THE EFFECT OF TWO POTYVIRUSES ON DEVELOPMENT AND YIELD IN TROPICAL PUMPKIN AND THE INHERITANCE OF RESISTANCE TO *PAPAYA RINGSPOT VIRUS*

by

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ABSTRACT

Viruses of the Potyviridae family can infect all cucurbit crops. General symptoms are well-described, but there are no known studies documenting yield and fruit quality loss in tropical pumpkin (*Cucurbita moschata*). Papaya ringspot virus (PRSV) is the most common of the potyviruses found in Puerto Rico. Two sources of resistance are well known in *C. moschata*: 'Nigerian Local' (NL) and 'Menina' (MEN). The inheritance of resistance from NL has been previously studied, however no inheritance studies have been reported for MEN, nor is it known if resistance to PRSV in NL is allelic to that in MEN. Therefore, the objectives of this research were to study (1) the impact of potyvirus on tropical pumpkin at the field level and (2) the inheritance of PRSV resistance in mechanically inoculated F_2 populations derived from both sources of resistance, and test for allelism in a NL x MEN F_2 population. Control plants and plants inoculated with PRSV, ZYMV or PRSV+ZYMV of six different genotypes were transplanted to the field. Number of fruit and fruit weight per plant, and average fruit size were reduced up to 50% in virus-inoculated plants. In the inheritance study susceptible genotypes were 'Verde Luz' (VL), 'Taina Dorada' (TD) and 'TP411' (TP). The third to fifth leaf of inoculated seedlings were rated on a 0 to 4 scale for disease severity and scores were combined to convert to a 0 to 12 scale. F_2 populations using NL as the source of resistance had a nearly normal distribution with an average disease severity of 5.23 in NL x TD and 6.25 in VL x NL. In contrast, the F_2 populations with MEN were strongly skewed towards resistance with an average severity of 3.38 in MEN x TD, 2.27 in VL x MEN and 2.80 in TP x MEN. The NL x MEN F_2 population was highly skewed, with an average combined severity of 0.840. It segregated 224:14 (R:S) when a combined severity of ≤ 4 was considered resistant. Segregations in resistant x susceptible F_2 populations were variable, depending on how severity scores were combined into

the resistant versus susceptible classes. However, most segregations suggested that at least two genes are involved in the inheritance of resistance to PRSV for both NL and MEN. The data clearly indicate that at least some of the genes for resistance in NL and MEN are different. Considering the level of resistance conferred by both NL and MEN, both sources, either alone or combined, will be useful in a breeding program.

EL EFECTO DE DOS POTYVIRUS EN EL DESARROLLO Y RENDIMIENTO DE CALABAZA TROPICAL Y LA HERENCIA DE GENES DE RESISTENCIA AL VIRUS DE LA MANCHA ANULAR DE LA PAPAYA

RESUMEN

Los virus de la familia *Potyviridae* pueden infectar todas las cucúrbitas. Los síntomas generales han sido descritos, pero no hay estudios documentando la pérdida de rendimiento y calidad de fruto en la calabaza (*Cucurbita moschata* Duchesne). El *virus de la mancha anular de papaya* (PRSV) es un potyvirus comúnmente encontrado en Puerto Rico. Para *C. moschata*, existen dos fuentes de resistencia que han sido reportadas, son los genotipos: 'Nigerian Local' (NL) y 'Menina' (MEN). Explicaciones sobre la herencia de los genes de resistencia de NL han sido presentadas por investigadores, pero no se han realizado estudios de este tipo para MEN, ni se ha reportado si la resistencia de ambos genotipos mencionados es alélica o son genes diferentes. Los objetivos de este estudio fueron (1) documentar el impacto de PRSV sobre calabaza sembrada al nivel de campo y (2) estudiar la herencia de resistencia a PRSV en poblaciones F_2 derivadas de ambas fuentes de resistencia, NL y MEN y realizar una prueba de alelismo con una población F_2 de MEN x NL. Se trasplantaron plantas no inoculadas e inoculadas con PRSV, ZYMV y PRSV+ZYMV de seis diferentes genotipos en el campo. El número y peso de fruto por planta y el peso promedio por fruto se disminuyeron hasta 50% en plantas inoculadas. En el estudio de herencia, los genotipos susceptibles utilizados para los cruces fueron: 'Verde Luz' (VL), 'Taina Dorada' (TD) y 'TP411' (TP). La tercera, cuarta y quinta hoja de las plántulas inoculadas fueron clasificadas de 0 a 4 en una escala de severidad de enfermedad, estos valores fueron combinados para realizar una escala combinada de 0 a 12. En poblaciones F_2 de NL se pudo observar una distribución aproximadamente normal con promedios de severidad de enfermedad de 5.23 para

NL x TD y 6.25 para VL x NL. Por otro lado, poblaciones F_2 con MEN fueron bastante sesgadas hacia mayor grado de resistencia con promedios de severidad de 3.38 para MEN x TD, 2.27 para VL x MEN y 2.80 para TP x MEN. La población F_2 de NL x MEN fue fuertemente sesgada, con una severidad combinanda promedio de 0.840. Segregó 229 resistentes y 9 susceptibles al considerar valores de $\leq$4 (en la escala combinada) como resistente. Las segregaciones observadas en poblaciones F_2 de cruces entre parentales resistentes y susceptibles variaban dependiendo como se clasificaba entre resistente y susceptible en referencia a la escala combinada. La mayoría de las segregaciones observadas en las diferentes poblaciones sugieren la presencia de más de un gen responsable de la resistencia a PRSV para ambos NL y MEN. La segregación en la F_2 de NL x MEN indica que el o los genes de resistencia a PRSV de MEN y NL no son alélicos. Al tomar en consideración el nivel de resistencia que proveen ambos NL y MEN, y considerando que sus genes son al menos parcialmente diferentes, ambos son útiles para un programa de mejoramiento.

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I dedicate this work to my niece Naia Solè Hernandez-Seda, who has begun her journey in life at about the same time I started to really fall in love with research and experimentation in the plant sciences field. I love her and wish her a life full of happiness and love.

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1. INTRODUCTION

Pumpkin are part of the family of plants named *Cucurbitaceae*. In this family there are many plants that have been domesticated for human consumption including melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb) Matsum. & Nakai), squash/pumpkin (*Cucurbita moschata* and *C. pepo* L.), and cucumber (*Cucumis sativus L.*). *Cucurbita spp*. is a genus of plants that have originated in the Americas (Whitaker and Robinson, 1986). Evidence suggest that *Cucurbita pepo, C. moschata* (including the tropical pumpkin "calabaza" in Puerto Rico), *Cucurbita argyrosperma* C Huber, *C. maxima* Lam. and *C. ficifolia* Wall., were commonly cultivated more than five hundred years ago in Mesoamerica. Pumpkin, (or squash – the common name does not refer to a particular *Cucurbita* species) is a slightly-lignified herbaceous vine plant with leaves, flowers and fruit that are edible. The pumpkin is valued in agriculture for its economic potential, nutritional value, and high availability throughout the year (Saeleaw and Schleining, 2011). Pumpkin is grown for several purposes around the world: fruit, seed, and the flowers are all edible parts of the plant that people harvest and they are considered important in their area of production (Vučurović, et al. 2012). Compared to agronomic crops, *Cucurbita* spp. are not considered major crops in the agricultural systems of the United States, but it is an important vegetable cash crop in the Americas. By 2016 in the United States there was a total of 71,400 acres of pumpkin harvested, which is a big increase compared to the previous fifteen years with an average of 44,200 acres harvested (U.S, Department of Agriculture, 2016). From 2009 to 2014, in Puerto Rico pumpkin has consistently been the second to third most important vegetable crop in terms of amount harvested and economic value of $4.7 million US dollars in 2014-15 (PR Departamento de Agricultura, 2015).

At the University of Puerto Rico at Mayaguez (UPRM) there is a pumpkin improvement program led by Dr. Linda Wessel-Beaver. The objectives of this program include developing resistance to pests and adding value to an already economically important crop. Collaborations between governmental and academic research organizations are being done in the United States as well as other countries, with the objective of developing genomic approaches for breeding and assessment of economic impact in production and disease control. Since 2015, UPRM, along with 10 other institutions across the U.S., has participated in a USDA "Coordinated Agricultural Project" (CAP) titled "CucCAP: Leveraging Applied Genomics to Increase Disease Resistance in Cucurbit Crops". The research presented in this thesis is part of the effort of the CucCAP project.

There are some challenges to growing pumpkin successfully in a tropical climate. Among them, the most common challenges are viral and fungal infections. More than 35 viruses have been isolated in cucurbits and some of these represent a high threat to production worldwide. These viruses can cause up to a 100% economic loss of production because of their high level of virulence (Menenzes et al., 2015). Viral infections of cucurbit crops were identified in Puerto Rico as early as the 1930's by a survey/study by Cook (1936). Adsuar and Cruz-Miret (1950) first reported and investigated what they called virus A and virus B. They characterized the symptoms and infection processes of these viruses in Puerto Rico. The study clearly demonstrated the existence of two separate viruses, although the identity of these viruses using modern virus nomenclature is not known. Studies of virus incidence in 1982 showed there was a high incidence of viral diseases around commercial cucurbit farms in Puerto Rico (Escudero, 1992). In 2001 and 2002, a survey of cucurbit crops in Puerto Rico showed that 69% of all materials surveyed to be infected by *Zucchini yellow mosaic virus* (ZYMV) and 59% of materials infected with *Papaya ringspot virus* (PRSV) (Paz-Carrasco and Wessel-Beaver, 2002). Interestingly, another finding was that about

62% of all material sampled were infected by more than one of the viruses surveyed. Infection with PRSV and ZYMV appeared to be severe, likely lowering yield output and causing economical loss to farmers who cultivated this crop although no data is available to document this observation.

Provvidenti et al. (1986) reported on evaluations of several populations of cucurbits resistant to viruses like ZYMV, *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus-1* (WMV-1, which is synonymous to PRSV), and *Watermelon mosaic virus-2* (WMV-2, also known simply as WMV). An accession of *C. moschata* obtained from Nigeria ('Nigerian Local') was confirmed to have resistance to some of these most important viruses. Provvidenti obtained this seed from Dr. Igwegbe, University of Nigeria, Nsukka, Anabra State in 1983 according to written correspondence between Provvidenti and Wessel-Beaver dated 24 April 2000. Thus Provvidenti gave this accession the name 'Nigerian Local' (L. Wessel-Beaver, personal communication).

Lecoq et al. (1998) released an important article in 1998 called "Cucurbit Viruses – The Classic and the Emerging" in which viruses from all vectors were described, and virus resistant strains released were evaluated. The paper mentions that potyvirus like PRSV (referred to as WMV-1 in the paper), ZYMV and WMV had already managed to spread and infect cucurbits all around the world. In response to this problem, plant breeders have been trying to develop strains that are resistant to these viruses. Other viruses not in the *Potyviridae* family have also been reported as harmful to cucurbit production in the United States during past years, including *Tobacco mosaic virus* (TMV) and *Beet pseudo yellows virus* (BpYV) (Wintermantel, 2004).

2. OBJECTIVES

Objective 1. Assess the impact of virus infection (PRSV & ZYMV) on growth, development and production of tropical pumpkin.

Objective 2. Study the inheritance of resistance to PRSV.

3. LITERATURE REVIEW

3.1 Potyvirus

Potyvirus is a genus of viruses known largely for affecting plants. They are defined and contrasted to other viruses by their particle size, with a measurement of approximately 730-760 nm in length (Shukla and Ward, 1989). The length measurement limits have been subjected to changes in consideration by the taxonomical investigations done on potyviruses through the years. Because of the difficulty of identification and classification of this genus of viruses, Shukla and Ward (1989) suggested the use of sequence data of the coat proteins as a better means for identification. The identification of potyviruses is often done utilizing serological methods such as Double Antibody Sandwich (DAS) Enzyme-Linked Immunosorbent Assay (ELISA). Further diagnosis of isolates of the same kind of virus can be done with RNA extraction, RT-PCR and sequencing.

These viruses are generally (if not always) spread from plant to plant through aphids as vectors in a non-persistent manner. Several common aphid species, known for mainly affecting other crops, have been reported to spread potyvirus like ZYMV and PRSV to cucurbits. This can be attributed to the aphid's wide range of hosts. Some of the genera of aphids known to vector potyviruses in Puerto Rico are the following: *Aphis, Aulacorthum, Lipaphis, Macrosiphum, Myzus, Rhodobium, Rhopalosiphum, Toxoptera* and, *Uroleucam* (Escudero, 1992). Several host plants to these viruses have been reported in Puerto Rico and some of these are actively growing throughout the whole year, meaning that the virus has a host in which to live until a planting of crops is made where these weeds are growing. Some reported host plant families for ZYMV, WMV, and PRSV

in a study in Puerto Rico include Cucurbitaceae, Chenopodiaceae, Leguminosae, Malvaceae, and Solanacea (Escudero, 1992). The largest number of hosts occurs in the Cucurbitaceae family.

Potyviridae are believed to cause high yield loss in watermelon, pumpkin, zucchini, melon and squash. Lecoq et al. (1998) explains how some potyviruses have worldwide distribution affecting several cucurbits, while others have a more regional distribution. There are many unreported and uncharacterized potyviruses in the world. A study that sampled various cucurbit species in Puerto Rico between 2006 and 2011 showed that two potyviruses, PRSV and ZYMV, are the most frequent virus species affecting cucurbits, especially tropical pumpkin, around the island (Paz-Carrasco and Wessel-Beaver, 2002). Ever-present wild cucurbit species on the island are known to be infected with potyviruses and can provide a source of inoculum for cultivated crops and the other way around (Rodrigues et al., 2012). The UPRM cucurbit breeding program uses locally isolated and characterized strains of potyvirus to conduct their experiments. Some of these were identified in a study by Rodrigues et al. (2012) through RNA extraction and RT-PCR.

3.2 Virus Control Methods

A solution to the detrimental effect of virus infection in cultivated cucurbit crops is the development of resistant varieties. Some of the ones that have already been released share a common source of resistance with different genes adapted to where the variety is released. Breeding programs develop these varieties after extensive research and evaluations specific for the crop in question. Resistance to viruses of the *Potyviridae* family have been incorporated into cultivar lines through several breeding programs and can be found in several cucurbits including squash, cucumber, pumpkin and melons (Gal-On et al., 2000; Brown et al., 2003; Kung et al., 2009; Pachner et al., 2011; Menenzes et al., 2015). Proposed models for inheritance of these

resistance genes have resulted and are becoming more complex with the proliferation of investigative technology and tactics; but there already exists commercial varieties of these crops with resistance from different sources and these have been attained through traditional breeding (L. Wessel-Beaver, personal communication).

While traditional selective breeding has proven to produce efficient resistance in important commercial cultivars, selection is likely to be assisted by the use of molecular markers and genomics information. More recent studies have been made with transgenes of plants and viruses to attenuate symptom severity, and to develop types of tolerance and systemic resistance (see for example, Gal-On et al., 2000). Furthermore, posttranscriptional gene silencing is an important natural aspect of the plant that can be further studied for this situation.

Other methods of control for the detrimental situation of virus's effect in crops exist; several of these are related to the control of the vector. As with other viruses, the immediate disposal of any infected plant can help control the spread of the virus; like other aphid-borne virus, the elimination, disorientation or prevention of the aphid provides better results. Studies have shown that plastic covers can be effective in the reduction of aphid populations in crop plantations. In a study done in California by Nameth et al. (1986) using reflective plastic covers for the planting beds, results showed aphid populations being reduced by up to 96%, and as a response, reduced virus incidence by 85 to 90%. This is a significant reduction and it is a useful measure of control to implement, especially integrated with other measures like the use of resistant varieties. Another, but less effective, measure of control experimented in this study was the use of aromatic and petrochemical oils for the disorientation and extermination of the aphids; this measure was proven very much less effective (17 to 33% reduction of vector population). Intercropping pumpkins with barrier crops is another control method that has been observed. Damicone et al. (2007)

experimented with intercropping pumpkins with several crops. Grain sorghum intercropping resulted in a control (43 to 96% less infection) of a two aphid-transmitted virus: PRSV and WMV-2. The methods of control listed above have a wide range of positive impact on disease control and should be assessed to see which are the ones that yield the best results.

3.3 *Papaya ringspot virus* - PRSV

PRSV belongs to the genus *Potyvirus* and family *Potyviridae,* with a positive sense RNA genome of about ten thousand nucleotides. Among plants, this virus is easily transmitted in a non-persistent manner via aphid feeding on many hosts including commercial crops of the families *Caricaceae* and *Cucurbitaceae* (Tripathi et al., 2008). There are two major strains of PRSV that affect cucurbits: strains P and W. PRSV is the cause of a destructive disease and a major limiting factor for papaya and cucurbit cultivation around the world (Zhao et al. 2015). There are several other potyvirus that are related to PRSV such as *Watermelon mosaic virus* (WMV-2), *Zucchini yellow fleck virus* (ZYFV), and other viruses that have only been partially studied and characterized.

Various studies have been made to evaluate and survey PRSV. Non-persistent aphid vectors can efficiently transmit PRSV from one plant to another in a manner of minutes or less (Kalleshwaraswamy, 2007). This study also concludes that the aphids make a very efficient vector because of their rapid population growth. This is a clear example as to why aphid prevention as a tactic for crop protection is hard to manage. Studies of aphid-borne viral incidence commonly found double and triple infection in pumpkin in squash (Paz-Carrasco and Wessel-Beaver, 2002; Vucurovic et al., 2012), thus viral incidence is observed frequently in the form of several virus infecting the same plant.

Brown et al. (2003) determined the inheritance of resistance to four cucurbit viruses in *C. moschata*. The study was carried out on in a single F_2 population of the cross between the susceptible cultivar 'Waltham Butternut' and the resistant cultivar 'Nigerian Local'. In separate experiments, F_2 populations were inoculated with each of the following viruses: CMV, WMV (=WMV-2) PRSV and ZYMV. Since the ratio of resistant to susceptible plants when infected with PRSV was 1:3, a single recessive gene model was proposed and the resistant gene named *prs*.

Mcphail-Medina et al. (2012) conducted a broader study on inheritance of resistance to PRSV in tropical pumpkin in multiple populations. The experiments observed F_2 populations developed by crossing 'Nigerian Local' with three susceptible tropical pumpkin cultivars previously developed in the UPRM breeding program for tropical pumpkins: 'Soler', 'Taina Dorada', and 'Verde Luz'. The plants infected were rated on a severity scale of 0 (few or no symptoms) to 3 (very severe symptoms) and the results were analyzed contrasting different groups of severity score combinations. A 13:3 (susceptible:resistant) ratio was found to be the model that best fit the results of severity in the different populations. This was interpreted by the investigators as a two-gene model with dominant suppression epistasis.

Inheritance of resistance to PRSV has also been studied in other cucurbits. In watermelon, Acevedo et al. (2012) concluded that there was an oligogenic inheritance of resistance to PRSV of about 2.61 genes with an additive behavior, suggesting that in this species, several genes are working together to produce a resistant phenotype.

A historical achievement in transgenes was the early development of genotypes of *Carica papaya* resistant to PRSV. Results of a study made by Kung et al. (2009) show that double-virus resistance in transgenes of papaya expressing the coat protein (CP) gene for PRSV is mediated by RNA-mediated post transcriptional gene silencing.

Both Brown et al. (2003) and Mcphail et al. (2012) studied a single source of resistance, 'Nigerian Local'. Another genotype, 'Menina', has also been reported to carry resistance to PRSV (Paris et al. 1988) but whether or not it is allelic to 'Nigerian Local' has not been studied. Even though the inheritance of resistance to PRSV still needs further study, there are already successful breeding programs selecting for resistance to PRSV; the Agricultural Experiment Station (AES) of UPRM has been successful in creating F_4 lines of tropical pumpkin with resistance to PRSV (L. Wessel-Beaver, personal communication).

3.4 *Zucchini Yellow Mosaic Virus* - ZYMV

In various regions, a very serious threat to production of pumpkins is ZYMV (Basky et al., 2001). This virus is widely distributed and causes devastating epidemics in a wide range of cucurbit crops. It was described first in Italy by Lisa et al. (1981), observed in a zucchini plant showing symptoms different from the symptoms associated to CMV, WMV and PRSV. It has, since then, been reported in all cucurbit growing areas in every continent (Vučurović et al., 2012; Desbiez and Lecoq, 1997). Plants with ZYMV can present chlorosis, mosaic-like distortion, and wrinkling when inoculated at a young stage in a greenhouse; symptoms can appear in as little as 5 to 7 days post-inoculation. A further observation of these plants shows symptoms of severe mosaic, stunting and lack of production. Tropical varieties of pumpkin plants have stunted growth, premature flowering and loss of fruit and flowers (Pachner et al. 2011). In terms of yield, studies have concluded that in temperate climates, infections of ZYMV in *C. maxima* can cause losses of 26 to 84% (Fletcher et al, 2000). No similar studies are known from the tropics or in *C. moschata*.

Paris et al. (1988) evaluated 68 accessions of *Cucurbita* for resistance to ZYMV and identified *C. moschata* 'Menina', received from a cultivar developed in Portugal that bred true for resistance to the virus. Crosses were made between an inbred line of 'Menina' and a susceptible

variety ‘Waltham Butternut’. Results of this study showed the F_2 populations having the expected ratio of 3:1 (resistant:susceptible) ratio that is seen in an inheritance of single dominant gene. This single dominant gene was denominated *Zym.* Brown et al. (2003) used ‘Nigerian Local’ as their source of resistance to ZYMV in a cross with susceptible ‘Waltham Butternut’ and also concluded that a single dominant gene controlled resistance.

Several years later Pachner and Lelley (2004) reported a different model for resistance to ZYMV evaluating resistant genotypes‘Menina’ and ‘Nigerian Local’, semi-resistant ‘Soler’ and susceptible ‘Waltham Butternut’. The studies they made resulted in the proposal of a three-gene segregation model for resistance. In a later study, Pachner et al. (2011) did further studies on the inheritance of ZYMV resistance. They concluded that at least five genes (*Zym-1, Zym-2, Zym-4, Zym-5* and *zym*-6) confer resistance to ZYMV and that some of these genes have epistatic relationships and that some of the loci may be linked. Resistance in ‘Nigerian Local’ (from *Zym-0* and *Zym-4*) was not allelic to that of ‘Menina’ (from *Zym-1*). ‘Soler’ carried a recessive gene (*zym-6*) for resistance. This resistance was considerably weaker than that of ‘Nigerian Local’ or ‘Menina’.

There are some studies done with ZYMV where resistance has been obtained by attenuating viral cDNA of ZYMV (Gal-On et al., 2000). A clone of infectious nature was processed by developing an attenuated virus. After constructing this clone, particle bombardment of the isolate was done to several cucurbits including squash. The stable engineered virus changed the symptoms significantly from severe to mild in pumpkin and eradicated symptoms in melon and cucumber. This AG1 mutated strain also proved to behave as a protectant to cucurbits against infection with ZYMV.

Since the sources of resistance to ZYMV are the same as observed in PRSV ('Nigerian Local' and 'Menina'), and both of these viruses belong to the *Potyviridae* family, it is very likely that resistance to PRSV is controlled by multiple genes as has been observed in ZYMV.

4. MATERIALS & METHODS

4.1 Preparation of virus inoculum / Mechanical inoculation

Inoculum, originally selected at Plant Virology Laboratory – EEA, was prepared with infected lyophillized tissue that was maintained frozen (-20 C°) in the Plant Breeding Laboratory of Dr. Linda Wessel-Beaver, Department of Agro-environmental Science, UPRM. Dried infected tissue was macerated in phosphate buffer (pH 7) added to a cold mortar (mortar and pestle stored at -20 °C) in a proportion of 0.12 g tissue to 10 mL buffer. A small amount of large grit carborundum was added to aid the maceration of the tissue. The macerated tissue was gently but firmly wiped onto 5 to 6 day-old cotyledons of 'Waltham Butternut' (Mountain Valley Seeds, Salt Lake City, Utah) seedlings using a piece of folded cheesecloth. After the inoculation, plants were lightly washed with water to remove the remaining carborundum. Plants used as inoculum were kept under artificial lights for 8 hours a day in the Plant Breeding Laboratory. To avoid cross-contamination, plants of PRSV were kept separate from plants with ZYMV. Approximately 25 days after inoculation, when the seedlings already had a fully expanded fourth true leaf, the plants were tested for concentration of virus (titer) with a Double Antibody Sandwich – Enzyme-Linked Immunosorbent Assay (DAS-ELISA) test using a virus-specific (PRSV and ZYMV) commercial kit (Agdia, Elkhart, Indiana). If a plant was found to have a reading of less than 0.4 for virus titer, it was discarded. Once a plant was confirmed to be infected (score of 0.4 or more) with a single

virus (and not cross-contaminated), leaves from that plant could be used as inoculum in experiments.

DAS-ELISA was performed to detect and quantify virus in several steps during experimentation. Commercial buffers, reagent sets and controls were bought (Agdia, Elkhart, Indiana) and the tests were realized by following Agdia's protocol. The samples of tissue from the evaluated plants were assayed in 96-well microplates; the readings were made at 405 nm by a microplate absorbency reader (Multiskan FC 357, Fisher Scientific, Hampton, New Hampshire). The readings were separated into two classes, an absorbance reading of less than 0.400 was considered a resistant plant, a reading of 0.400 or greater was considered a susceptible plant.

4.2 Methods for Objective 1: Assess the impact of virus infection (PRSV and ZYMV) on growth, development and production of tropical pumpkin

Six genotypes of pumpkin were planted and evaluated throughout their life cycle: 'Waltham Butternut' (Mountain Valley Seeds, Salt Lake City, Utah), 'Mos166' (AES-UPRM), 'Soler' (AES-UPRM), 'Taina Dorada' (AES-UPRM), 'Menina' (original seed stock from Dr. T. Lelley, University of Natural Resources and Life Sciences, Vienna, Tulln, Austria), and 'Nigerian Local' (original seed stock from Dr. R. Provvidenti, Cornell University, Ithaca, New York). Seed from these genotypes were planted in the greenhouse in 10 cm pots filled Promix BX® (Premier Tech, Quakertown, Pennsylvania) that had been previously wetted. When the germinated seedlings had fully expanded cotyledons (5 to 6 days), one of four treatments was applied to the cotyledons: (1) inoculation with PRSV, (2) inoculation with ZYMV, (3) inoculation with both viruses and, (4) mock-inoculation with buffer (control). Plants were inoculated as described above, except that fresh, rather than dried tissue was used at a rate of 1.0 g fresh tissue in 10 mL of phosphate buffer.

For plants inoculated with both viruses, one cotyledon was inoculated with PRSV and the other with ZYMV. Seedlings were kept in an enclosed greenhouse, well-watered and fertilized with a dose of 1 tablespoon of soluble fertilizer per gallon (20-20-20 N-P-K) approximately every 5 days. Greenhouse temperature varied from about 28 °C (night) to about 40 °C (day). About 25 days after planting the plants were assayed for virus infection through ELISA on a fully-expanded fourth true leaf. At approximately 4 weeks after planting, seedlings were transplanted to the field at the UPRM-AES in Lajas in April of 2016 (Experiment 1) and February of 2017 (Experiment 2). Both experiments were arranged in a completely randomized design. There was one plant per plot, and plots were 1.8 m apart within and between rows. Plantings were done on gray plastic-covered banks with drip irrigation. During their development, fertilizing (via the drip irrigation), regular monitoring and control of pests was done when necessary.

In Experiment 1 (2016) and Experiment 2 (2017) plants were periodically photographed and were assayed with ELISA at 20, 55 and 90 days post-inoculation (dpi) and 18, 54 and 98 dpi in 2016 and 2017, respectively. Flowering dates of the first staminate and pistilate flowers were recorded in both experiment years. In Experiment 2 (2017) mature fruit was harvested and taken to the laboratory. Total weight, fruit diameter, and pulp thickness were measured. Pulp color was measured as L*, a* and b* space (coordinates in the color space defined by the International Commission of Illumination) using a Colorflex EZ® (HunterLab, Reston, Virginia) colorimeter. °Brix was measured by a hand-held refractometer (Atago Co., Minato, Tokyo, Japan). Percentage of dry matter was determined. For pulp color and soluble solids measurements (°Brix) a 2cm × 2 cm by 5 cm sample of pulp was cut from each fruit. Fresh weight was determined, the colorimeter reading was taken and the sample was then frozen in a plastic bag at -20°C. The sample was later thawed. The juice squeezed from the sample, was harvested and used to measure °Brix by pouring

some juice in the hand-held refractometer and reading the number observable through the device's reading side. The pulp sample was dried in an oven at 65℃ for 48 hours (or until completely dry). Percent dry matter was then calculated as: dry weight divided by the fresh weight and then multiplied by 100. Hue angle and chroma was calculated from a* and b* using formulas from McGuire (1992).

Factorial analysis of variance (5 genotypes × 4 inoculation treatments) was done for each trait evaluated. Means were compared using Fisher's Least Significant Difference at the 0.05 probability level. Additionally, single degree of freedom contrasts were made for comparisons such as PRSV and ZYMV versus control, PRSV versus control, and ZYMV versus control. Pearson's correlation was used to compare ELISA readings between greenhouse and field results, and between field results taken of different dates.

4.3 Methods for Objective 2: Study the inheritance of resistance to PRSV

Previous to this study resistant genotypes 'Nigerian Local' and 'Menina' were crossed with susceptible genotypes 'Taina Dorada', 'Verde Luz' and 'TP411' in the breeding program of Dr. Linda Wessel-Beaver. At regular intervals, 60 to 120 F_2 plants, along with their respective parents and F_1 populations were planted in the greenhouse, at a rate of about 5 parental or F_1 plants per every 60 F_2s planted. Between September 2016 and May 2018 a total of 11 greenhouse plantings of F_2 populations were made in the greenhouse (Table 1).

Table 1. List of inheritance tests carried out in the greenhouse with dates planted and inoculated, populations tested, source from where the population's seed was taken, and number of seed planted.

Test	Planting date	Inoculation date	Population tested[1]	Seed source[2]	Number of seed planted
B	22-Oct-2016	28-Oct-2016	NL (res) TD (sus) F_1 F_2	E1406-NL-A⊗ E1305-22⊗ E1406-(47*52) E1512-36-1⊗	5 5 5 50
C	16-Feb-2017	22-Feb-2017	NL (res) TD (sus) F_1 F_2	E1406-NL-A⊗ E1305-22⊗ E1406-(47*52) E1512-36-1⊗	5 5 5 50

Table 1. (Continued)

Test	Planting date	Inoculation date	Population tested[1]	Seed source[2]	Number of seed planted
E	22-Oct-2017	28-Oct-2017	NL (res) TD (sus) F_1 F_2	E1406-NL-A⊗ E1305-22⊗ E1406-(47*52) E1512-36-1⊗	5 5 5 50
G	1-Dec-2017	5-Dec-2017	MEN (res) VL (sus) F_1 F_2	E1406-69⊗ E1602-7⊗ E1406-(38*63) E1512-4-1⊗	10 10 10 120
H	13-Dec-2017	19-Dec-2017	NL (res) MEN (res) F_1 F_2	E1406-A⊗ E1406-69⊗ E1406-(53*63) E1512-45-1⊗	10 10 10 120
I	24-Jan-2018	30-Jan-2018	NL (res) MEN (res) F_1 F_2	E1406-A⊗ E1406-69⊗ E1406-(53*63) E1512-45-1⊗	10 10 10 120

J	4-May-2018	10-May-2018	VL (sus) NL (res) F_1 F_2	E1602-9⊗ E1711-NL-2⊗ E1406-(36*NL) E1602-63A⊗	10 10 10 120
K	4-May-2018	10-May-2018	TP411 (sus) MEN (res) F_1 F_2	E1602-43⊗ E1711-MEN-2⊗ E1512-(74*85)-1 E1602-36-A⊗	10 10 10 120

[1]NL - 'Nigerian Local', TD – 'Taina Dorada', MEN – 'Menina', VL – 'Verde Luz', res – resistant line, sus – susceptible line.
[2] Experimental designations in the University of Puerto Rico, Mayagüez Campus, tropical pumpkin breeding program.

Seedlings were inoculated as previously described. The fully expanded fourth leaf was sampled and tested with ELISA as described above. When plants were 25 to 35 days old the third, fourth and fifth leaf (or on some occasions the fourth to sixth leaf) of each plant was evaluated for leaf symptom severity using the 0 to 4 severity scale in Figure 1. All leaf ratings of a plant were summed to obtain a single combined severity rating per plant. The combined severity scale had a range of 0 to 12. ELISA readings were converted into two classes: an absorbance reading of less than 0.400 was considered a resistant plant, a reading of $\geq$ 0.400 was considered a susceptible plant. Models of inheritance were tested by means of a chi squared test (χ^2) to evaluate how well the observed segregation fit with different gene models. Various models for one, two and three genes were tested using Fehr (1993, Table 3.3) as a guide. For both the 0 to 12 combined severity rating fit to models was tested by combining classes in various ways.

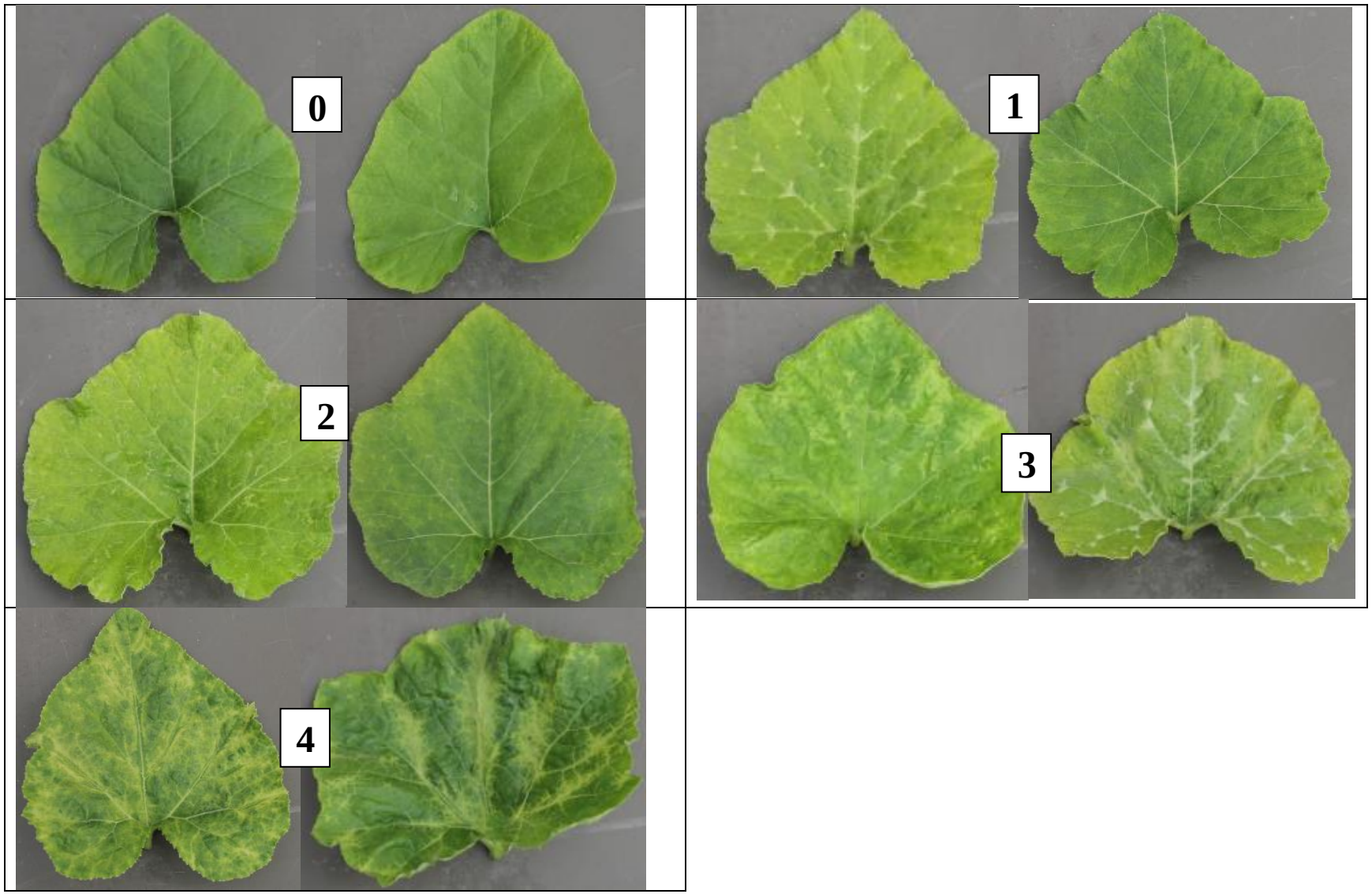

Figure 1. Visual scale (0 to 4) used to evaluate virus severity symptoms observed in plants of tropical pumpkin (*Cucurbita moschata*) inoculated with *Papaya ringspot virus*.

5. RESULTS

5.1 Impact of virus infection on performance of tropical pumpkin

Flowering occurred somewhat earlier in 2016 compared to 2017. The first male flower appeared before the first female flower, regardless of the virus infection treatment or genotype (Table 2). The average number of days after transplanting (DAT) to the opening of the first staminate flower of both treated and untreated plants averaged 21.7 days in 2016 and 29.0 days in 2017. For pistillate flowers, the number of DAT was 41.1 in 2016 and 42.6 in 2017. In both years 'Waltham Butternut', 'Nigerian Local' and 'Menina' generally were the earliest to flower, 'Taina Dorada' was intermediate and 'Soler', and 'Mos 166' flowered the latest.

In Experiment 1 (2016) inoculation treatment had no effect on flowering of either male or female flowers (Table 2). In Experiment 2 (2017), staminate flowering in plants inoculated with PRSV was later (33.1 DAT) compared to plants with other treatments (26.3 to 29.7 DAT). DAT of male flowering in plants inoculated with ZYMV or with both PRSV+ZYMV was not different from that of the uninoculated control. Days to pistillate flowering was delayed by nearly a week in plants inoculated with PRSV+ZYMV (46.2 DAT) compared to control plants (39.5 DAT). DAT in female flowering in plants inoculated with ZYMV or PRSV+ZYMV did not differ from control plants with a difference of less than 4.362 between means.

Table 2. Mean number of days from transplanting to anthesis of first male and female flower of six *Cucurbita moschata* genotypes inoculated with *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), PRSV+ZYMV (double inoculation – Experiment 2 only), and uninoculated (control) in Experiment 1 (2016) and Experiment 2 (2017).

	Flowering (days from transplanting)							
	Experiment 1 (2016)				Experiment 2 (2017)			
Factor	Male		Female		Male		Female	
Genotype (G)								
Menina	15.13	A	39.58	AB	28.7	C	48.2	C
Nigerian Local	20.83	B	37.92	AB	23.3	B	47.2	C
Mos166	27.72	D	50.39	C	38.4	D	39.1	B
Taina Dorada	22.17	BC	39.75	AB	27.2	C	39.7	B
Soler	26.50	CD	46.50	BC	37.6	D	51.6	C
Waltham Butternut	17.83	AB	32.67	A	18.9	A	29.9	A
F test probability	0.0001		<0.0001		<0.0001		<0.0001	
F-LSD (0.05)	5.0174		5.347		3.675		5.347	
Inoculation (I)								
PRSV	22.65	A	36.72	A	33.1	C	42.2	AB
ZYMV	22.00	A	41.25	A	26.3	A	42.6	AB
PRSV+ZYMV	--		--		29.7	B	46.2	B
Control	21.58	A	44.08	A	27.0	AB	39.5	A
F test probability	0.4955		0.2723		0.0001		0.0259	
F-LSD (0.05)	--		--		2.986		4.362	
G × I (F test probability)	0.5820		0.2568		0.0069		0.6172	

F-LSD (0.05) = Fisher's Least Significant Difference at the 0.05 probability level. When the probability of the F value is greater than 0.05 no F-LSD value is reported and means are considered to be not significantly different.
Within a column, means followed by a common letter are not significantly different at the 0.05 probability level according to Fisher's Least Significant Difference at the 0.05 probability level.

Among the fruit traits evaluated, a significant genotype x inoculation treatment interaction was observed only for number of fruit (Table 3). However, since the interaction was ordered, the effect of inoculation treatments was the same for all genotypes. Number of fruits per plant varied from 0.9 to 5.0, depending on the genotype. Fruit weight varied from 0.3 kg to 7.2 kg per plant, with 'Soler' and 'Nigerian Local' producing the highest yields.

There were significant differences among inoculation treatments for the three fruit traits (Table 3). Plants that were not inoculated with virus (controls) had a significantly greater number of fruit, higher yield (total fruit weight per plant), and greater average fruit weight when compared to plants inoculated with PRSV or PRSV+ZYMV, although for number of fruit, plants with the double inoculation did not vary significantly from controls and plants inoculated with ZYMV. The reduction in number of fruit in inoculated versus control plants varied from 25% to 36%. A similar reduction (25% to 39%) occurred in average fruit weight per plant. The greatest impact of virus inoculation occurred in total fruit weight (yield) which exhibited a reduction of 53% to 56% compared to control plants.

There were no significant genotype x inoculation treatment interactions for diameter, pulp width and dry matter (Table 4) indicating that the effect of inoculation treatment was the same for all genotypes. Among the genotypes, diameters ranged from 7.04 to 30.66 cm. 'Soler' and 'Taina Dorada' had the higher means for diameter (30.66 and 20.65 cm) and pulp width (6.35 and 5.43 cm) among the genotypes, traits of agronomical importance. There was a slight reduction of 19% to 14% in the diameter of the fruit of inoculated plants when compared to control plants that were not inoculated with virus. For pulp thickness and percent dry matter there were few or no significant differences between inoculated and control plants. However, for pulp thickness, there was a consistent trend for pulp to be thicker in fruits from control plants compared to virus-inoculated plants.

Table 3. Mean number of fruit, total fruit weight, and average fruit weight per plant of six tropical pumpkin (*Cucurbita moschata*) genotypes inoculated with *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), PRSV+ZYMV (double inoculation), and uninoculated (control). Experiment 2 (2017)

Factor	Number of fruit		Total fruit weight (kg)		Average fruit weight (kg)	
Genotype (G)						
Menina	0.9	A	2.8	B	2.2	B
Nigerian Local	4.0	B	7.0	D	1.7	B
Mos 166	5.0	C	3.3	B	0.5	A
Taina Dorada	1.6	A	5.3	C	3.8	C
Soler	0.8	A	7.2	D	6.7	D
Waltham Butternut	1	A	0.3	A	0.2	A
F test probability	<0.0001		<0.0001		<0.0001	
F-LSD (0.05)	0.847		1.560		0.921	
Inoculation (I)						
PRSV	1.8	A	3.5	A	2.7	A
ZYMV	2.1	A	3.7	A	2.3	A
PRSV+ZYMV	2.1	A	3.4	A	2.2	A
Control	2.8	B	7.8	B	3.6	B
F test probability	0.0512		<0.0001		0.0728	
F-LSD (0.05)	0.692		1.269		0.750	
G × I (F test probability)	0.0264		0.0896		0.9069	

F-LSD (0.05) = Fisher's Least Significant Difference at the 0.05 probability level. When the probability of the F value is greater than 0.05 no F-LSD value is reported and means are considered to be not significantly different.

Within a column, means followed by a common letter are not significantly different at the 0.05 probability level according to Fisher's Least Significant Difference at the 0.05 probability level.

Table 4. Mean diameter, pulp width and percentage of dry matter of six tropical pumpkin (*Cucurbita moschata*) genotypes inoculated with *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), PRSV+ZYMV (double inoculation), and uninoculated (control) (Experiment 2, 2017)

Factor	Diameter (cm)		Pulp thickness (cm)		Dry matter (%)	
Genotype						
Menina	15.54	C	2.52	C	3.12	A
Nigerian Local	17.32	C	2.79	C	5.01	B
Mos166	10.26	B	1.89	B	6.22	BC
Taina Dorada	20.65	D	5.43	D	7.01	C
Soler	30.66	E	6.35	E	5.73	B
Waltham Butternut	7.04	A	1.09	A	3.05	A
F test probability	<0.0001		<0.0001		<0.0001	
F-LSD (0.05)	1.810		0.5054		1.2800	
Inoculation (I)						
PRSV	17.04	A	3.32	AB	4.91	A
ZYMV	16.71	A	3.32	AB	5.25	A
PRSV+ZYMV	16.00	A	3.28	A	4.91	A
Control	19.86	B	3.68	B	4.99	A
F test probability	0.068		0.301		0.667	
F-LSD (0.05)	1.4800		0.4120		1.0428	
G × I (F test probability)	0.6100		0.8603		0.4164	

F-LSD (0.05) = Fisher's Least Significant Difference at the 0.05 probability level. When the probability of the F value is greater than 0.05 no F-LSD value is reported and means are considered to be not significantly different.
Within a column, means followed by a common letter are not significantly different at the 0.05 probability level according to Fisher's Least Significant Difference at the 0.05 probability level.

Color variables (L, chroma and hue) and soluble solids readings (°Brix) obtained in the Experiment 2 had differences among the means of the six genotypes. This was not the case for the means of the different treatments, where no differences were observed (Table 5). There was no interaction among genotypes and inoculation treatments for chroma, hue angle, and °Brix. Virus infection did not seem to have any effect on these three variables in the evaluated plants.

Table 5. Mean L, chroma, hue and brix of six tropical pumpkin (*Cucurbita moschata*) genotypes inoculated with *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV), PRSV+ZYMV (double inoculation), and uninoculated (control) (Experiment 2, 2017)

Factor	L		Chroma		Hue angle		°Brix	
Genotype								
Menina	74.30	C	49.41	A	70.19	C	5.33	BC
Nigerian Local	81.19	D	51.32	AB	79.05	E	4.96	B
Mos166	64.05	A	55.78	B	71.89	D	6.9	D
Taina Dorada	70.19	B	69.96	D	67.56	B	7.26	D
Soler	73.34	C	61.62	C	67.88	B	5.75	C
Waltham Butternut	72.19	BC	67.93	D	65.1	A	2.42	A
F test probability	<0.0001		<0.0001		<0.0001		<0.0001	
F-LSD (0.05)	2.700		4.790		1.618		0.603	
Inoculation (I)								
PRSV	71.43	A	60.77	B	68.32	A	5.87	B
ZYMV	72.37	AB	57.88	B	69.73	B	5.53	AB
PRSV+ZYMV	71.45	A	61.42	B	70.45	B	5.06	A
Control	74.35	B	53.8	A	73.07	C	5.76	B
F test probability	0.3758		0.4006		0.2257		0.1250	
F-LSD (0.05)	2.196		3.89676		1.31664		0.49048	
G × I (F test probability)	0.7333		0.4263		0.9768		0.3861	

F-LSD (0.05) = Fisher's Least Significant Difference at the 0.05 probability level. When the probability of the F value is greater than 0.05 no F-LSD value is reported and means are considered to be not significantly different.
Within a column, means followed by a common letter are not significantly different at the 0.05 probability level according to Fisher's Least Significant Difference at the 0.05 probability level.

5.2 Evaluation of virus infection during plant development

In both Experiment 1 (2016) and Experiment 2 (2017) ELISA readings were taken to monitor virus infection in plants both in the greenhouse and in the field. In the greenhouse in Experiment 1 (2016), PRSV-inoculated plants of 'Nigerian Local' 'Soler' and 'Menina' had low

readings that were considered negative for the presence of PRSV. ‘Waltham Butternut’, ‘Taina Dorada’, and ‘Mos166’ also had low readings but above the threshold considered positive for PRSV. In the field at 55 dpi the resistant genotypes ‘Nigerian Local’ and ‘Menina’ still had negative readings for PRSV (less than 0.400). Susceptible genotypes ‘Waltham Butternut’ and ‘Mos166’ had very high positive readings for concentration of virus while ‘Taina Dorada’ and ‘Soler’ were positive for infection of virus but with a rather low concentration. At 90 dpi mature plants of all genotypes in the field tested positive for infection to PRSV although the reading for ‘Nigerian Local’ was very close to the threshold. Readings for resistant genotype ‘Menina’ was quite high in this experiment.

For ZYMV in Experiment 1 (2016) (Figure 3) ‘Menina’ and ‘Nigerian Local’ presented no infection after 20 and 55 dpi. After 90 days, however, both had clearly high readings considered positive for ZYMV infection. ‘Waltham Butternut’ and ‘Taina Dorada’ present high virus titer values for most cases, be it in the greenhouse or the field. Susceptible genotype ‘Mos166’ had mean positive readings during the first two ELISA tests but then had a low virus titer negative for ZYMV during the second field test at 90 dpi. ‘Soler’ kept a low mean virus titer around the critical value of 0.4. During the test made at 55 dpi, ‘Soler’ had negative results overall.

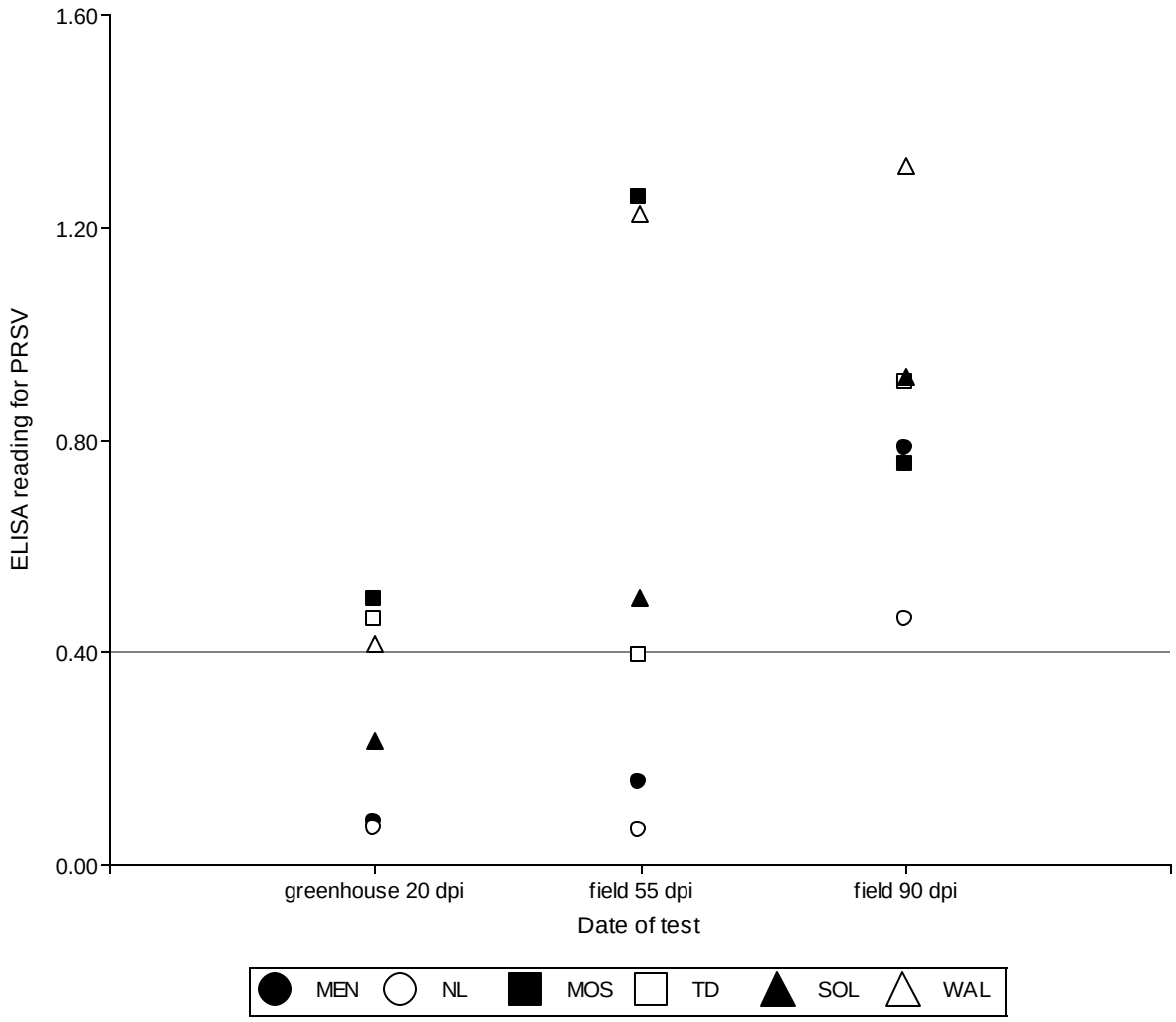

Figure 2. Experiment 1 (2016): Mean enzyme-linked immunosorbent assay (ELISA) readings for *Papaya ringspot virus* (PRSV) from plants of six genotypes of tropical pumpkin (*Cucurbita moschata*) inoculated with PRSV. Plants were assayed in the greenhouse at 20 days post-inoculation (dpi) and in the field (Lajas, Puerto Rico) at 55 and 90 dpi. MEN=’Menina’, NL=’Nigerian Local’, MOS=’Mos166’, TD=’Taína Dorada’, SOL=’Soler’, WAL=’Waltham’. Readings above the horizontal line were considered to be positive readings for the presence of virus.

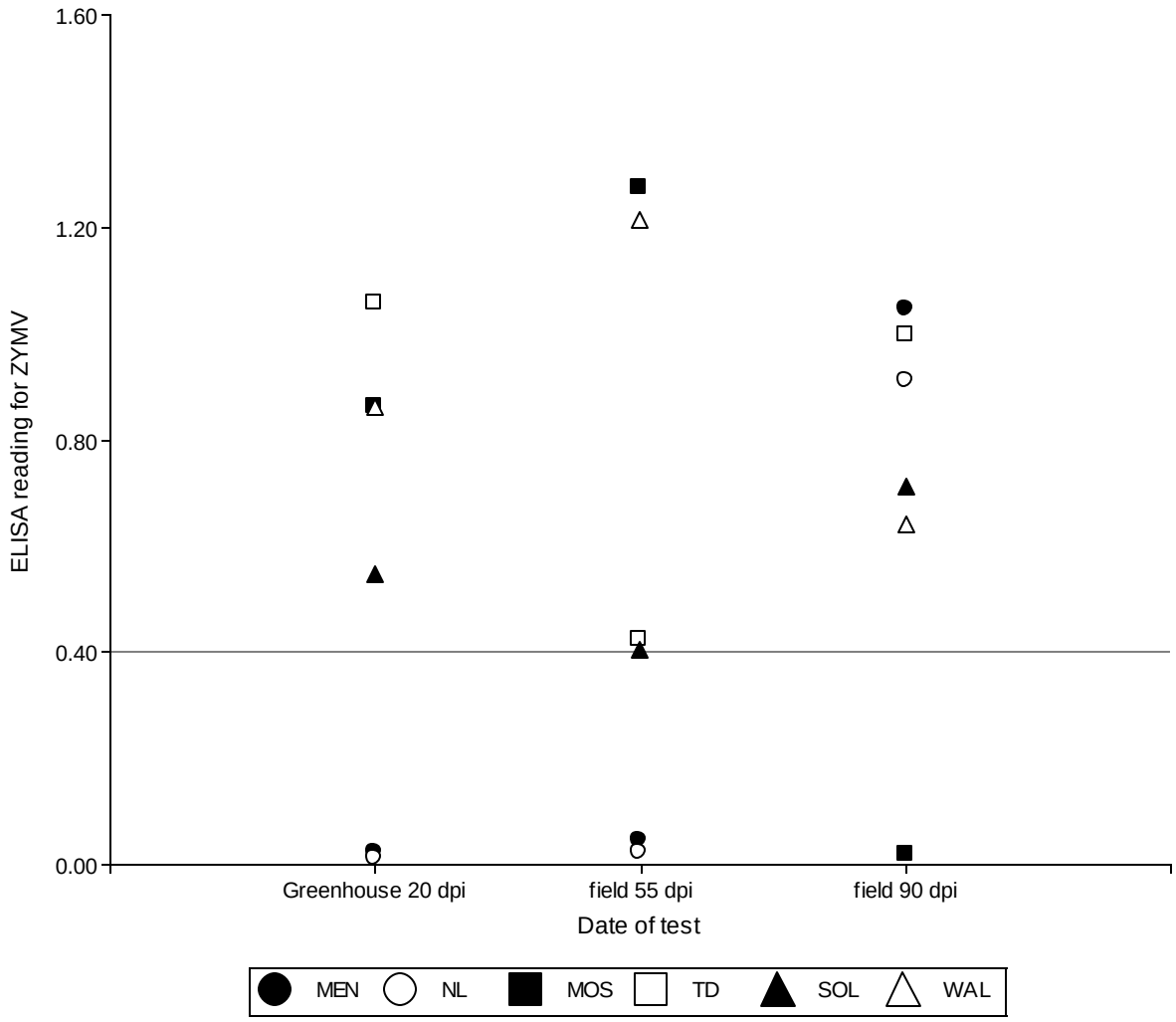

Figure 3. Experiment 1 (2016): Mean Enzyme-linked immunosorbant assay (ELISA) readings for *Zucchini yellow mosaic virus* (ZYMV) from plants of six genotypes of tropical pumpkin (*Cucurbita moschata*) inoculated with ZYMV. Plants were assayed in the greenhouse at 20 days post-inoculation (dpi) and in the field (Lajas, Puerto Rico) at 55 and 90 dpi. MEN=’Menina’, NL=’Nigerian Local’, MOS=’Mos166’, TD=’Taína Dorada’, SOL=’Soler’, WAL=’Waltham’. Readings above the horizontal line were considered to be positive readings for the presence of virus.

In Experiment 2 conducted during 2017, somewhat different results were obtained. Figure 4 shows that at 18 dpi when the test was conducted in the greenhouse, all genotypes inoculated with PRSV had readings above the critical value for virus infection. In the field at 54 and 98 dpi, resistant genotypes ‘Nigerian Local’ and ‘Menina’ had values lower than that of the

critical value of a 0.4 absorbency. For all tests, ‘Mos166’ and ‘Waltham Butternut’ had the highest mean values of ELISA readings. Also for the three tests, ‘Soler’ averaged a considerably low positive reading. ‘Taina Dorada’ was tested for virus titer at 18 and 54 dpi. The results of both tests show this genotype had mean values above the critical level of virus concentration of PRSV.

Among plants inoculated with ZYMV in 2017, ‘Menina’ and ‘Nigerian Local’ presented no viral infection either in the greenhouse or in the field (Figure 5). ‘Waltham Butternut’ and ‘Mos166’ were the only two genotypes with consistent readings above the critical level during all the ELISA tests. The commercial variety ‘Soler’ presented high values of infection to ZYMV at 18 dpi in the greenhouse. Afterwards in the field, the mean ELISA readings of ‘Soler’ were either at or just above the critical level considered to be a positive reading. Similarly, ‘Taina Dorada’ had high readings in the greenhouse but values considered negative for the virus in the field 54 dpi with ZYMV.

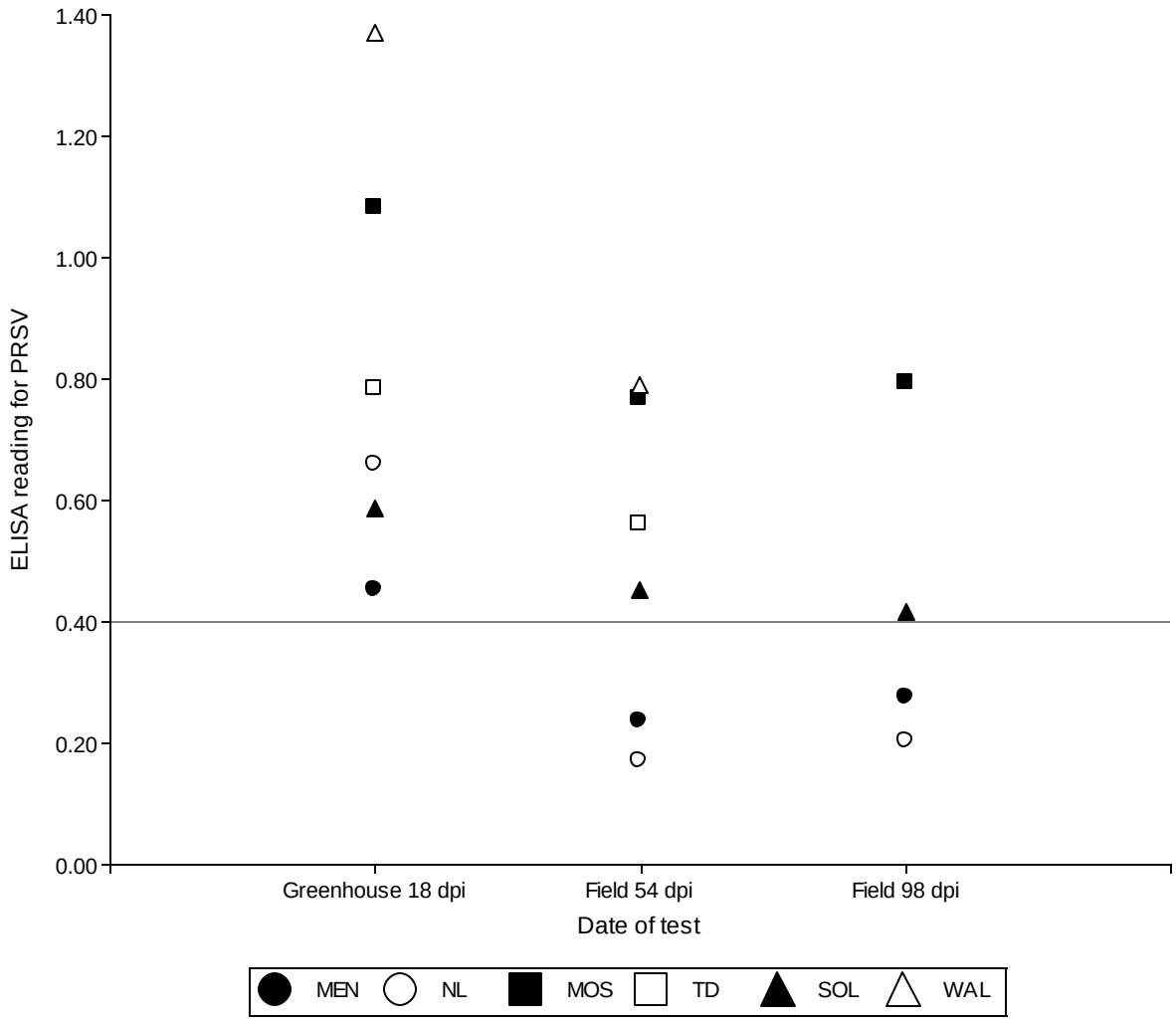

Figure 4. Experiment 2 (2017): Mean enzyme-linked immunosorbent assay (ELISA) readings for *Papaya ringspot virus* (PRSV) from plants of six genotypes of tropical pumpkin (*Cucurbita moschata*) inoculated with PRSV. Plants were assayed in the greenhouse at 18 days post-inoculation (dpi) and in the field (Lajas, Puerto Rico) at 54 and 98 dpi. MEN=’Menina’, NL=’Nigerian Local’, MOS=’Mos166’, TD=’Taína Dorada’, SOL=’Soler’, WAL=’Waltham’. Readings above the horizontal line were considered to be positive readings for the presence of virus.

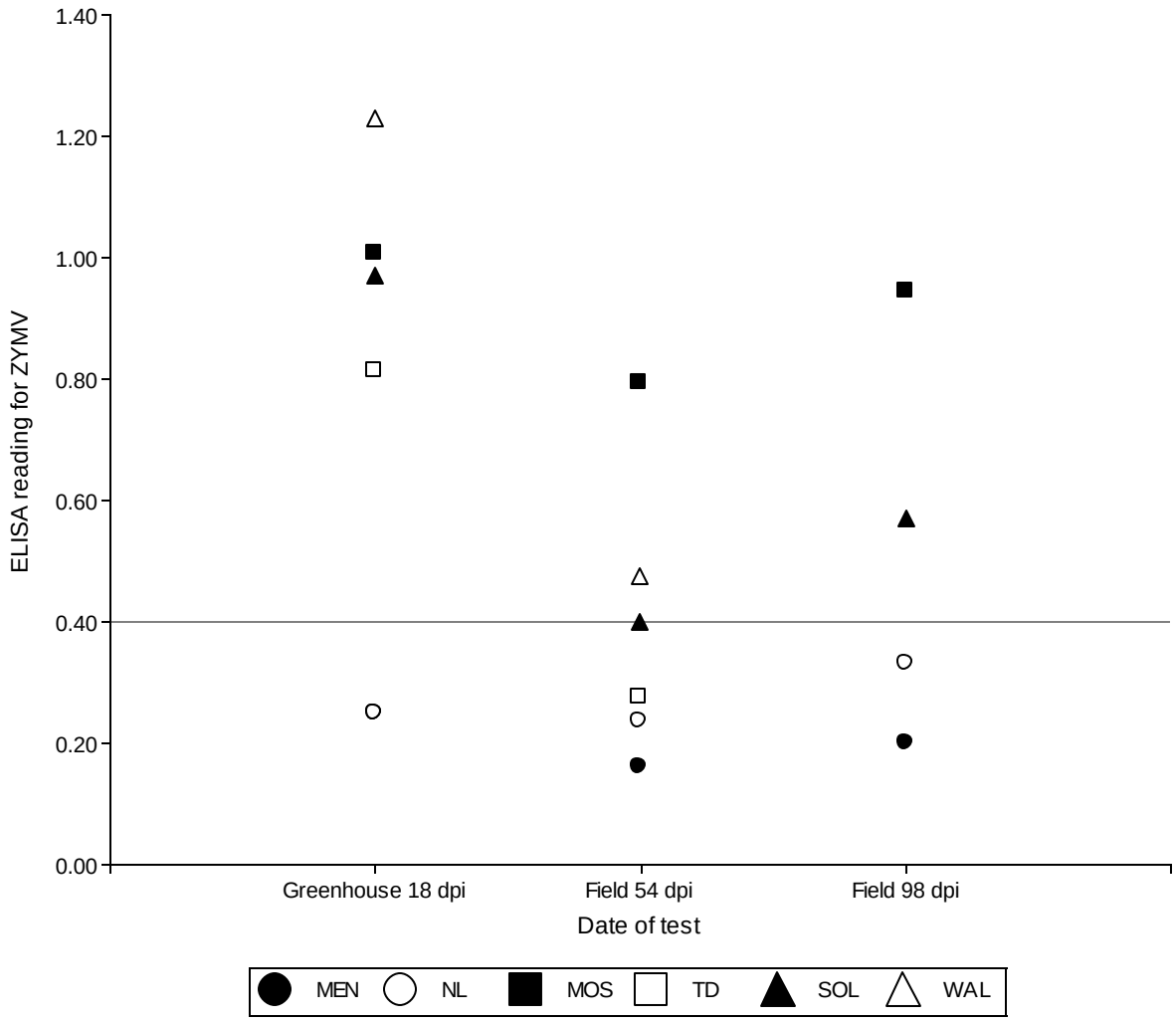

Figure 5: Experiment 2 (2017) Mean enzyme-linked immunosorbent assay (ELISA) readings for *Zucchini yellow mosaic virus* (ZYMV) from plants of six genotypes of tropical pumpkin (*Cucurbita moschata*) inoculated with ZYMV. Plants were assayed in the greenhouse at 18 days post-inoculation (dpi) and in the field (Lajas, Puerto Rico) at 54 and 98 dpi. MEN='Menina', NL='Nigerian Local', MOS='Mos166', TD='Taína Dorada', SOL='Soler', WAL='Waltham'. Readings above the horizontal line were considered to be positive readings for the presence of virus.

5.3. Associations between ELISA readings taken on different dates

Figures 6 to 11 show associations between single plant ELISA readings taken in the greenhouse and in the field. Readings that fall in the lower left-hand quadrant of each scattegram are negative for the presence of virus at both sampling dates. Readings in the upper

right-hand quadrant are positive for the presence of virus at both sampling dates. Readings that did not fall in either of these two quadrants were either positive for the virus at one date or negative for the virus at the other date, or vice versa. Only results from Experiment 2 (2017) are presented.

For PRSV ELISA readings at 18 and 54 dpi (Figure 6), all plants of 'Taína Dorada', 'Mos166' and 'Waltham' and one plant of 'Soler' fell into the upper right-hand quadrant indicating that plants classified as positive for the virus at 18 dpi were also classified as positive at 54 dpi. Only two plants (one of each genotype of: 'Menina' and 'Nigerian Local') had readings negative for the presence of PRSV at both 18 and 54 dpi. In general, PRSV-infected plants had higher values for PRSV infection at 18 dpi in the greenhouse when compared to the results at 54 dpi. 'Waltham Butternut' and 'Mos166' had some of the highest values of infection for both dates. Virus titer in 'Nigerian Local' and 'Menina' was noticeably reduced at 54 dpi, when compared to 18 dpi. A similar case to the latter happened for most plants of genotypes 'Taina Dorada' and 'Soler'. When evaluating the second field test at 98 dpi and comparing it with the test made in the greenhouse at 18 dpi (Figure 7) most plants had a lower ELISA reading in the field than in the greenhouse. Readings of the plants of the genotype 'Menina' did not seem to vary as much as all the genotypes together did. These plants had, overall, negative readings in the field at 98 dpi and mostly low readings just above the critical level in the greenhouse.

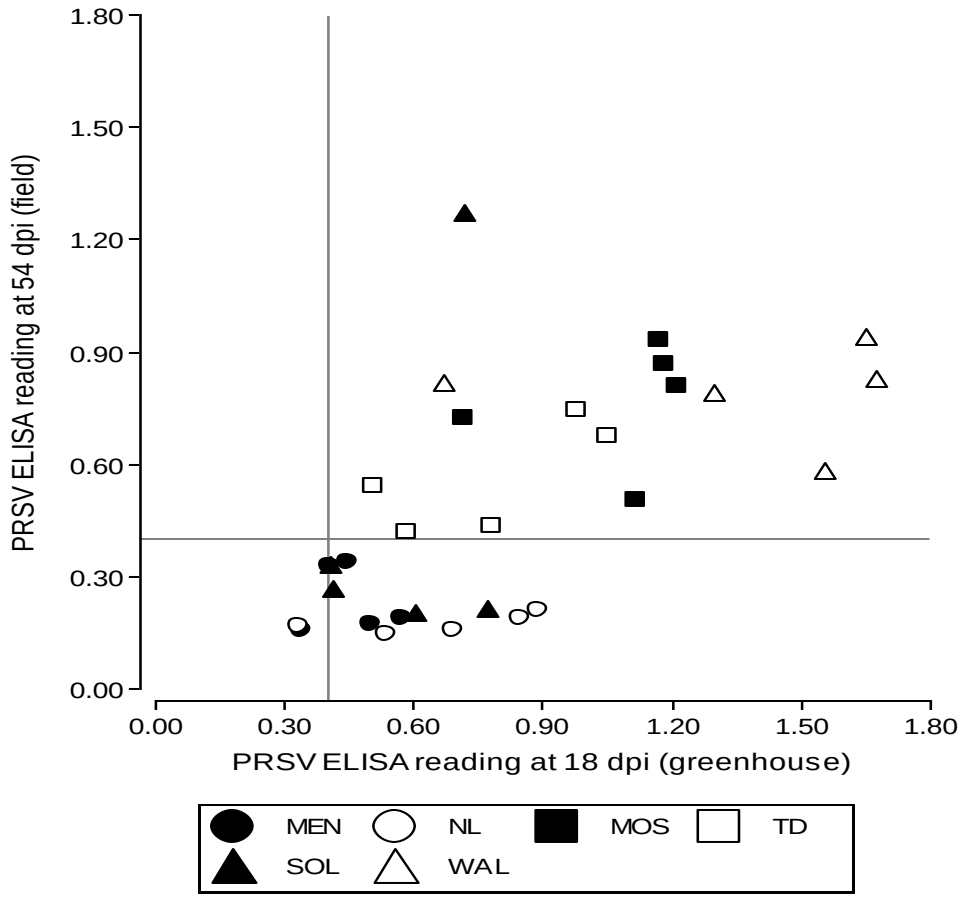

Figure 6. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Papaya ringspot virus* (PRSV) in six genotypes of tropical pumpkin inoculated with PRSV. Each data point represents readings for a single plant at 18 days post-inoculation (dpi) and 54 dpi. MEN='Menina', NL='Nigerian Local', MOS='Mos166', TD='Taína Dorada', SOL='Soler', WAL='Waltham'. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (18 dpi) or above the horizontal line (54 dpi) were considered positive for the presence of PRSV.

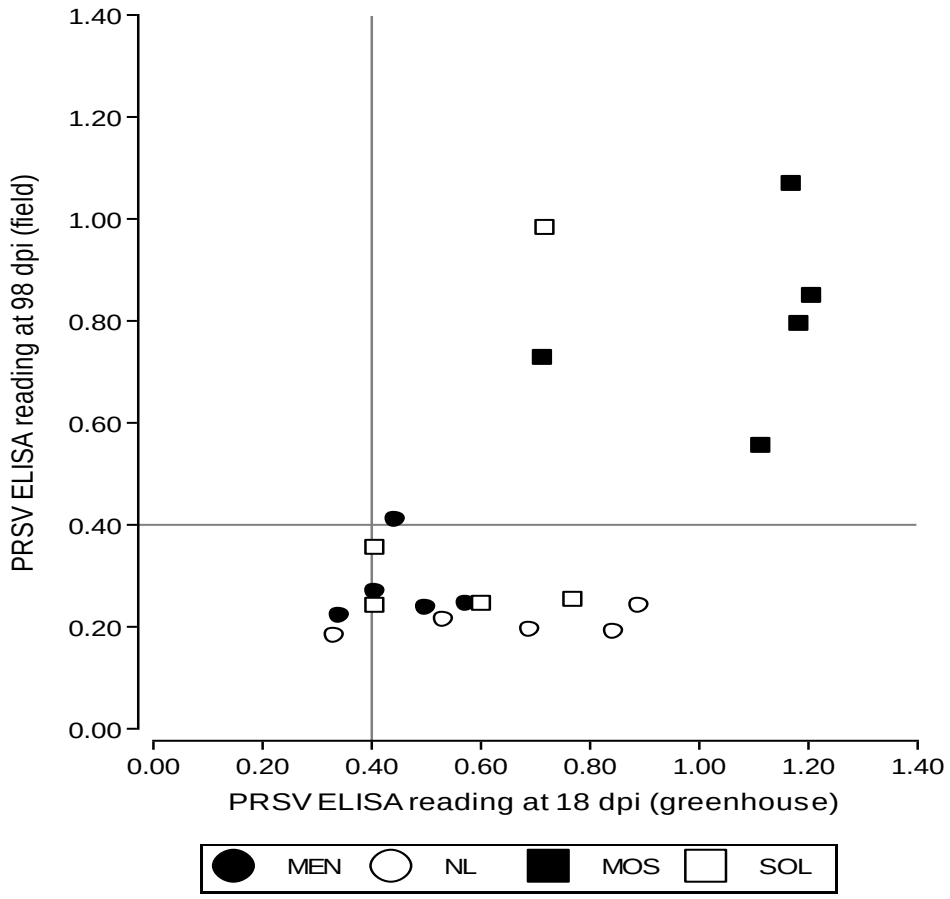

Figure 7. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Papaya ringspot virus* (PRSV) in six genotypes of tropical pumpkin inoculated with PRSV. Each data point represents readings for a single plant at 18 days post-inoculation (dpi) and 98 dpi. MEN=’Menina’, NL=’Nigerian Local’, MOS=’Mos166’, SOL=’Soler’. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (18 dpi) or above the horizontal line (98 dpi) were considered positive for the presence of PRSV.

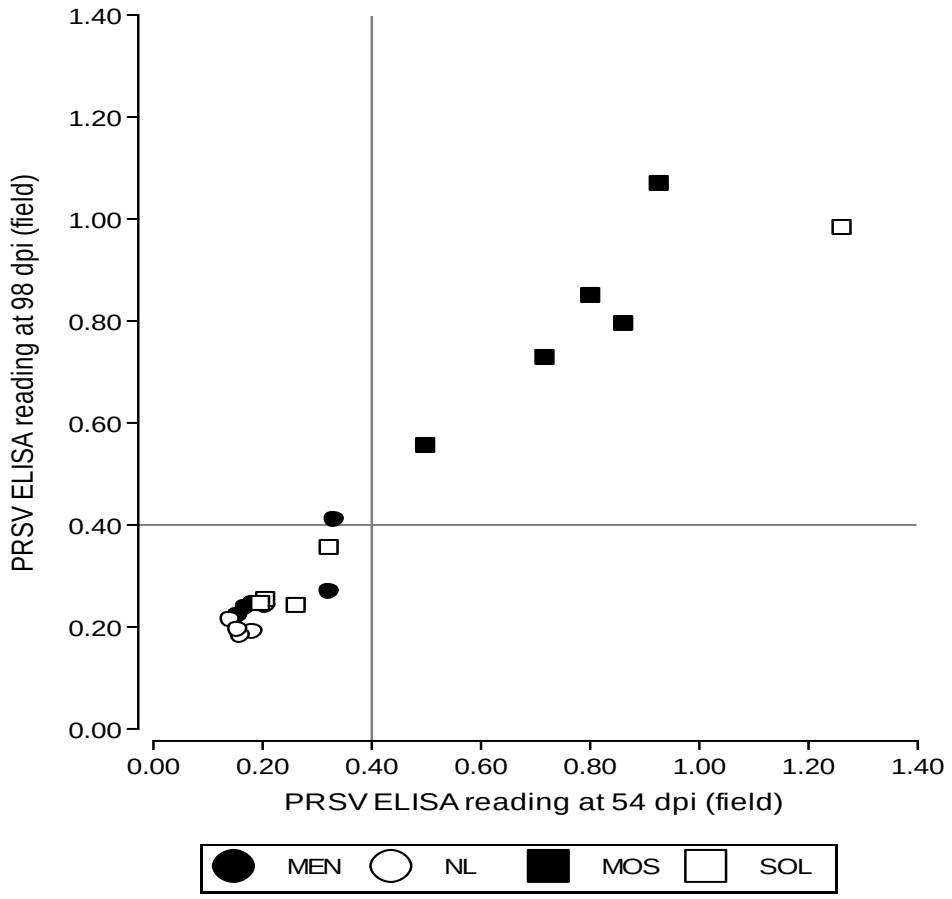

Figure 8. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Papaya ringspot virus* (PRSV) in six genotypes of tropical pumpkin inoculated with PRSV. Each data point represents readings for a single plant at 54 days post-inoculation (dpi) and 98 dpi. MEN='Menina', NL='Nigerian Local', MOS='Mos166', SOL='Soler'. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (54 dpi) or above the horizontal line (98 dpi) were considered positive for the presence of PRSV.

Field readings at 54 dpi almost perfectly predicted how those same plants would be classified (infected or not infected with PRSV) at 98 dpi (Figure 8). 'Nigerian Local' and 'Menina' plants inoculated with PRSV had negative or very low values of infection for both tests realized at the field. Plants of susceptible line 'Mos166' have high positive readings for the tests realized at both dates and there was not much difference in the values they had. Most PRSV-

infected ‘Soler’ plants had low ELISA readings in both tests, although one plant of this genotype had a very high positive value in tests at both dates.

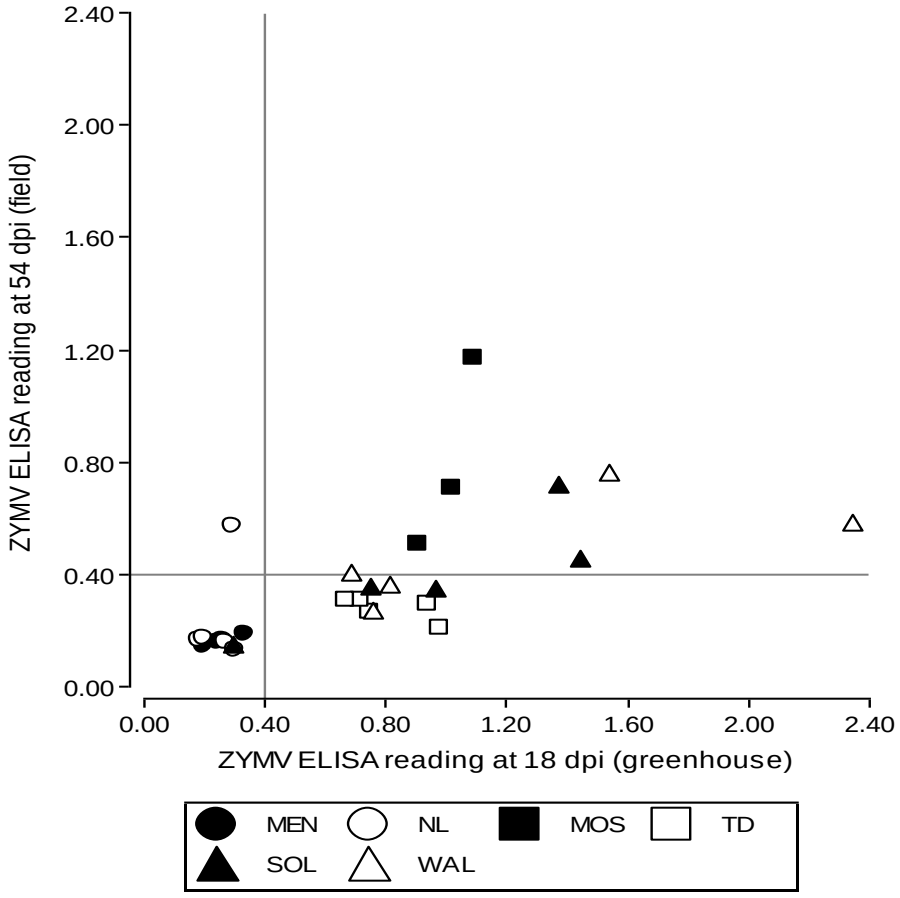

Figure 9. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Zucchini yellow mosaic virus* (ZYMV) in six genotypes of tropical pumpkin inoculated with ZYMV. Each data point represents readings for a single plant at 18 days post-inoculation (dpi) and 54 dpi. MEN=’Menina’, NL=’Nigerian Local’, MOS=’Mos166’, TD=’Taína Dorada’, SOL=’Soler’, WAL=’Waltham’. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (18 dpi) or above the horizontal line (54 dpi) were considered positive for the presence of ZYMV.

Readings for ZYMV infection for plants of ‘Menina’ and ‘Nigerian Local’ were very similar at 18 dpi and 54 dpi; nearly all readings fell into the lower left-hand quadrant (Figure 9). Greenhouse ZYMV ELISA readings were, in most cases, higher than in the field (54 dpi), with

the exception of ‘Nigerian Local’. In one case, a ‘Nigerian Local’ plant, with a negative reading in the greenhouse, had a positive reading for the first test realized in the field. ‘Waltham Butternut’ plants had high readings. Moreover, ‘Taina Dorada’ initially tested positive for most plants at 18 dpi, then tested negative for virus infection at 54 dpi. Readings of ZYMV infection at 54 and 98 dpi were generally lower for several genotypes (Figure 10). ‘Menina’ and ‘Nigerian Local’ plants mostly had values negative for ZYMV for both tests. A positive value for one plant of the genotype ‘Nigerian Local’ at 98 days after infection contrasts with the negative value it had at 18 dpi. Virus infection was slightly reduced in ‘Soler’ at 98 dpi compared to 18 dpi, although plants of this genotype generally maintained a reading positive for ZYMV at 98 dpi. Of the tests done in the field (Figure 11), one ‘Nigerian Local’ plant had readings that were positive for ZYMV; the reading of the test done at 98 dpi was higher than that of the other field test at 54 dpi. All other plants of this genotype, as well as of ‘Menina’, ‘Mos166’, and ‘Soler’ were fairly similar between field tests. Susceptible genotype ‘Mos166’ had the highest readings for ZYMV infection in the two tests, while ‘Menina’ and ‘Nigerian Local’ had the lowest readings.

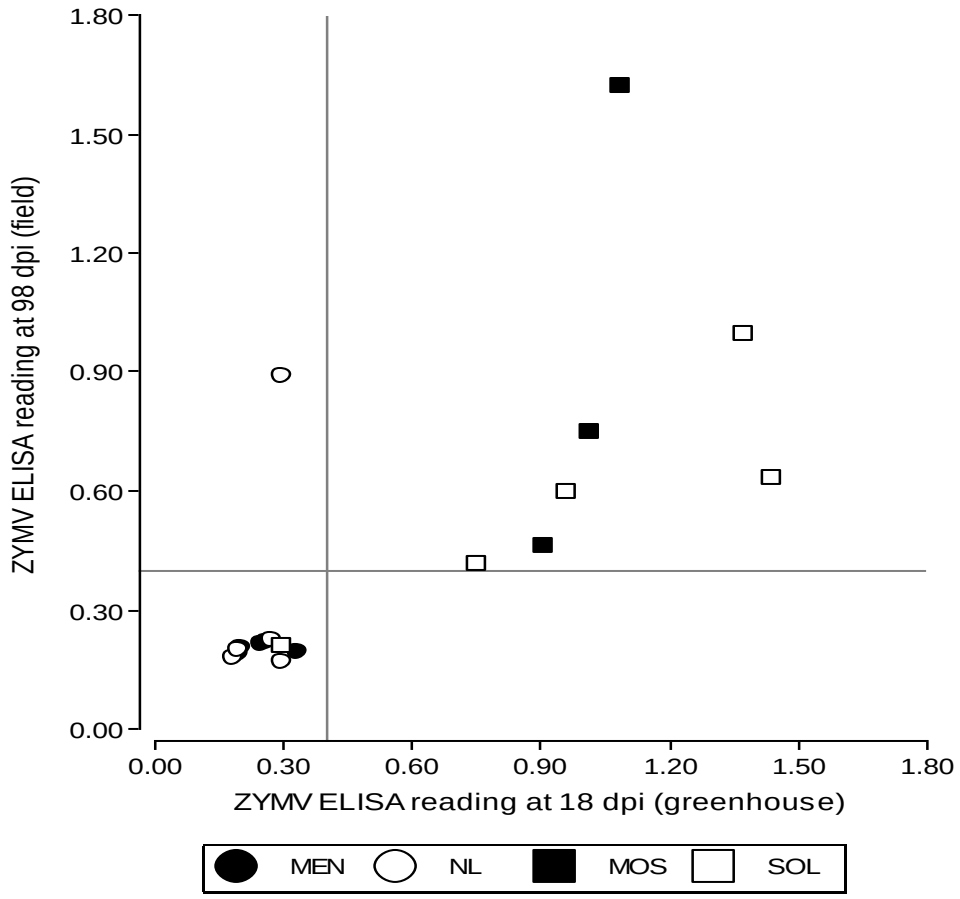

Figure 10. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Zucchini yellow mosaic virus* (ZYMV) in six genotypes of tropical pumpkin inoculated with ZYMV. Each data point represents readings for a single plant at 18 days post-inoculation (dpi) and 98 dpi. MEN='Menina', NL='Nigerian Local', MOS='Mos166', SOL='Soler'. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (18 dpi) or above the horizontal line (98 dpi) were considered positive for the presence of ZYMV.

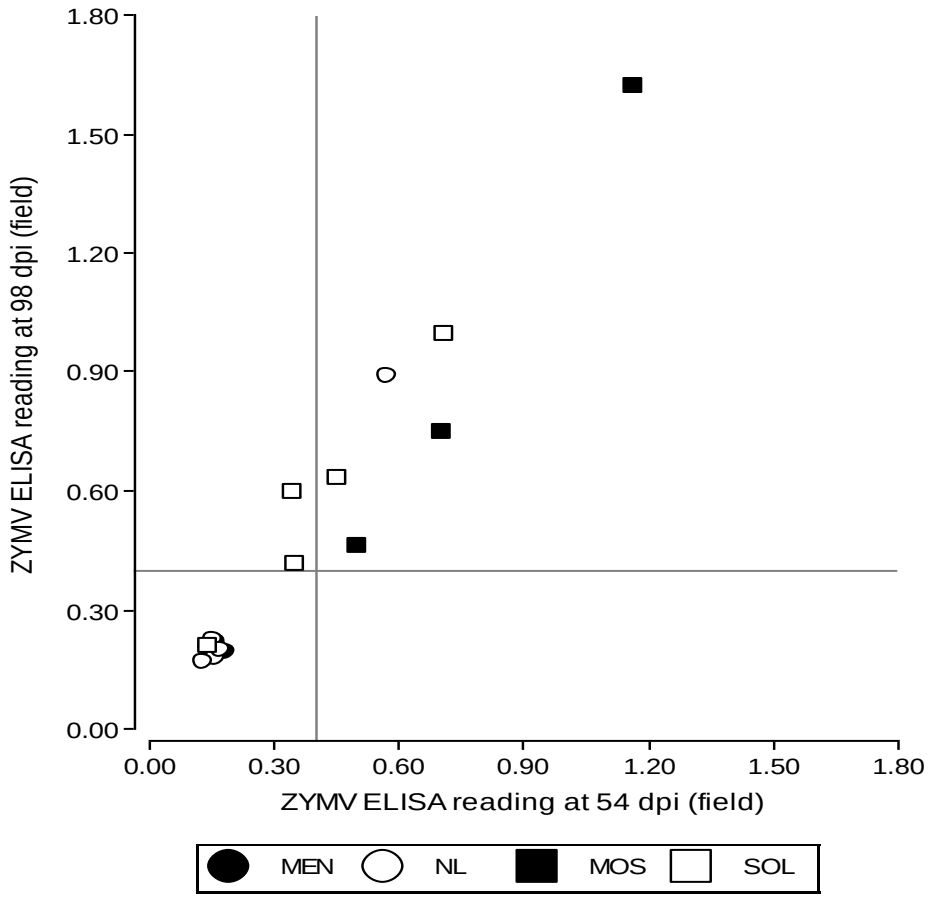

Figure 11. Experiment 2 (2017) Scattergram of enzyme-linked immunosorbent assay (ELISA) readings for *Zucchini yellow mosaic virus* (ZYMV) in six genotypes of tropical pumpkin inoculated with ZYMV. Each data point represents readings for a single plant at 54 days post-inoculation (dpi) and 98 dpi. MEN='Menina', NL='Nigerian Local', MOS='Mos166', SOL='Soler'. Readings above the horizontal line were considered to be positive readings for the virus. Readings to the right of the vertical line (54 dpi) or above the horizontal line (98 dpi) were considered positive for the presence of ZYMV.

5.4 Inheritance of resistance to PRSV

5.4.1 F_2 populations with 'Nigerian Local' as the source of resistance

Mean severity ratings and ELISA readings for PRSV-inoculated parental, F_1, and F_2 plants of the different crosses appear in Table 6. The distribution of relative frequencies of combined disease severity (0 to 12 scale) in resistant x susceptible (or susceptible x resistant) F_2

populations inoculated with PRSV can be observed in figures 12 to 17. Disease severity in the F_2 population Nigerian Local × Taína Dorada ranged in disease severity from no symptoms of infection (class=0) to very highly infected symptoms (class=12) (Figure 12). The F_2 population approximated a normal distribution and had an average severity rating of 5.23. The mean of ELISA readings was 0.87.

The F_2 populations Verde Luz × Nigerian Local, also had plants with low, intermediate and high severity ratings, although no plants fell into class 0 and 1 (Figure 13). There was an excess of plants falling into class 4 (and to a lesser extent class 9), resulting in a distribution that was reasonably symmetrical, but not very normal. Half or more of the F_2 plants screened for resistance had severity ratings over 5. The mean severity rating for this cross was 6.5 among the 110 plants evaluated. The mean ELISA reading for this cross was 1.17 (Table 6).

Table 6. Mean severity rating and ELISA readings for five parental genotypes ('Nigerian Local', 'Taina Dorada', 'Verde Luz', 'Menina', and 'TP411'), 'Waltham Butternut', and F1, and F2 plants of six crosses of *Cucurbita moschata,* including the mentioned parentals, inoculated with *Papaya ringspot virus.*

Genotype	Mean combined severity rating	Mean ELISA reading
Menina (resistant parent)	0.10	0.40
Nigerian Local (resistant parent)	0.09	0.40
Taína Dorada (susceptible parent)	6.90	0.98
Verde Luz (susceptible parent)	5.10	0.78
TP411 (susceptible parent)	5.56	0.76
Waltham Butternut (susceptible check)	8.21	1.53
Nigerian Local × Taína Dorada (F_1)	2.80	0.84
Menina × Taína Dorada (F_1)	1.30	0.65
Verde Luz × Nigerian Local (F_1)	7.30	0.97
Verde Luz × Menina (F_1)	1.30	0.37
TP411 × Menina (F_1)	1.90	0.88
Nigerian Local × Menina (F_1)	0.15	0.42
Nigerian Local × Taína Dorada (F_2)	5.23	0.87
Menina × Taína Dorada (F_2)	3.38	1.01
Verde Luz × Nigerian Local (F_2)	6.25	1.17
Verde Luz × Menina (F_2)	2.27	0.44
TP411 × Menina (F_2)	2.80	0.72
Nigerian Local × Menina (F_2)	0.84	0.40

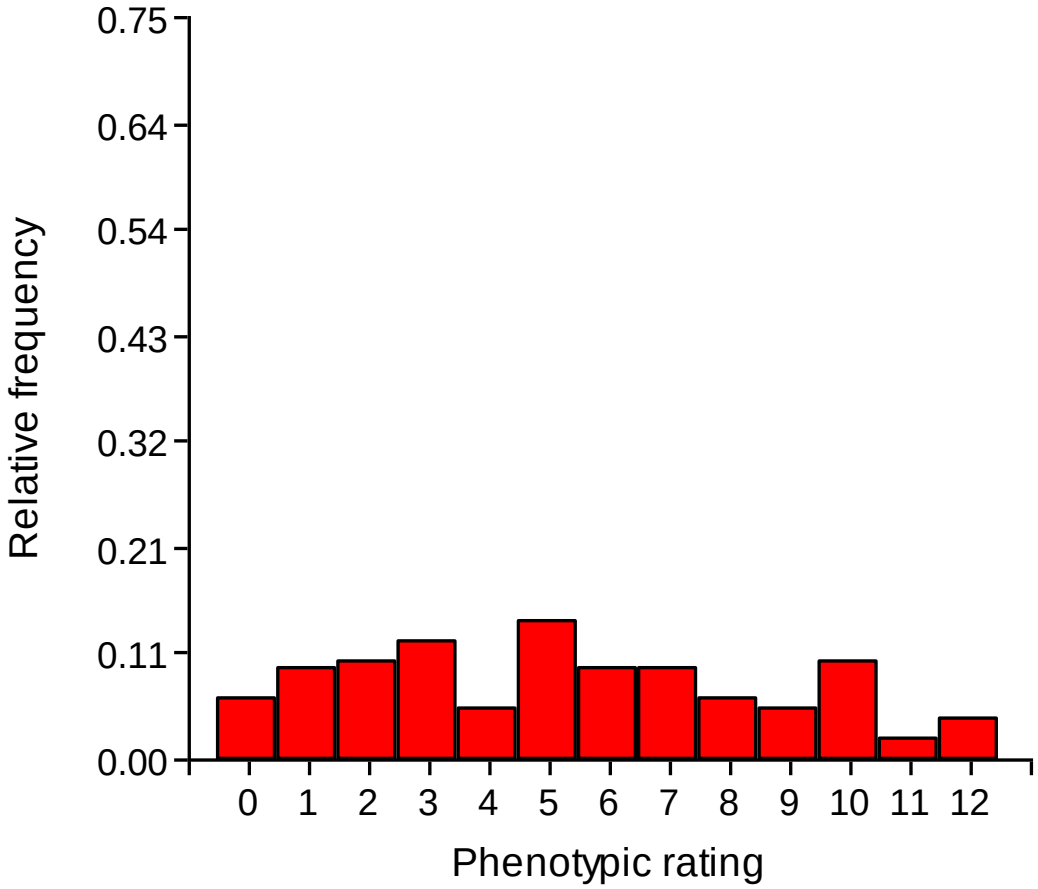

Figure 12: Distribution of combined severity ratings of plants (n=111) from the Nigerian Local × Taína Dorada F_2 population inoculated with *Papaya ringspot* virus (PRSV).

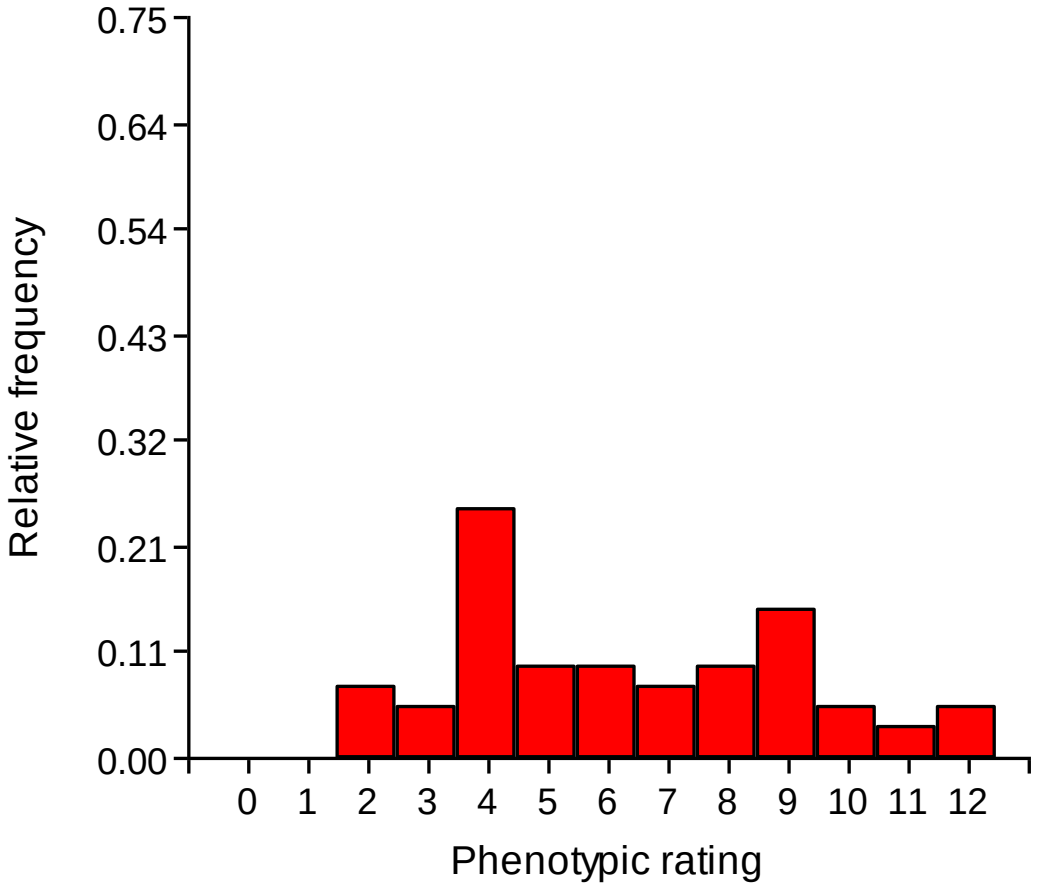

Figure 13: Distribution of combined severity ratings of plants (n=110) from the Verde Luz × Nigerial Local F_2 population inoculated with *Papaya ringspot* virus (PRSV).

5.4.2 F_2 populations with 'Menina' as the source of resistance

In the case of the second filial generation of the cross of Menina × Taina Dorada, around 85% of plants screened for virus resistance had a severity rating of 5 or less in the Menina × Taína Dorada F_2 population (Figure 14). The distribution was clearly skewed to the left (towards lower disease severity). The average severity rating for these 120 F_2 plants was 3.38 and the mean ELISA reading was 1.01 (Table 6).

Figure 15 presents the relative frequencies of severity ratings of F_2 plants from the cross of Verde Luz × Menina. Again, the distribution was strongly skewed to the left. Of 118 plants

screened for resistance to PRSV, about 88% had a disease severity rating value of 5 or less. No symptoms were observed in around 30% of the plants mechanically inoculated with the virus. The average severity rating was 2.27 and the mean ELISA reading was 0.44, a very similar value to the mean ELISA readings of resistant parent ‘Menina’ (Table 6). In this cross, very few F_2 plants exhibited very severe symptoms of infection.

The third F_2 population with of ‘Menina’, TP411 × Menina, again had a very skewed distribution with over 85% of plants with little to no symptoms, a severity rating of 5 or less (figure 16). A fifth of the observed plants had no symptoms in any of the first six leaves after infection and development. The severity rating that was most frequently seen in this population was 2, about 30% of the plants screened. The average severity rating was 2.82 and the average ELISA reading was 0.72 (Table 6).

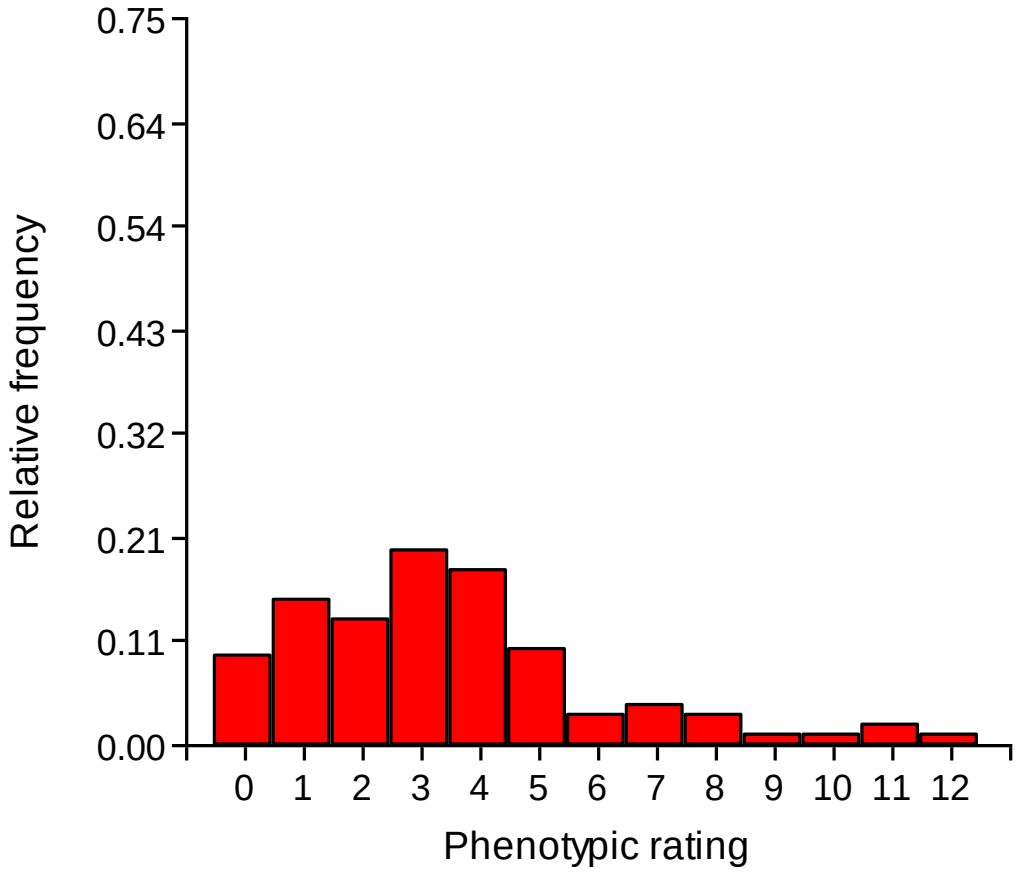

Figure 14. Distribution of combined severity ratings of plants (n=120) from the Menina × Taína Dorada F_2 population inoculated with *Papaya ringspot* virus (PRSV).

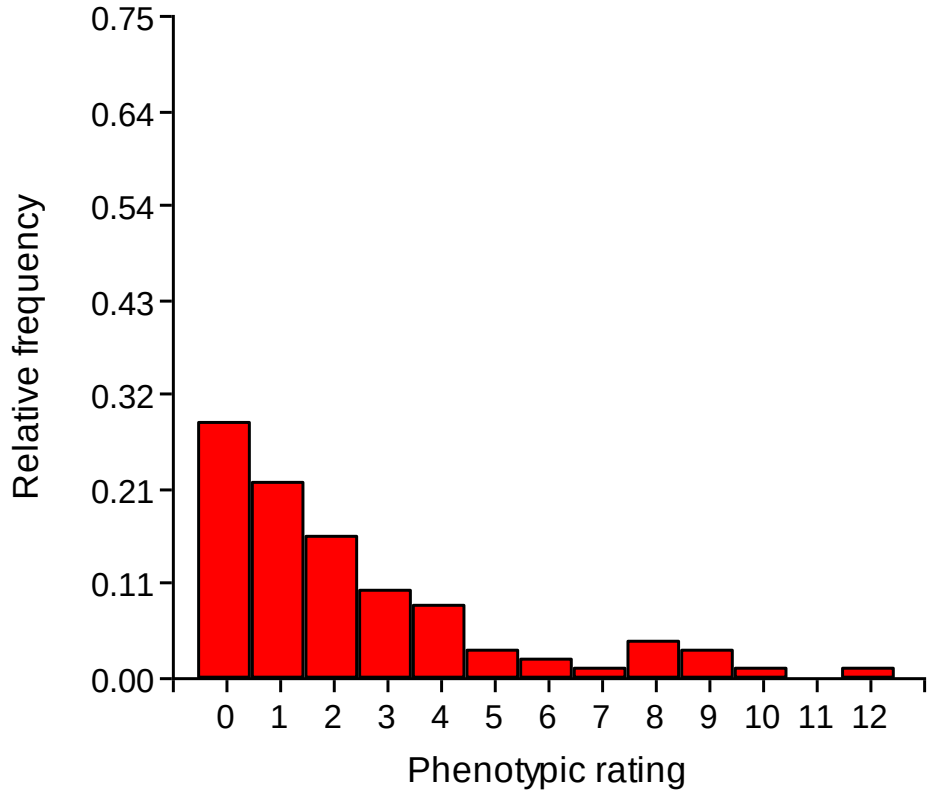

Figure 15. Distribution of combined severity ratings of plants (n=118) from the Verde Luz × Menina F_2 population inoculated with *Papaya ringspot* virus (PRSV).

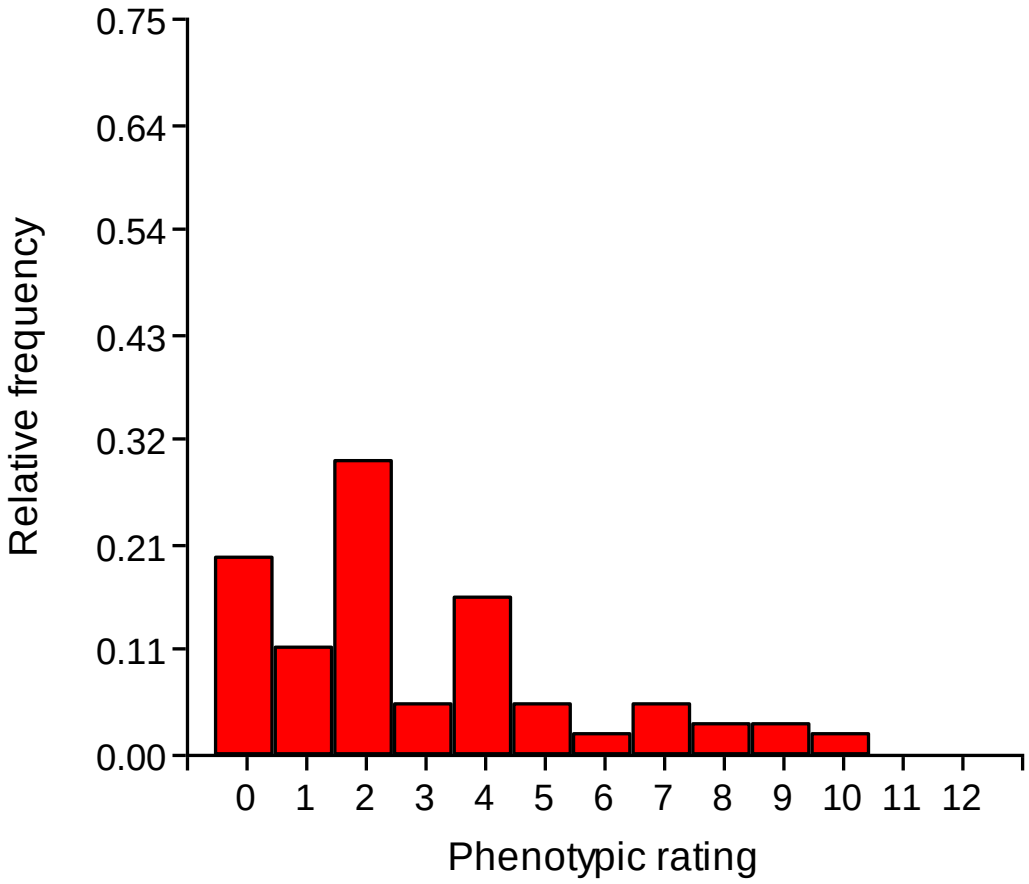

Figure 16. Distribution of combined severity ratings of plants (n=111) from the TP411 × Menina F_2 population inoculated with *Papaya ringspot* virus (PRSV).

5.4.3 Resistant x resistant F_2 population (Menina × Nigerian Local)

The cross of Menina × Nigerian Local is a cross of two resistant parents (Figure 17). Out of 238 F_2 plants evaluated, 171 of them had no symptoms of infection after having been infected with PRSV. Of these, only nine plants had symptoms that were considered definitive of infection. Over 95% of the plants screened for virus symptoms have values of five or lower. ELISA readings of the parents, the F_1 and the F_2 generations averaged approximately 0.4 and the average severity was very low (0.84) (Table 6).

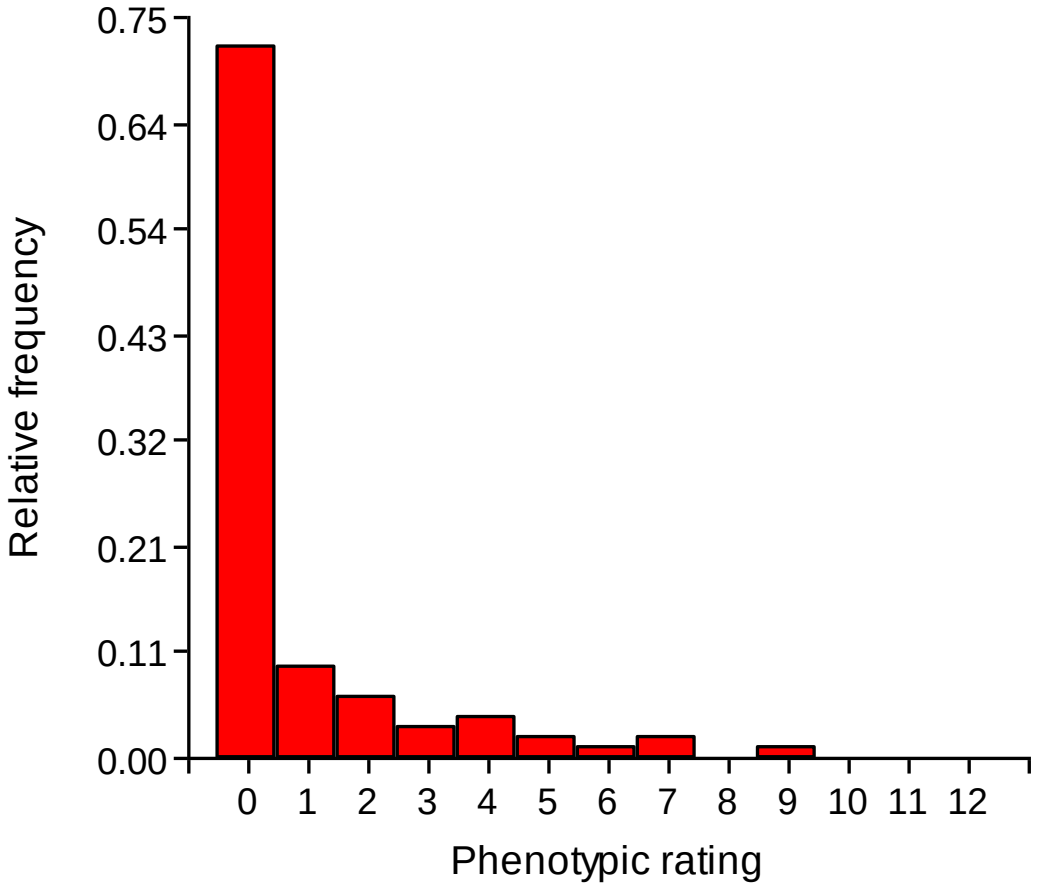

Figure 17. Distribution of combined severity ratings of plants (n=238) from the Nigerian Local × Menina F_2 population inoculated with *Papaya ringspot* virus (PRSV).

5.4.4 Goodness-of-fit tests

The previously presented F_2 frequency distributions were used as a guide to group plants into two phenotypic classes: resistant versus susceptible. All possible two-class groupings of the 13 severity classes (0 to 12) were considered, for a total of 13 possible groupings. For example, the first grouping placed plants with a severity of 0 in the resistant class and plants with a severity greater than 0 in the susceptible class. The seventh grouping placed plants with a severity $\leq$6 in the resistant class and plants $\geq$7 in the susceptible class. For each grouping, one or

more gene segregation models were considered based on the observed ratios of resistant to susceptible plants. Chi-square goodness-of-fit tests were carried out to determine if segregation of resistance of the populations was in accordance to the proposed gene model. The most consistent results were obtained when plants with severity ≤ 4 were grouped into the resistant class and plants with severity ≥ 5 were grouped into the susceptible class. Those results are presented here.

Crosses concerning the resistant genotype 'Nigerian Local' appear in table 7. Out of the 111 F_2s of the cross of 'Nigerian Local' and 'Taina Dorada', 47 of them had a severity rating of 4 or less, considered resistant; 64 plants were susceptible. A p-value of 0.7950 confirms that the segregation observed fits the model of duplicate recessive epistasis with an expected ratio of 7:9 (resistant:susceptible). A similar case was observed for the F_2 of the cross between 'Nigerian Local' and 'Taina Dorada'; of the 110 plants infected, grown and evaluated, 42 were resistant and 68 were susceptible. With a p-value of 0.2391, the observed ratio for resistant to susceptible in this cross provides an excellent fit of the 7:9 ratio of the genetic model of duplicate recessive epistasis. Neither of the crosses mentioned adjusted well when compared to the 3:1 model involving only one gene.

'Menina', the other parental genotype considered for its resistance to the virus, was crossed with several susceptible genotypes: 'Taina Dorada', 'Verde Luz', and 'TP411'. The F_2 TP411 × Menina population agreed with a 13:3 (resistant:susceptible) (p=0.1285) gene model of dominant and recessive epistasis (Table 8). The F_2 Verde Luz × Menina also fit 13:3 (resistant:susceptible) genetic model (p=0.2268). The Menina × Taína Dorado F_2 population also fitted the 13:3 model (p=0.8434).

An allele test of the resistance genes in ‘Nigerian Local’ and ‘Menina’ was carried out by evaluating the F_2 cross of these two resistant parents. The F_2 segregated 224 resistant to 14 susceptible suggesting that resistance genes in ‘Nigerian Local’ and ‘Menina’ are not allelic (Table 9). The fit to a 15:1 model of duplicate dominant epistasis was excellent ($p=0.8147$).

Table 7. Number of plants evaluated and observed segregations in parental, F_1 and F_2 populations. 'Nigerian Local' was the resistant parent in the F_1 and F_2 crosses. Goodness-of-fit in F_2 populations was tested with chi-square.

Genotype	Population	Number of plants	Observed segregation R[1]	S[1]	Tested ratio (R:S)	$\chi 2$	Probability
Nigerian Local (NL)	Resistant parent	34	34	0	n/a	n/a	n/a
Taina Dorada (TD)	Susceptible parent	20	2	18	n/a	n/a	n/a
Verde Luz (VL)	Susceptible parent	20	4	16	n/a	n/a	n/a
Nigerian Local × Taína Dorada	F_1	10	8	2	n/a	n/a	n/a
Verde Luz × Nigerian Local	F_1	10	10	0	n/a	n/a	n/a
Nigerian Local × Taína Dorada	F_2	111	47	64	7:9	0.0894	0.7950
Verde Luz × Nigerian Local	F_2	110	42	68	7:9	1.3859	0.2391

[1]For each plant, disease severity in leaves 3 to 5 was evaluated on a 0 to 4 scale (0=no symptoms), then values were summed to produce an overall severity index of 0 to 12. Plants were then categorized R for resistant (overall severity rating of ≤ 4) or S for susceptible (overall severity rating of ≥ 5).

n/a = not applicable

Table 8. Number of plants evaluated and observed segregations in parental, F_1 and F_2 populations when 'Menina' was the resistant parent in the F_1 and F_2 crosses. Goodness-of-fit in F_2 populations was tested with chi-square.

Genotype	Population	Number of plants	Observed segregation		Tested ratio (R:S)	$\chi 2$	Probability
			R[1]	S[1]			
Menina	Resistant parent	50	50	0	n/a	n/a	n/a
Taína Dorada	Susceptible parent	20	2	18	n/a	n/a	n/a
Verde Luz	Susceptible parent	20	4	16	n/a	n/a	n/a
TP411	Susceptible parent	9	0	9			
Menina × Taína Dorada	F_1	10	10	0	n/a	n/a	n/a
Verde Luz × Menina	F_1	10	10	0	n/a	n/a	n/a
TP411 × Menina	F_1	10	10	0	n/a	n/a	n/a
Menina × Taína Dorada	F_2	120	91	29	13:3	2.3110	0.1285
Verde Luz × Menina	F_2	118	101	17	13:3	1.4611	0.2268
TP411 × Menina	F_2	111	91	20	13:3	0.0390	0.8434

[1]For each plant, disease severity in leaves 3 to 5 was evaluated on a 0 to 4 scale (0=no symptoms), then values were summed to produce an overall severity index of 0 to 12. Plants were then categorized R for resistant (overall severity rating of $\leq$4) or S for susceptible (overall severity rating of $\geq$5).
n/a = chi-square test not applicable

Table 9. Number of plants evaluated and observed segregations in parental, F_1 and F_2 populations of Nigerian Local × Menina. Goodness-of-fit in the F_2 population was tested with chi-square.

Genotype	Population	Number of plants	Observed segregation R[1]	S[1]	Tested ratio (R:S)	$\chi 2$	Probability
Nigerian Local	Resistant parent	34	34	0	n/a	n/a	n/a
Menina	Resistant parent	50	50	0	n/a	n/a	n/a
Nigerian Local × Menina	F_1	20	20	0	n/a	n/a	n/a
Nigerian Local × Menina	F_2	110	224	14	15:1	0.0549	0.8147

[1]For each plant, disease severity in leaves 3 to 5 was evaluated on a 0 to 4 scale (0=no symptoms), then values were summed to produce an overall severity index of 0 to 12. Plants were then categorized R for resistant (overall severity rating of ≤ 4) or S for susceptible (overall severity rating of ≥ 5).
n/a = not applicable

6. DISCUSSION

Impact of virus infection on performance of tropical pumpkin

Experiments 1 and 2 were carried out in different years and plants of these experiments were taken to the field at different times. Transplanting to the field for Experiment 1 was done later in the spring and virus incidence was high, along with very low fruit yield. In a study of aphid-borne virus incidence on pumpkin and squash in Serbia, Vucurovic and collaborators (2012) found double and triple infections of virus as well as higher virus incidence later in the season of planting. This could be a partial explanation for the differences observed among the two experiments realized at different moments in time. Experiments 1 and 2 had very different results when evaluating the effect of virus on flowering time of the genotypes evaluated. There are no differences among treatments for mean days to flowering in Experiment 1 while Experiment 2 on the other hand had significant differences that set some of the treatments apart for both pistillate and staminate flowers. In particular, anthesis of pistillate flowers was delayed in virus-inoculated plants compared to the controls. Flowering in plants is regulated by internal plant factors (usually under genetic control) or by environmental factors such as day length, temperature and both abiotic and biotic stress. Takeno (2016) reviewed the topic of stress-induced flowering, including flowering in virus-infected plants. This review concludes that stress-induced flowering is a response to retarded growth that promotes a change in plant development from vegetative growth to flowering and reproduction. The delayed flowering observed in virus-infected plants in Experiment 2 was the opposite of what Takeno (2012) noted occurred in many plant species infected with a variety of pathogens.

Virus infection of susceptible genotypes had a negative effect on several traits important for production of tropical pumpkin fruit including fruit number, yield (fruit weight per plant),

and average fruit weight and, to a lesser extent, pulp diameter. Symptoms of infection to PRSV and ZYMV were comparable to those mentioned in other reports on these viral diseases (Mcphail-Medina et al., 2012; Brown et al., 2003; Pachner et al., 2011; Escudero, 1992). In contrast with the control (uninoculated) plants, all inoculation treatments affected two or more of the observed traits related to yield and development. The combined infection of PRSV+ZYMV had a very similar effect to single infections of just one of the two viruses, reducing fruit number, yield and average fruit weight. Surveys realized in and outside of Puerto Rico (Paz-Carrasco and Wessel-Beaver, 2012; Vucurovic et al., 2012) found mixed infections of PRSV and ZYMV to be commonly found in the open field. Quemada et al. (2008) observed that mixed infections of ZYMV and other potyviruses are common in natural (wild) populations of *C. pepo*,

In the case of yield and development, mixed and single viral infections had a similar negative effect on plants compared to uninfected plants. A genotype by treatment interaction was observed for the number of fruit but the interaction was ordered. In the case of number of fruit, total fruit weight and average fruit weight, results demonstrate that uninfected plants of the evaluated six genotypes had higher means for these three economically important traits when compared to infected ones (PRSV, ZYMV, PRSV+ZYMV). The highest number of fruit observed for any genotype was for 'Mos166'. This was expected because it is a plant that produces many small fruit that are mostly inedible. 'Nigeran Local' and 'Soler' had the highest values for total fruit weight, but 'Soler' alone had the highest average fruit weight. These two genotypes share the highest value of total fruit weight because 'Nigerian Local' produces many medium sized fruit and 'Soler' produces fewer but much larger fruit. The latter mentioned genotype also had the highest value for fruit diameter and pulp width for this very reason. 'Waltham Butternut' had the worst yield, development and fruit quality among the group of

evaluated genotypes. This was true even for control plants of ‘Waltham’ because viral and vector presence in the field meant that this highly susceptible genotype would become infected once it was transplanted to the field.

Variables such as color and soluble solids (as observed with hue angle, chroma and °Brix) or with dimensional traits like fruit diameter and pulp width were not as affected by virus infection. Color breaking of the rind and outer colors of the fruit was a symptom observed in infected fruit but the color of the pulp, as defined by L*, chroma and hue angle, was not significantly affected by the viruses. Virus infection did not seem to have any effect on percentage of dry matter in the fruits of plants of the six genotypes evaluated. This might not be the case for other genotypes of pumpkin or for other viral infections.

Evaluation of virus infection during plant development

When evaluating virus infection throughout the experiments mentioned above, ELISA readings made on the same plants at different dates generally produced the same conclusions as to whether a plant was classified as virus-infected or not infected. Studies of viral incidence agree that virus infection in the field is common, especially with the presence of the vector, the aphid. This is congruent with what was observed in the field, where some plants inoculated with one virus tested positive for the presence of the other virus, or where control plants became infected in the field. In experiment 1, plants inoculated with PRSV had higher values, in general, for ELISA readings for the test realized in the field when compared to readings in the greenhouse.

Infection of susceptible plants always resulted in positive readings after 98 dpi. Infection of resistant genotypes ‘Nigerian Local’ and ‘Menina’ had more unexpected results because in

Experiment 1, resistant genotypes inoculated with either PRSV or ZYMV did not always have negative readings during the test realized in the greenhouse or in the field. On the other hand, in the case of Experiment 2, resistant genotypes inoculated with PRSV or ZYMV had negative readings in the field, regardless of the presence aphids or virus.

There was some indication in this study that 'Soler' might carry some level of resistance to PRSV and ZYMV. This cultivar seemed to have consistently low positive (close to 0.400) and sometimes even negative ELISA readings (less than 0.400) for virus infection even after 98 days in the field. 'Soler' does not seem to be completely resistant but definitely tolerates the effect of the virus infection better than known susceptible genotypes like 'Waltham Butternut'. Several studies have talked about 'Soler' and how it has some kind of resistance to ZYMV (Pachner et al., 2004, 2011). It may have a similar level of resistance to PRSV.

Unexpectedly in the case of Experiment 2, the first test for ELISA realized in the greenhouse had all genotypes infected with the virus, regardless of resistance or susceptibility. In a much related thesis study, Miranda-Vélez (2018) observed this same phenomenon. The first leaves (1 to 3) of an inoculated plant often produce a high virus titer, even in resistant genotypes. Miranda-Vélez determined that it takes about 20 days for the 4th leaf to be fully expanded and that this leaf is the most helpful in screening for resistance because it is when resistant genotypes begin to show considerable lower ELISA readings than do susceptible genotypes. In the research presented here, ELISA tests were carried out at 18 dpi which might have been too early to properly evaluate the plants level of virus titer. Lastly, readings conducted in field material after transplanting the plants and letting them develop (at 54 or 55 dpi) have a good correlation with readings before leaving the greenhouse just 20 days after inoculation. This means ELISA tests

make a good assay for screening for resistance even when plants are young and in the greenhouse. The tendency for lower readings for ELISA in the field can be attributed to the presence of on-site infections with other virus. Acevedo Torres (2012) concluded that ELISA tests realized with cucurbit plants infected with PRSV and/or ZYMV will have significantly lower absorbency values if they have multiple virus infections when compared to plants with single infections.

Inheritance of resistance to PRSV and goodness of fit tests

Evaluation of the F_2 distributions of the combined severity ratings (0 to 12 scale) in various crosses was one of the techniques used to evaluate the inheritance of resistance to PRSV in tropical pumpkin. Plants of the crosses made with 'Nigerian Local' as the resistant parent had a more normal F_2 distribution than plants of crosses made with 'Menina'. Crosses made with 'Menina' had F_2 distributions very skewed towards resistance. This suggests that 'Menina' either has more genes for resistance and these genes have an additive effect, or the gene or genes that 'Menina' carries provide more effective resistance to PRSV strain used in this study than the gene or genes of 'Nigerian Local'.

The F_2 distribution of the cross between the two parents (Nigerian Local × Menina) was evaluated for the purpose of determining if the gene or genes of 'Nigerian Local' and 'Menina' are allelic. The F_2 distribution of this cross was extremely skewed, that is to say, most plants were grouped in the most resistant severity rating, 0. When both resistant parents carry the same gene or genes (alleles), no segregation is expected in the F_2. The fact that at least a small number of plants were clearly symptomatic suggests that 'Nigerian Local' and 'Menina' do not carry the same genes for resistance to PRSV.

The second technique used to study the inheritance of resistance to PRSV was to carry out goodness-of-fit tests for different ratios of resistant vs. susceptible plants observed in the F_2 populations. The challenge was to determine the best way to group plants originally classified into a total of 13 severity classes into only two classes of resistance (resistant and susceptible) in order to carry out goodness-of-fit tests. For each of the 13 classes (0 to 12) cumulative absolute frequencies were determined for the resistance and the susceptible category. For example (see Appendix, Table A1), for the F_2 Nigerian Local × Taína Dorada, class 4 had a cumulative frequency of 47 resistant plants (the sum of plants in classes 0, 1, 2, 3, and 4). Plants in the remaining classes (5, 6, 7, 8, 9, 10, 11 and 12) were grouped into the susceptible class with a cumulative frequency of 64. This segregation of 47:64 was then used to calculate a ratio by dividing the highest value by the lowest value: 1.36 in this example. Finally, this ratio was used as a guide to determine which gene models might be expected to fit the observed two phenotypic class segregation (9:7 corresponds to a ratio of 1.29, 3:1 corresponds to 3.0, 13:3 corresponds to 4.33, 15:1 corresponds to 15.0 and 63:1 corresponds to 64.0). As can be seen in the Appendix (Tables A1 to A6), there was a good fit ($p \geq 0.05$) to one or more genetic models for most groupings of resistant to susceptible plants in each of the F_2 distributions. However, very few of the groupings resulted in consistency between F_2 populations that used the same resistant parent. Grouping severity ratings 0 to 4 as resistant and 5 to 12 as susceptible presented the most consistent results of gene models that fit the observed ratios.

For the case of Nigerian Local, crosses made with susceptible genotypes ‘Taína Dorada’ and ‘Verde Luz’ adjust to the model of duplicate recessive epistasis. F_1s of the crosses made were not all resistant, something expected mainly for resistance dependent on complete

dominance of one gene. For the crosses realized with ‘Menina’, the observed segregations nicely adjust to a model of dominant and recessive epistasis.

The cross between both resistant parents did not exhibit allelism. Rather, this cross shows that the genes for resistance to PRSV in these resistant genotypes evaluated are different genes. The model of duplicate dominant epistasis, with a ratio of 15:1 adjusted well to the observed ratio of 224 resistant: 14 susceptible. It is clear that more than one gene is involved in the determination of resistance to PRSV in tropical pumpkin. This is because none of the crosses realized adjusted well to segregations observed with traits dependent on one gene (a ratio of 3 resistant to 1 susceptible). The possibility of more than two genes being involved in the resistance to PRSV is not a far-fetched notion. In the case of ZYMV, Pachner et al. (2004, 2011) evaluated tropical pumpkin genotypes and observed the segregations of crosses of four resistant parents and susceptible genotype ‘Waltham Butternut’. In the case of those at studies, six different genes are described as being involved in the resistance for ZYMV and ‘Menina’ and ‘Nigerian Local’ were found to have different genes of resistance to ZYMV.

7. CONCLUSIONS

Infection with PRSV, ZYMV, or a combination of the two can have a significant effect on the period of time it takes for the plants to flower, which in turn affects the uniformity of harvest.

Virus infection of these viruses or a combination of the two can cause pumpkins to have a high reduction of yield, and plants infected will have a lower amount of fruit set, as well as a lower average fruit weight. In the case of the genotypes evaluated, for much of the fruit traits

observed (fruit diameter, pulp width, percentage of dry matter, percentage of soluble solids, and color) infection with PRSV, ZYMV or PRSV+ZYMV had no significant effect.

There were good positive correlations between ELISA readings realized at different moments of the infected plants' development. This suggests an early greenhouse ELISA test realized on pumpkin plants that have been inoculated just 20 days earlier is highly indicative of the resistance or susceptibility this plant has towards infection with the evaluated viruses. In the case of using a severity rating, plants rated at about 26 days after inoculating will have 4 to 5 leaves, enough to screen for resistance. Classification of resistant or susceptible for PRSV-inoculated plants through severity ratings correlated with the classification realized with ELISA readings. Both methods of determining resistance to infection seem to be effective. When evaluating a complicated trait like inheritance of resistance, it would be pertinent to use both methods together for a more precise result. Screening for resistance to PRSV or ZYMV in pumpkin is simplified this way and costs of this process are reduced.

The inheritance of resistance to PRSV is still unclear. Crosses of susceptible and resistant plants segregate for resistance and display different degrees of disease severity. The allele test realized between 'Menina' and 'Nigerian Local' suggests that the gene or genes involved in resistance are different. Results obtained from evaluating the F_2s of these crosses possibly point to several genes being involved in resistance. Goodness of fit tests realized give the idea that two-gene models can be used to explain all the crosses evaluated. It is important to consider both 'Menina' and 'Nigerian Local' because they have different genes for resistance. A program for developing a new variety will benefit from the use of both for the best of results.

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9. APPENDIX

Table A1. Number of plants by severity rating in the Nigerian Local × Taina Dorada F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	model with best fit			Alternative models		
					Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	7	7	104	14.86	1:15	0.0006	0.9804			
1	10	17	94	5.53	3:13	0.8596	0.3539			
2	11	28	83	2.96	1:3	0.0030	0.9563	3:13	3.0550	0.0805
3	13	41	70	1.71	7:9	2.0937	0.1479			
4	6	47	64	1.36	7:9	0.0894	0.7950			
5	15	62	49	1.27	9:7	0.0070	0.9333			
6	10	72	39	1.85	9:7	3.3475	0.0673			
7	10	82	29	2.83	3:1	0.7051	0.7841			
8	7	89	22	4.05	13:3	0.0834	0.7728	3:1	1.5886	0.2075
9	5	94	17	5.53	13:3	0.8596	0.3539			
10	11	105	6	17.50	15:1	0.1351	0.7132			
11	2	107	4	26.75	15:1	1.3267	0.2494			
12	4	111	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable

Table A2. Number of plants by severity rating in the Verde Luz × Nigerian Local F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	model with best fit			Alternative models		
					Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	0	0	110	xx	xx	xx	xx			
1	0	0	110	xx	xx	xx	xx			
2	8	8	102	12.75	1:15	0.1964	0.6577			
3	6	14	96	6.86	3:13	2.6191	0.1056			
4	28	42	68	1.62	7:9	1.3859	0.2391			
5	10	52	58	1.12	7:9	0.5547	0.4564	9:7	3.6023	0.0577
6	10	62	48	1.29	9:7	0.0006	0.9808			
7	8	70	40	1.75	9:7	2.4387	0.1184			
8	10	80	30	2.67	3:1	0.3030	0.5820			
9	17	97	13	7.46	13:3	3.4695	0.0625			
10	5	102	8	12.75	15:1	0.1964	0.6577			
11	3	105	5	21.00	15:1	0.5455	0.4602			
12	5	110	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable

Table A3. Number of plants by severity rating in the Menina × Taína Dorada F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

					model with best fit			Alternative models		
Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	11	11	109	9.91	1:15	1.7422	0.1869			
1	18	29	91	3.14	1:3	0.0444	0.8330	3:13	2.3110	0.1285
2	16	45	75	1.67	7:9	1.9048	0.1675			
3	24	69	51	1.35	9:7	0.0762	0.7825			
4	22	91	29	3.14	3:1	0.0444	0.8330	13:3	2.3110	0.1285
5	12	103	17	6.06	13:3	1.6547	0.1983			
6	4	107	13	8.23	15:1	4.3022	0.0381			
7	5	112	8	14.00	15:1	0.0356	0.8504			
8	3	115	5	23.00	15:1	0.8889	0.3458			
9	1	116	4	29.00	15:1	1.7422	0.1869			
10	1	117	3	39.00	15:1	2.8800	0.0897			
11	2	119	1	119.00	xx	xx	xx			
12	1	120	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable

Table A4. Number of plants by severity rating in the Verde Luz × Menina F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	model with best fit			Alternative models		
					Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	34	34	84	2.47	1:3	0.9153	0.3387			
1	26	60	58	1.03	9:7	1.3995	0.2368			
2	19	79	39	1.03	3:1	4.0791	0.0434			
3	12	91	27	1.03	3:1	0.2825	0.5951	13:3	1.3220	0.2502
4	10	101	17	1.03	13:3	1.4611	0.2268			
5	4	105	13	1.03	15:1	4.5763	0.0324			
6	2	107	11	1.03	15:1	1.9006	0.1680			
7	1	108	10	1.03	15:1	0.9966	0.3181			
8	5	113	5	1.03	15:1	0.8158	0.3664			
9	3	116	2	1.03	63:1	0.0135	0.9077			
10	1	117	1	1.03	63:1	0.3923	0.5311			
11	0	117	1	1.03	63:1	0.3923	0.5311			
12	1	118	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable

Table A5. Number of plants by severity rating in the TP411 × Menina F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	model with best fit			Alternative models		
					Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	22	22	89	4.05	3:13	0.0834	0.7728			
1	12	34	77	2.26	1:3	1.8769	0.1707			
2	33	67	44	1.52	9:7	0.7620	0.3827			
3	6	73	38	1.92	9:7	4.0842	0.0423			
4	18	91	20	4.55	13:3	0.0390	0.8434			
5	5	96	15	6.40	13:3	1.9979	0.1575			
6	2	98	13	7.54	13:3	3.6094	0.0575			
7	5	103	8	12.88	15:1	0.1736	0.6770			
8	3	106	5	21.20	15:1	0.5772	0.4474			
9	3	109	2	54.50	63:1	3.7483	0.0529			
10	2	111	0	xx	xx	xx	xx			
11	0	111	0	xx	xx	xx	xx			
12	0	111	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable

Table A6. Number of plants by severity rating in the Nigerian Local × Menina F_2 distribution. Within a severity rating class the number of plants in that class and all previous classes (resistant plants) or all higher classes (susceptible plants) were totaled. Gene models were hypothesized and tested with chi-square.

Severity rating	Number of plants in severity class	Cumulative number of plants classified as **resistant**[1]	Cumulative number of plants classified as **susceptible**[2]	Ratio of number of plants from largest class divided by number of plants from smallest class	model with best fit			Alternative models		
					Gene model **(R:S)**	χ^2	Probability	Gene model (R:S)	χ^2	Probability
0	171	171	67	2.55	3:1	1.2605	0.2616			
1	21	192	46	4.17	13:3	0.0521	0.8194			
2	15	207	31	6.68	13:3	5.1200	0.0237			
3	8	215	23	9.35	15:1	4.7339	0.0296			
4	9	224	14	16.00	15:1	0.0549	0.8147			
5	5	229	9	25.44	15:1	2.4751	0.1157			
6	3	232	6	38.67	63:1	1.4216	0.2331			
7	4	236	2	118.00	63:1	1.1242	0.2890			
8	0	236	2	118.00	63:1	1.1242	0.2890			
9	2	238	0	xx	xx	xx	xx			
10	0	238	0	xx	xx	xx	xx			
11	0	238	0	xx	xx	xx	xx			
12	0	238	0	xx	xx	xx	xx			

[1]Within a severity rating class, the value corresponds to the sum of the number of plants in that class and all previous classes.
[2]Within a severity rating class, the value corresponds to the sum of the number of plants in the higher (more susceptible) classes.
xx = not applicable