readings (A_{405nm}) of susceptible genotypes 'Waltham' and 'Moschata 166' inoculated with sap from plants of 'Menina' and 'Nigerian Local' inoculated with ZYMV

Inoculum Source	**Tested Genotype**	**ZYMV ELISA Reading**
Menina	Waltham	0.226 a
	Moschata 166	0.276 a
Nigerian Local	Waltham	0.245 a
	Moschata 166	0.254 a
Mean		0.250
Interaction F-test		0.4606
LSD (0.05)		0.087
Genotype F-test		0.2974
Source of inoculum F-test		0.9559

The fourth leaf was sample at 20 days after inoculation.

LSD= Least Significant Difference at α=0.05 for the inoculum source x tested genotype combination of treatments.

Within a column, means followed by a common letter are not significantly different according to Fisher's Least Significant Difference (α=0.05).

5. DISCUSSION

Four individual experiments were conducted with the goal of studying PRSV and ZYMV serological detection in seedlings of *C. moschata*, develop efficient methods to identify resistance among genotypes of tropical pumpkin for both PRSV and ZYMV, determine if PRSV, ZYMV, and the interaction of both viruses in the same plant can affect seedling development of *C. moschata*, and to evaluate if 'Menina' can be used as a source of resistance for both viruses. Analysis of the virus concentration in the first four leaves using ELISA tests were performed in two different ways: sampling all leaves simultaneously (Experiment 1) and sampling leaves as they emerged (Experiment 2). An ELISA test was also performed in the fifth leaf (Experiment 3). The main purpose of these three experiments was to determine what is the earliest leaf stage where ELISA can be used to differentiate between susceptible and resistant genotypes. Experiment 3 also served to compare fresh and dry weight (biomass) in susceptible and resistant genotypes to determine if there were significant differences in seedling development of tropical *C. moschata* when uninoculated (control) or inoculated with PRSV, ZYMV or PRSV+ZYMV. Finally, in Experiment 4, inoculation of susceptible genotypes with sap of inoculated resistant genotypes 'Menina' and 'Nigerian Local' was performed to determine if these genotypes have the capacity to transmit viable viral particles to susceptible genotypes.

'Menina Brasilera' was first reported to be resistant to WMV-1 by Costa et al (1974). WMV-1 is now known as PRSV (Zitter et al., 1996). Maluf et al. (1986) performed an experiment with the goal of screening thirty *Cucurbita spp.* accessions, composed of *C. moschata*, *C. pepo*, *C. maxima*, and *C. ecuadorensis* to determine which species were resistant to WMV-1 and confirmed that 'Menina Brasilera' was resistant to this virus. Previous research also demonstrated that the resistance of 'Menina Brasilera' combined with pre-immunization is an effective way of

controlling PRSV-W disease under greenhouse and field condition, increasing fruit yield (Rezende et al., 1999). By performing a multiple plate trapped-enzyme linked immunosorbent assay (PTA-ELISA) test for PRSV-W in 'Menina Brasilera', Pacheco et al. (2003) suggested that low virus concentration might be associated with resistance to PRSV-W attribute to this genotype.

It is not know whether the seed of 'Menina' used in the research presented here is the same genotype as 'Menina Brasilera' studied by the above authors working in Brazil. The seed used in this research is derived from seed obtained from Tamas Lelley (University of Natural Resources and Applied life Science, Vienna, Austria). Lelley is a co-author of the study by Pachner et al. (2011), where 'Menina' was found to be resistant to ZYMV. L. Wessel-Beaver (personal communication) believes the two genotypes are similar or the same.

Small leaf development, intervenal chlorosis, leaf deformation, curled leaves, and mosaic were some of the symptomatology observed in susceptible genotypes inoculated with PRSV (Figure 2) and ZYMV (Figure 3). These symptoms were not seen in 'Menina' and 'Nigerian Local'. However, plants of 'Menina' inoculated with virus had leaves that where somewhat chlorotic and the plants themselves appeared to be smaller compared with the uninoculated (control) plants (Figure 4). No symptoms were observed in 'Nigerian Local'. These observations were the motivation for later conducting Experiment 3 where fresh and dry weight of inoculated and inoculated plants were compared.

For PRSV ELISA, both methods (sampling all leaves simultaneously and sampling leaves as they emerged) required sampling of the fourth leaf to find a significant difference between resistant and susceptible genotypes. Nevertheless, there were some differences among genotypes in the virus concentration of the first three leaves that need to be considered for further analysis. In Experiment 1, virus serological titer of the first three leaves was high, giving positive readings

(>0.400) for all genotypes including resistant genotypes ‘Menina’ and ‘Nigerian Local’ (Table 1). In the susceptible genotypes, mean virus titer increased as leaf position increased (a higher leaf position corresponds to younger leaves). For resistant genotypes ‘Menina’ and ‘Nigerian Local’, virus serological titer decreased at higher leaf positions (in younger leaves), giving negative readings (<0.400) in leaf 4. This pattern did not occur in Experiment 2 where leaf positions were tested as the leaf emerged (in other words, each leaf was tested at a similar growth stage: as the leaf expanded). All genotypes, including susceptible genotypes ‘Waltham’ and ‘Moschata 166’, gave negatives readings (<0.400) in leaf 1 and positive ELISA readings (>0.400) in leaf 2, leaf 3, and leaf 4 (Table 5). Only resistant genotypes ‘Menina’ and ‘Nigerian Local’ had negative readings in all leaves. Positive ELISA readings were observed only in leaf 4 for the genotype ‘Soler’. In general, virus titer increased at higher leaf positions (in younger leaves) in all genotypes except for ‘Menina’ and ‘Nigerian Local’ where virus titer was relatively the same in leaf 1 to leaf 4. In Experiment 2, leaf 4 was tested between 9 and 12 days after leaf 1 was tested.

A different tendency in ELISA readings was observed for ZYMV compared with PRSV. In Experiment 1, where all leaves were sampled at 21 days post-inoculation, all leaves could be used to differentiate between resistant with susceptible genotypes in the ELISA readings for ZYMV (Table 3). All genotypes, except resistant ‘Menina’ and ‘Nigerian Local’, had positive readings in all leaves. The same general tendency was observed in Experiment 2, were leaves were sampled as each leaf fully expanded (Table 7). In this experiment there were significant difference among genotypes for ZYMV ELISA readings in leaf 1, but already in this leaf position there was a tendency towards differentiating the susceptible versus the resistant genotypes. ‘Nigerian Local’ gave negative readings at all four leaves positions while ‘Menina’ was negative for ZYMV in leaf 2, 3, and 4 (in leaf 1 the reading was weakly positive). In this experiment ‘Waltham’ exhibited

some low readings in leaf 1 and leaf 2 and thus could not be distinguished from both resistant genotypes.

When samples were taken from leaf 1 at 20 days post-inoculation (such as was the case in Experiment 1), there were a high PRSV viral protein titer in cells resulting in a high absorbency in PRSV ELISA readings. But when leaf 1 was sampled three to four days post-inoculation (as it emerged such as was the case in Experiment 2), PRSV ELISA readings were negative (had low absorbance) meaning that there were no viral particles in the tissue or the number of viral titer was so low that the PRSV ELISA test could not detect them. Because of virus replication, the number of viral particles is expected to increase over time resulting in more viral particles when the first leaf is sampled 20 days post-inoculation versus when that same leaf is sampled three to four days post-inoculation. Leaf 4 was sampled at approximately 20 days post-inoculation in both Experiment 1 and Experiment 2 (leaf 4 was emerging approximately 20 days post-inoculation). As expected, PRSV ELISA readings in leaf 4 for a particular genotype were similar in both experiments. Readings were high in three of the susceptible genotypes ('Waltham', 'Moschata 166', and 'Taína Dorada') intermediate in 'Soler' and negative for PRSV in 'Menina' and 'Nigerian Local'. In general, for PRSV more than three or four days and less than 20 days are needed to have high viral replication in susceptible genotypes, to distinguishing them from resistant genotypes.

For genotypes susceptible to ZYMV, there were a high number of viral particles in cells (and thus a high ELISA absorbances reading) in leaf 1 whether it was sampled 3 to 4 days post-inoculation (Experiment 1, Table 3) or at 20 days post-inoculation (Experiment 2, Table 7). This status continued thru leaf 4. In contrast, to PRSV, ZYMV readings in resistant genotypes 'Menina' and 'Nigerian Local' remained low in all four leaves no matter when they were sampled.

To confirm what would happen to PRSV and ZYMV readings after leaf 4, Experiment 3 included readings from leaf 5. Leaves were sampled every three to four days as each leaf fully expanded, starting with leaf 1 three to four days after inoculation. The susceptible genotypes gave strongly positive PRSV readings on leaf 5 while the resistant genotypes 'Menina' and 'Nigerian Local' had slightly positive readings (0.414 to 0.454). 'Menina' and 'Nigerian Local' had readings that were negative for PRSV on the first and forth leaves (Table 9). Only 'Menina' inoculated with PRSV+ZYMV gave negative PRSV ELISA readings (<0.400) in all leaves.

For ZYMV, all leaves of resistant 'Menina' and 'Nigerian Local' (expect the first leaf of 'Menina' inoculated with ZYMV) had negative readings while susceptible 'Waltham' and 'Moschata 166' gave positive ZYMV ELISA readings (>0.400) on leaf 1, 4, and 5 (Table 9). For ZYMV, there was no detectable virus particles (via ELISA) in 'Menina' and 'Nigerian Local', and both genotypes could be differentiated from susceptible genotypes in the first five leaves. This suggests that resistant genotypes can be distinguished from susceptible genotypes since the first or second leaf for ZYMV, but the fourth leaf, or waiting few days once the fourth leaf has fully expanded, is required to be able to distinguish between resistant and susceptible genotypes for PRSV. In general, it is possible to differentiate between genotypes that are susceptible or resistant to ZYMV much earlier than it is possible to do the same for PRSV.

In previous research, a similar experiment was performed. Pacheco et al. (2003) inoculated three genotypes, including 'Menina Brasilera', with mild and severe strains of PRSV-W. PTA-ELISA test for *Papaya ringspot virus* was performed in these genotypes for 35 days, sampling plants days 7, 14, 21, 28, and 35 post-inoculation. They found that after 21 to 28 days post-inoculation, the virus concentration of the different strains inoculated on 'Menina Brasilera' was dramatically reduced and viral particle were not detected by the PTA-ELISA test, although low

positive PTA-ELISA readings were obtained in the first 14 days post-inoculation. A similar tendency was observed in Experiment 1 (sampling all leaves simultaneously), were resistant genotypes 'Menina' and 'Nigerian Local' virus concentration decreased in higher leaf positions (higher leaf position corresponds to younger leaves), giving negative readings (<0.400) in leaf 4 (20 days post-inoculation). In Experiment 2, even though 'Menina' and 'Nigerian Local' gave negative ELISA readings in all the leaves, susceptible genotypes gave positive ELISA readings after 6 to 8 days post-inoculation (leaf 2, 3, and 4) and mean virus concentration increased as leaf position increased (younger leaves). This research suggests that for PRSV, more than three or four days and less than 20 days are needed so that virus concentration is reduced to a point that viral particle would not be detected by ELISA test in resistant genotypes. In susceptible genotypes, viral concentration increases through time, and it is not necessary to wait between 21 to 28 days post-inoculation to see a viral serological titer decrease as happens in resistant genotypes.

Testing the effect of single infection with PRSV or ZYMV versus double inoculation with PRSV+ZYMV was an additional objective of Experiment 3. In general, no significant differences were found between single and double inoculation for PRSV ELISA tests among genotypes. Only susceptible 'Waltham' presented, in the first and fourth leaves, a significant difference between single and double inoculation PRSV ELISA readings, being greater in plants with PRSV than in plants with PRSV+ZYMV (Table 9). This significant difference was not observed in leaf 5. For ZYMV, significant differences were not found in ZYMV inoculated versus PRSV+ZYMV inoculated plants on the first, fourth, and fifth leaves (Table 10). In a double inoculation of ZYMV with *Cucumber mosaic virus* (CMV), Fattouh (2003), found that in the double inoculation of ZYMV+CVM, ZYMV titer did not significantly increases compared with the single inoculation.

Although the lowest fresh and dry weight among genotypes was obtained from the double inoculation, in general, there were not significant differences between uninoculated (control) and inoculated (PRSV, ZYMV, and PRSV+ZYMV) plants. The same tendency was observed in previous research, that compared the fresh weight (25 days after inoculation) between uninoculated (healthy), single inoculated (PRSV-W, ZYMV, WMV, and CMV), and mixed infection in 'Caserta' (*Cucurbita pepo*) (Barbosa et al., 2016). No significant differences were observed in the fresh weight between single, double, triple, and quadruple infection. However, significant differences were found in fresh weight between uninoculated and inoculated plants (Barbosa et al. 2016), in contrast to what was observed in the study presented here.

Despite previous observations, documented in photographs, that show that growth of 'Menina' is affected by these viruses, no consistent effect of virus inoculation was observed when comparing fresh and dry seedling weight (Table 11). Significant differences were found in 'Menina' but the mean of inoculated plants for both fresh and dry weight was similar or higher than the weight of control plants. In the study presented here, it appears that viral replication did not affect seedling growth. For susceptible genotype 'Waltham', there was a tendency for plants inoculated with both PRSV and ZYMV to have the lowest fresh and dry weight and for control plants to have the greatest weight. However, in general, no significant differences were found between plants of 'Waltham' inoculated with PRSV or ZYMV with uninoculated plants (control). For susceptible genotypes 'Moschatta 166' and resistant genotype 'Nigerian Local' the type of inoculation had no effect on fresh or dry weight.

Pacheco et al. (2003) reported mild mosaic symptoms in 'Menina Brasilera' infected with a severe strain of PRSV. However, they found no statistical difference between the biomass of 'Menina Brasilera' infected with mild and severe strains compared to the control plants (inoculated

with buffer), 40 days after infection. Two individual experiments were performed. In general, fresh and dry weight means of the aerial part of inoculated plants with mild and severe virus strains had higher weight than healthy plants in both experiments. Pacheco et al. (2003) reported that only in experiment 1, 'Menina Brasilera' inoculated with mild strains had a biomass reduction of 0.3%, but for the rest of the treatments, in experiment 1 and experiment 2, inoculated plants had higher biomass than control plants.

Finally, Experiment 4 was designed to determine if resistant genotypes 'Menina' and 'Nigerian Local' inoculated with PRSV or ZYMV have the capacity of transmitting viable viral particles to susceptible genotypes 'Waltham' and 'Moschata 166' via mechanical inoculation. This research demonstrated that previously inoculated plants of 'Menina' and 'Nigerian Local' could not be used as a source of inoculum to infect susceptible 'Waltham' and 'Moschata 166'. This confirms that both 'Menina' and 'Nigerian Local' mechanically inoculated with PRSV and ZYMV are not suitable host for replication of these two viruses.

6. CONCLUSION

Viral diseases are a major problem in crops of the *Cucurbitacea* family. The most efficient way to diminish or eradicate viral incidence is by using resistant genotypes developed in plant breeding programs. Breeders depend on the development of efficient and reliable methods of assessing resistance. The resistance of 'Nigerian Local' is to both PRSV and ZYMV is well documented in the literature. There is a necessity to identify new genotypes that are resistant to viral diseases and have good agronomical characteristics, so breeders can used them in their breeding programs.

Although 'Menina' inoculated with PRSV and ZYMV exhibit smaller leaves and weakening of green color symptomatology, it was found to also be highly resistant to PRSV and ZYMV. 'Soler' may have some intermediate resistance to both viruses. This research also demonstrated that ELISA can be used as an efficient way of assessing resistance. Mechanical inoculation of both PRSV and ZYMV can be used to test *C. moschata* for resistance to these viruses. For PRSV ELISA readings from leaf 4 can give valuable information to the breeder to determine whether a genotype is resistant or not. For ZYMV, ELISA readings from the 1st leaf, sampled as it emerges can usually differentiate between susceptible and resistant genotypes.

Symptomatology combine with ELISA test on the fourth leaf for PRSV and in any of the first four leaves for ZYMV, it is a reliable and efficient method of assessing resistance. Both 'Menina' and 'Nigerian Local' provide the plant breeder with a good source of genes for resistance to both potyviruses.

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