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Comparison of two Near-Isogenic Lines of Bell Pepper (Capsicum annuum): One Endornavirus-Infected and the Other Endornavirus-Free

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COMPARISON OF TWO NEAR-ISOGENIC LINES OF BELL PEPPER *(Capsicum annuum)*: ONE ENDORNAVIRUS-INFECTED AND THE OTHER ENDORNAVIRUS-FREE

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To my loved family:

In especial my parents Raquel Guardado and Juan Escalante, my sister Iris, my bothers Vidal, David, Jaime, Misael and Oswaldo for their prayers and for providing me with unfailing support and continuous encouragement throughout my years of study.

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ABSTRACT

Bell pepper (*Capsicum annuum*) is an economically important food crop cultivated worldwide. So far, all tested commercial cultivars have been shown to be infected with *Bell pepper endornavirus* (BPEV). Although BPEV does not cause apparent disease, the effect of this virus on bell pepper has not been investigated.

A comparative study that included plant phenotype and some physiological characteristics was conducted with two near-isogenic lines (NIL) of the bell pepper cv. Marengo: one infected with BPEV and the other BPEV-free. The interaction of BPEV with a disease-causing virus of pepper, *Pepper mild mottle virus* (PMMoV), was also investigated.

Differences in the overall phenotypic characteristics between the two bell pepper lines were not observed. Comparisons of the vegetative growth which included plant growth habit, plant height, stem thickness, fruit size, and percentage of dry matter did not yield statistically significant differences. The BPEV-free line showed significantly higher percentage of seed germination and radicle length, and the total fruit weight obtained from the BPEV-negative line was significantly higher than the fruit weight from the BPEV-infected line.

A field isolate of PMMoV was characterized and used to conduct an interaction study between BPEV and PMMoV. Mechanical inoculations of PMMoV to the bell pepper NILs resulted in less severe symptoms in the BPEV-infected line than in the BPEV-free line. The BPEV-infected line also yielded lower virus titer and viral RNA accumulation. Although the virus titer and RNA

accumulation data analyses did not result in statistically significant differences, the negative effect of BPEV on PMMoV was consistent in the various tests, suggesting that BPEV has an antagonistic effect on PMMoV.

The overall results of this investigation suggest that infections of bell pepper by BPEV could have a negative effect on bell pepper production. However, more comparative studies involving biotic and abiotic agents should be conducted to determine other effects that BPEV may have on bell pepper.

CHAPTER I. LITERATURE REVIEW

Peppers (*Capsicum* spp.) are native plants from the Americas. The genus *Capsicum* belongs to the family *Solanaceae*. Currently, there are 38 reported *Capsicum* species (USDA-IRS, 2011) and the five domesticated are: *C. annuum, C. baccatum, C. chinense, C. frutescens* and *C. pubescens*; however, *C. annuum* is the most commonly cultivated species (Pickersgill, 1989; DeWitt and Bosland, 1996; Eshbaugh, 1993; Pickersgill, 1997).

The genus *Capsicum* has a diploid genome that consists of 12 chromosomes (Moscone *et al*., 2003; Arumuganathan and Earle, 1991). The sequence of the whole-genome of both wild and domesticated pepper revealed that a large percentage of the pepper genome consists of transposons (Qin *et al*., 2014). During the domestication process, several commercial traits have been selected. They include compact architecture, increased efficiency of self-pollination and fruit set, early flowering and non-deciduous, pendant fruits (Hill *et al*., 2013). Peppers have diverse uses, which have allowed the development of *C. annuum* genotypes that fit various consumers' needs (Hill *et al*., 2013). It is thought that the continued selection during domestication of pepper has allowed the development of lines with larger, non-pungent fruit with better shape and fruit mass (USDA-IRS, 2011).

Peppers are considered an important cash crop for small farmers in developing countries (Lin *et al*., 2013). The fruits are consumed fresh or dehydrated in a broad scale worldwide and in some

cases fruit extracts are used by the pharmaceutical and cosmetic industries or as a lachrymator (Pernezny *et al*., 2003; Hill *et al*., 2013; Bosland and Votava, 2000). In 2007, the United States had 21,974 Ha of peppers (USITC, 2008), however, in 2014 the total area decreased to 18,818 Ha (NASS, 2015).

1.1 *Capsicum annum*

Capsicum annuum is cultivated worldwide (Lin *et al*., 2013). This species was domesticated in the central region of Mexico and it has been suggested that it originated from a wild progenitor containing two pairs of acrocentric chromosomes (Pickersgill, 1989). There are several *C. annuum* horticultural types that include bell, cayenne, jalapeño, ancho, serrano, poblano and others; the bell type is the most commonly grown in the United States (Bosland *et al*., 1996; USDA, 2013). In 2012, in the United States, per capita consumption of bell pepper reached 5.0 Kg per person (USDA, 2013). Bell pepper plants yield a sweet fruit, and marketable cultivars include those with red, orange or yellow fruits (Jovicich *et al*., 2004); however, fruits of other colors such as brown, white and purple can also be found.

1.2 Plant Viruses

Viruses are obligate parasites and depend on the host's cellular machinery for their replication. A plant virus is a group of single or double DNA or RNA template molecules that code for a few proteins surrounded by a coat capsid, and having the ability to replicate only within a suitable host cell (Hull, 2014). The study of plant viruses began in the 1890s when Dmitri Iwanowski demonstrated that a plant disease could be transmitted by plant sap, and the agent was called virus (Reviewed by Roossinck, 2010). Since that time many plant viruses that infect

economically important crops have been discovered (Fauquet *et al*., 2005). Plant viruses are studied for different reasons, for example the bromovirus *Brome mosaic virus* has been broadly studied in molecular plant virology from a scientific point of view whereas the African cassava mosaic disease caused by a begomovirus complex has been studied due to the severe losses caused in cassava, a major crop grown in Africa (Rybicki, 2015).

Viruses are present in all forms of life and in crop plants are considered a limiting factor causing severe losses (Boualem *et al*., 2016). Virus symptoms are diverse and include foliar mosaic, mottling, yellowing, curling, stunting, ringspots, plant stunting, flower abortion and fruit and root malformations (Hull, 2014). Virus movement from cell-to-cell occurs through plasmodesmata and long distance movement through the plant's vascular system. Transmission of plant viruses is carried out mostly by biological vectors which include insects, mites, nematodes and fungi. Insects represent the majority of these vectors (Whitefiled *et al*., 2005; Hogenhout *et al*., 2008; Brown *et al*., 1996). Plant viruses are also transmitted by direct introduction of the virus into the cell (mechanical transmission). Transmission can be achieved also through seed, pollen, vegetative propagation and grafting (Card *et al*., 2007; Hull, 2014).

There are several methods used for the detection of plant viruses. These methods involve biological and physical properties of the virus particle, and properties of the viral protein and nucleic acid (Hull, 2014). Properties of the viral coat protein are among the most widely used methods for virus detection, and in some cases, have been also used for relative quantification of

plant viruses. ELISA (enzyme-linked immunosorbent assay) is a serological procedure based on the interaction between an antigen (target protein) and an antibody (Vainionpää and Leinikki, 2008). This interaction can be visualized using an enzyme-labeled antibody and quantified by measuring the optical absorbance emitted by the label.

Other virus detection and quantification methods rely on properties of the viral nucleic acids. Polymerase Chain Reaction (PCR) has become a widely used technique for viral nucleic acid detection. This technique consists of hybridization of synthetic; viral specific oligonucleotides primers which allow the amplification of the target sequence and after 30-40 cycles, multiple copies of the viral DNA are generated by the enzyme Taq polymerase (Innis, 1990). The amplicon of the reactions can be analyzed by gel electrophoresis.

A variant of PCR is reverse transcription PCR (RT-PCR); in this case the enzyme reverse transcriptase is used to generate a copy DNA of the viral RNA. Another variant is real-time PCR or quantitative PCR (qPCR). In the latter approach, in addition to primers, a fluorescently labeled probe is used. During the amplification process, the probe emits a signal, allowing accurate quantification of the target sequence and the amplicon is analyzed in real time. The data obtained can be analyzed by calculating absolute or relative quantification using an endogenous control most commonly known as a reference gene (Wong and Medrano, 2005; Applied Biosystems, 2014). In the last two decades, the combination of improved nucleic acid sequencing and bioinformatics has helped to increase the reliability of detection, identification and characterization of plant viruses. Bioinformatics includes the genome sequence analyses, statistics, literature analyses and other tools that are available online (Lysholm, 2012).

1.3 Acute and Persistent Plant Viruses

The most commonly studies plant viruses are those that cause symptoms, which have been designated acute viruses (Roossinck, 2010). Acute viruses are transmitted horizontally and in some cases vertically. They code for a cell-to-cell movement protein which gives them the ability to spread from the point of initial infection. Acute viruses can be transmitted mechanically or by vectors (Blanc, 2007). During the past three decades, another group of plant viruses called persistent viruses have been discovered (Fukuhara and Moriyama, 2008; Pfeiffer, 1998; Boccardo *et al*., 1987). These viruses do not cause symptoms, they lack cell-to-cell movement and are transmitted only vertically via gametes (Blanc, 2007). Persistent plant viruses include members of the families *Amalgaviridae, Chrysoviridae, Endornaviridae* and *Partitiviridae* (Adams *et al*., 2014; King *et al*., 2012).

Persistent viruses infect economically important crops such as alfalfa, avocado, sugar beet, common bean, rice, pepper, melon, and tomato (Fukuhara and Moriyama, 2008; Roossinck *et al*., 2011; Sabanadzovic *et al*., 2016; Villanueva *et al*., 2012; Okada *et al*., 2011; King *et al*., 2012; Pfeiffer, 1998; Boccardo *et al*., 1987). Due to the lack of symptom induction and the lack of transmission by conventional methods, persistent viruses have been poorly studied (Valverde and Castillo 2013; Roossinck, 2010). Recently, there has been an interest in learning about the role that these viruses play in the plant and their interaction with the host under biotic and abiotic stresses (Roosinck, 2010; Roosinck *et al*., 2015).

1.4 Endornaviruses

Endornaviruses are linear ssRNA viruses that infect plants, fungi and oomycetes, with a replicative form ranging from 9.8 to 23.6 kbp (Fukuhara and Moriyama, 2008; Fukuhara and Gibbs, 2012; Ochoa *et al*., 2008; Ong *et al*., 2016). Endornaviruses are persistent viruses classified in a single genus, *Endornavirus*, in the family *Endornaviridae* and like other persistent viruses, they do not cause symptoms in plants (Fukuhara and Gibbs, 2012); although male sterility has been associated with the presence of *Vicia faba endornavirus* in *Vicia faba* (Pfeiffer, 1998; Moriyama *et al*., 1996).

In bell pepper, a dsRNA thought to be the genome of an unidentified virus was reported first in 1990 (Valverde *et al*., 1990). In 2011 the RNA was sequenced and shown to be the genome of a novel endornavirus named *Bell pepper endornavirus* (BPEV) (Okada *et al*., 2011). BPEV has a replicative form of 14.7 kbp, and a genome organization that contains four domains: methyltransferase (MTR), RNA helicase 1 (Hel-1), UDP-glucose-glycosyltransferase (UGT) and an RNA dependent RNA polymerase (RdRp) (Okada *et al*., 2011; Roossinck *et al*., 2011) Furthermore, the positive strand of the replicative form of some plant endornaviruses contains a discontinuity in the positive strand.

In 1991, after gel electrophoretic analyses, Valverde and Fontenot reported several genotypes of pepper with endornavirus-like dsRNA profiles. In a study conducted by Okada *et al*. (2011) todetermine the occurrence of BPEV in pepper, 18 bell pepper cultivars were tested for BPEV by

RT-PCR and gel electrophoretic analysis and all were found to be infected. This report suggests that most bell pepper cultivars in the United States may be infected with BPEV or closely related viruses.

As mentioned earlier, little is known about the effects that endornaviruses have on plants. Sela *et al*., (2012) reported the production of small RNAs in bell pepper infected with BPEV. This indicates the activation of host gene silencing and supports the hypothesis that endornaviruses have an active role in the infected plant.

1.5 Acute Viruses of Pepper

Acute viruses are a major problem affecting pepper production around the world. Peppers are infected by viruses when planted under open as well as protected conditions, causing yield and fruit quality losses (Rialch *et al*., 2015). Several plant viruses are known to infect peppers and these include: *Cucumber mosaic virus*, *Pepper mottle virus*, *Potato virus Y* (PVY), *Tomato yellow leaf curl virus*, *Pepper severe mosaic virus*, *Pepper golden mosaic virus*, *Pepper mild mottle virus* (PMMoV), *Pepper ring spot virus*, *Tomato spotted wilt virus* (TSWV), *Tobacco mosaic virus* (TMV), *Tobacco mild green mosaic virus*, *Tomato chlorotic spot virus*, *Pepper yellow mosaic virus*, *Tobacco etch virus* and *Groundnut ringspot virus* (Gracia *et al*, 1968; Villalon, 1975; Pernezny *et al*., 2003; Black *et al*., 1991).

The genus *Tobamovirus* belongs to the family *Virgaviridae* which contains 37 species. All of the members of this genus have four open reading frames that encode for five proteins, a gene that codes for the 126-kDa protein that contains MTR and Hel domains, a read-through of 183-kDa

protein containing the RdRp, the 30-kDa movement protein (MP) and the 17.5-kDa coat protein (CP) (Hull, 2014). They are rigid rod-shaped virions of approximately 312 nm long and 18 nm in diameter (Hull, 2014; Regenmortel, 1981; ICTV, 2017). The type species of this genus is TMV which is known to cause some problems in pepper production. PMMoV is another specie of this genus that has worldwide distribution causing severe diseases on pepper and in some cases resulting in severe losses (Pernezny *et al*., 2003). In Louisiana, PMMoV has been a threat especially for tabasco pepper production (R. A. Valverde, personal communication). In 1952, McKinney first recognized PMMoV in the United States as the latent strain of TMV while, in Europe, PMMoV was identified as another TMV strain (Wetter *et al*., 1984). PMMoV has a narrow host range in natural conditions; although under experimental conditions, PMMoV has a wide host range and several members of the families *Amaranthaceae*, *Chenopodiaceae* and *Solanaceae* are used in host range and symptomatology studies (Wetter *et al*., 1984). There are several strains of PMMoV, and in general, the criteria to characterize strains of PMMoV, is based on variation of the overall nucleotide sequence by less than 10% (ICTV, 2017).

Plants infected with PMMoV develop a mild foliar mosaic and sometimes also leaf crinkling. The fruits may show symptoms such as distortion, chlorotic rings and line patterns (Black *et al*., 1991). The virus has the capability to survive in crop debris, soil and contaminated equipment (Rialch *et al*., 2015). PMMoV does not have a biological vector, and its transmission is mostly mechanically and through seed contamination which is considered to be one of the main sources of infection (Black *et al*., 1991; Pernezny *et al*., 2003; Greenleaf, 1986). Planting “clean seed” is one of the most commonly recommended ways to control this virus, although resistant varieties have been developed (Özkaynak, 2014; Pernezny *et al*., 2003). Since the embryo of the pepper

seed is not infected by PMMoV, there are some procedures that result in efficient elimination of the virus from the coat. These methods are based on chemical treatments such as 10% sodium hypochlorite or 10% trisodium phosphate (Jarret *et al*., 2008).

Metagenomic studies have identified PMMoV in food products and human stools (Colson *et al*., 2010). A case-control study associated biological and clinical symptoms with the presence of PMMoV in the stools, suggesting a direct or indirect role of the pathogen in humans (Colson *et al*., 2010).

1.6 Interactions between Acute Plant Viruses

The result of double infections of plants by some acute viruses is synergism, antagonism, or no interaction (Chávez *et al*., 2016). During synergistic interactions, the measurable differences in replication, phenotypic and pathological changes in the cell, cellular tropism, within host movement and transmission rate of one of the two viruses or both increase (Mascia and Galliteli, 2016). In the antagonistic interaction there is reduction of replication or inhibition of one or more of these functions (Mascia and Galliteli, 2016).

There are several examples of synergism and antagonism occurring in economically important crops during double infection by plant viruses. A recent study of two unrelated viruses in papaya; *Papaya ringspot virus* (PRSV) and *Papaya mosaic virus* (PapMV) revealed differences in symptoms depending of the order or inoculation (Chávez *et al*., 2016). Synergism occurred when plant where infected first by PRSV or when infections with PRSV and PapMV occurred simultaneously. In contrast, antagonism occurred when plants were first inoculated with PapMV and later with PRSV. This was also observed by McGregor *et al*. (2009) with infections of *Sweet*

potato chlorotic stunt virus (SPCSV) and *Sweet potato feathery mottle virus* (SPFMV), they found that symptoms were significantly more severe in plants infected with SPCSV followed by SPFMV compared to plants infected with SPFMV followed by SPCSV. In tomato plants, double infection of PVY and *Potato virus X* (PVX) has resulted in more severe symptoms than single infection, indicating PVY-PVX synergism (Liang *et al*., 2016). Examples of synergism have been shown mostly in mixed infection of two or more unrelated viruses; however, a recent study with co-infection with two isolates of TSWV (non-resistance breaking and resistance breaking), caused synergism with systemic necrosis on the apical leaves on pepper chili (Aramburo *et al*., 2015). Mixed infection can result in cross protection. Cross protection is the process by which a mild virus strain activates the "plant immune system" protecting the plant from a more severe strain. A successful example of cross protection is the use of a mild strain of *Citrus tristeza virus* in citrus trees, to reduce the disease severity of more aggressive strains. (Folimonova, 2013).

1.7 Interactions between Persistent and Acute Viruses

Only a few preliminary studies on the interaction between persistent and acute viruses have been reported (Escalante and Valverde, 2016; Escalante *et al*., 2016). Research on the interactions between endornaviruses and other plant pathogens such as fungi or bacteria, or herbivores has not been reported. Like acute viruses, endornaviruses could affect the host response to infection by any of these pathogens. It is possible that endornaviruses could interact with acute viruses and result in more severe diseases than those caused by one acute virus alone. Alternatively, it is also possible that the "activation of the plant immune system" by endornaviruses could result in less severe diseases such as in the case of cross protection. Preliminary experiments with PMMoV,

have suggested that the latter may be true. Khankhum (2016) found that double infections of *Phaseolus vulgaris endornavirus* 1 and/or *Phaseolus vulgaris endornavirus* 2 with *Sunn-hemp mosaic virus* (persistent and acute virus) did not show any effect on the symptom expression caused by of the acute virus.

CHAPTER II. EVALUATION OF THE PHENOTYPE, VEGETATIVE GROWTH, FRUIT YIELD AND SEED GERMINATION OF TWO NEAR-ISOGENIC LINES OF BELL PEPPER: ONE INFECTED WITH *BELL PEPPER ENDORNAVIRUS* AND THE OTHER ONE ENDORNAVIRUS-FREE

2.1 Introduction

Peppers (*Capsicum* spp.) originated in the Americas. The genus *Capsicum* includes five domesticated species: *C. annuum, C. baccatum, C. chinense, C. frutescens* and *C. pubescens*. *Capsicum annuum* is the most commonly cultivated species (Pickersgill, 1989; DeWitt and Bosland, 1996). Peppers are considered an important cash crop for small farmers in developing countries (Lin *et al*., 2013). The fruit are consumed fresh or can be used as a condiment. In some countries, the fruit extracts are used by the pharmaceutical and cosmetic industries (Pernezny *et al*., 2003). More recently, capsaicin extracts from hot peppers have been used as lachrymator by law enforcement agencies. In 2007, the United States had a total of 21,974 Ha of harvested peppers (USITC, 2008). However, in 2014 the total area of harvested peppers decreased to 18,818 Ha (NASS, 2015).

Capsicum annuum is cultivated worldwide (Lin *et al*., 2013). This species was domesticated in the central region of Mexico, probably in northeast or east-central Mexico (Kraft *et al*., 2013). There are several different horticultural types of *C. annuum*, but in the United States, the bell pepper type is the most common (Bosland *et al*., 1996; USDA, 2013). Bell pepper plants yield a sweet fruit, and marketable cultivars include those with red, orange or yellow fruits (Jovicich *et al*., 2004); however, fruits of other colors such as brown, white and purple can also be found.

Plant viruses can cause severe crop loses because they interfere with many aspects of the physiology of the plant. The reduction of foliage area and total biomass caused by viruses usually translates in in low yield and poor quality of the fruit (Boualem *et al*, 2016; Hull, 2014). According to the symptomatology, viruses can be categorized as persistent or acute (Roossinck, 2010). Acute viruses cause distinctive symptoms and a variety of diseases. Unlike acute viruses, persistent viruses do not cause symptoms and thus little research has been conducted on these viruses.

Valverde *et al*. (1990), reported a dsRNA from symptomless Yolo Wonder bell pepper and suggested that it was the genome of an unidentified virus. In 2011, the dsRNA of Yolo Wonder bell pepper was sequenced and shown to be the genome of a novel endornavirus. The virus was named *Bell pepper endornavirus* (BPEV) (Okada *et al*., 2011). Many cultivars of bell pepper grown in the United States have tested positive for BPEV (Valverde and Fontenot, 1991; Okada *et al*., 2011). This suggests that most bell pepper cultivars in the United States may be infected with BPEV or closely related viruses. Another *C. annuum* horticultural type, hot pepper, has been reported infected with an endornavirus, Hot pepper endornavirus is closely related to BPEV (Lim *et al*., 2015).

Khankhum (2016), conducted experiments with two lines of common bean (*Phaseolus vulgaris*) cv. Black Turtle Soup; one infected with two endornaviruses and the other line endornavirus-free. He did not find differences between the two lines in terms of yield and phenotypic characteristics; although the chlorophyll content of the endornavirus infected line was lower than the endornavirus-free line.

2.2 Objective

The objective of this investigation was to compare the plant phenotype, percentage of dry matter, vegetative growth, fruit yield and seed germination of two bell pepper near-isogenic lines (NIL); one infected with BPEV and the other endornavirus-free.

2.3 Materials and Methods

2.3.1 Development of Bell Pepper Near-Isogenic Lines

The bell pepper line free of BPEV reported by Okada *et al*. (2011) was provided by M. J. Roossinck (The Pennsylvania State University). The backcross breeding method was used to generate a BPEV-infected NIL (R. A. Valverde, personal communication). This approach consisted of using a BPEV-positive plant as a pollen donor parent and the BPEV-negative plant as a recurrent parent. Backcrosses were carried out using the BPEV-positive F_1 plant as pollen donor. Five backcrosses were conducted to generate the BPEV-infected near-isogenic to the BPEV-negative line. A diagrammatic illustration is shown in Figure 2.1. After self-pollination the seed of these plants were used in all experiments conducted in this investigation.

2.3.2 Planting Conditions

Seeds of the two bell pepper NILs were planted in autoclaved clay pots (0.49-L) containing a soil mix that consisted of 1.5 parts of soil, 1.5 parts of sand and 3 parts of potting mix (Miracle-Gro® Lawn Products, Inc., Marysville, OH) (Fig. 2.2A and 2.2B). This soil mixture was used in all the experimental stages. Prior to planting, seeds were treated with 10% sodium phosphate tribasic dodecahydrate (Sigma-Aldrich Co., MO, USA). Seedlings were kept in the laboratory, under

artificial light (54W/120V 60Hz/4.0A Lamps) with 15 h photoperiod. Seedlings (15-days-old) were transplanted into 11.3-L pots filled with the soil mix described above and kept in a greenhouse located on the Campus of Louisiana State University, Baton Rouge (Fig. 2.2C). Greenhouse day/night temperatures averaged 25/18 ºC respectively. Plants were kept in the greenhouse for 10 days and then the pots were transferred outside (open field) (Fig. 2.2D).

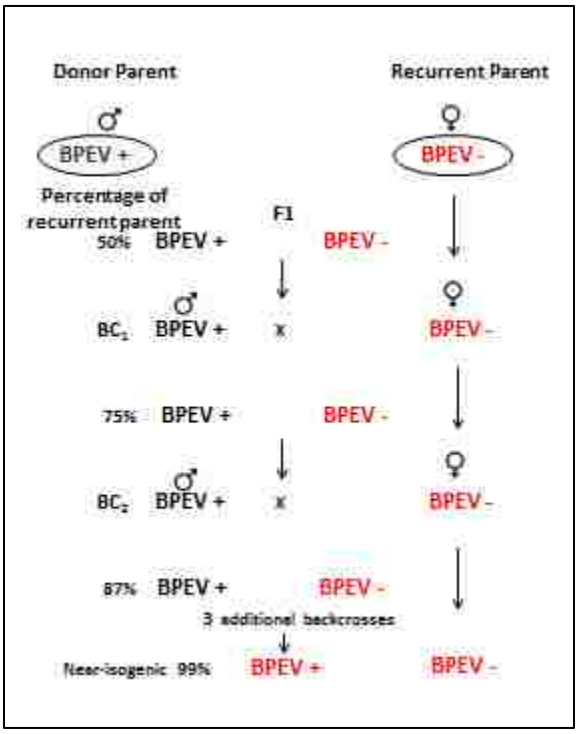

Figure 2.1 Diagrammatic illustration of the steps taken to develop near-isogenic lines of bell pepper cv. Marengo, one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). (R. A. Valverde, unpublished).

Insect control was carried out as needed using Imidacloprid (Bayer Environmental Science, Research Triangle PK, NC) and Avid (Syngenta Crop Protection, Inc., Greensboro, NC). Plants were fertilized every 30 days with (Osmocote® Smart-Release® Plant Food (19-6-3), The Scotts Company LLC, Marysville, OH). Fifteen plants per line (30 plants total) were used in each experiment. All comparative experiments were conducted four times (four plantings) during 2015 and 2016.

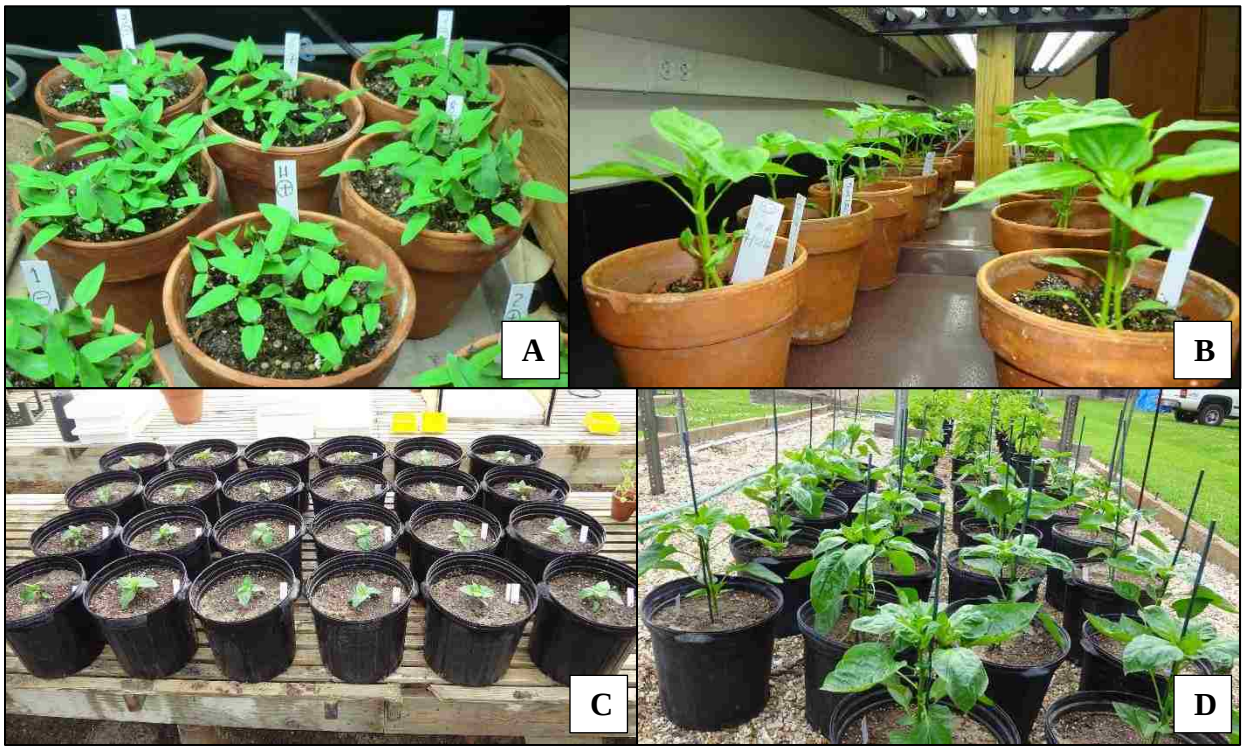

Figure 2.2 Illustration of the various planting, transplanting and plant growth stages. A, Seedlings growing in the greenhouse; B, Plants growing in the laboratory; B, Plants transplanted in 11.3-L pots in the greenhouse; C, Plants in open field.

2.3.3 Testing Plants for BPEV

To confirm the presence/absence of BPEV in experimental plants, foliar tissue was desiccated in silica gel as described by Khankhum *et al*. (2016). Desiccated tissue was ground with a mortar and pestle and 0.07 g used for dsRNA (replicative form of the viruses) extraction. Essentially the method consisted in phenol extraction followed by binding of viral dsRNA to fibrous cellulose in 16% ethanol. All extracted samples were treated with 1 unit of RNase-free DNase I (Fisher Scientific, Waltham, MA, USA) and analyzed in 1.2% agarose gel. Negative and positive controls consisted of tissue samples from plants known to be endornavirus-free and endornavirus-infected respectively.

2.3.4 Evaluation of the Plant Phenotype

The plant phenotype was evaluated by weekly visual inspections for plant size, leaf shape, leaf and stem color. This was conducted throughout all phenological stages of the plants grown in the laboratory, greenhouse and open field.

2.3.5 Fruit Yield

Number of fruits. Fruit were harvested two times for each planting, the first harvest was done when the fruits reached 7.0 cm long (only fruit that were approximately 7 cm or greater were collected). The second harvest was conducted three weeks after the first one; although in some cases the second harvest took longer time to reach the appropriate fruit size. At the end of each harvest, the total number of fruit per plant was recorded.

Fruit weight. After harvesting, fruit were and placed into Ziploc™ bags and transferred to the laboratory. The fruit were weighed immediately, using a digital balance (VWR® A-Series Balances). At the end of the second harvest, the weight of the fruit from both harvests was added and the average of yield per plant was determined.

Fruit size. The size of the fruit was determined by using the equation $Yi = 19.226859 + 0.139562Xi - 0.256142Zi + 1.429122Ti$; were, Yi = Size of the fruit (cm^3), Xi = Diameter of the fruit (mm), Zi = Length of the fruit (mm) and Ti = Weight of the fruit (g) (Kadri and Kilic, 2010). Five fruit per plant were randomly selected and all the fruit dimensions were individually measured using an electric digital caliper (784EC 6”). Individual fruit were also weighed.

Percentage of dry matter. This was determined by collecting the plants after the second harvest. The plants were cut at the soil level with a hand pruner, then cut into 10 cm pieces and placed in paper bags. The fresh plant material was weighed and then placed in an oven (Precision, UL® Jouan, Inc.) at 60 °C until the plants reached a constant weight (72 h) (Abu-Zahra, 2012). Once the material reached a constant weight, the percentage of dry matter was determined by the following formula: *Dry Matter (%)* = $\frac{Dried\ Tissue\ (g)}{Fresh\ Tissue\ (g)}$ *x 100.*

2.3.6 Seed Germination

For the seed germination experiments, seeds were collected from fruit of the two NILs that ripened at the same time. Fruit were dissected in the laboratory, seed were dried at room temperature for two days and stored in vials at 4 ºC. Prior to the germination test, seeds were immersed in 10% sodium hypochlorite solution for 10 min and then rinsed with sterile water. Thirty seeds per line were used in each petri dish (replicate). Two layers of brown paper and one folded kimwipe (Kemtech Science, Roswell, GA, USA) were placed in each petri dish plate. Ten seeds were arranged in the middle of each plate and 8 ml of deionized water were added to moist the paper (Fig. 2.3). Plates were incubated (Ambi-Hi-Lo® Chamber, Lab-Line Instruments, Inc., Ill, USA) at 23 ºC for 12 days. Beginning three days after placing the plates in the incubator, seed were inspected and germination recorded daily. Radicle length was measured at the end of the experiment (12 days).

2.3.7 Plant Height and Stem Thickness

Vegetative growth evaluations were conducted for each NIL at the end of the second harvest in all four plantings. Plant height was determined by measuring the length of the main stem from the top of the soil level to the apical bud of the plant. Thickness of the stem was measured at 1.0 cm above the site where the plant was cut (base of the stem). An electric digital caliper (784EC 6") was used to measure the diameter of the stem.

Figure 2.3 Illustration of the seed germination test design of the two bell pepper cv. Marengo near-isogenic lines.

2.3.8 Experimental Design and Statistical Analysis

A completely randomized design was used in the experiments performed. The averaged data from each pepper line was analyzed by One-Way ANOVA using SPSS (IBM© SPSS© Statistics Version 24) the analysis was performed through the General Desktop Virtual Lab of the Louisiana State University. Statistical analysis for phenotypic characteristics and fruit yield was carried out between NILs and between plantings (replicates). Post Hoc analysis (Tukey $P \leq 0.05$)

could not be performed between NILs because there were fewer than three treatments. In the case of analysis between seasons or replicates, Post Hoc analyses were performed when there were statistical differences. The comparisons were considered statistically significant at $P \leq 0.05$. The experiments on the evaluation of plant phenotype, percentage of dry matter, plant height, stem thickness and fruit yield were replicated four times. Seed germinations tests were replicated three times.

2.4 Results

2.4.1 Plant Phenotype

With the exception of fruit shape, differences were not observed between BEPV-positive and BPEV-negative lines throughout the different phenological stages (Fig. 2.4).

2.4.2 Fruit Yield

Fruit yield data are shown in Figure 2.5. For all four variables evaluated, the BPEV-negative line consistently yielded greater values than the BPEV-positive line. However, only the fruit weight showed significant differences, yielding 365.8 g and 290.6 g for the BPEV-negative and BPEV-positive line, respectively. The other variables evaluated (fruit size and number of fruit) had only minor differences which were not statistically significant.

The BPEV-negative line yielded greater average number of fruits per plant (10 fruits per plant) than the BPEV-positive line (Fig. 2.5A). Similarly, in the four experimental replicates this line yielded larger fruit than the BPEV-positive line; although the differences were not significant (Figs. 2.5B and 2.6). A One-Way ANOVA analysis among replicates was performed to

determine differences between replicates. All of the variables analyzed resulted in statistical differences among replicates.

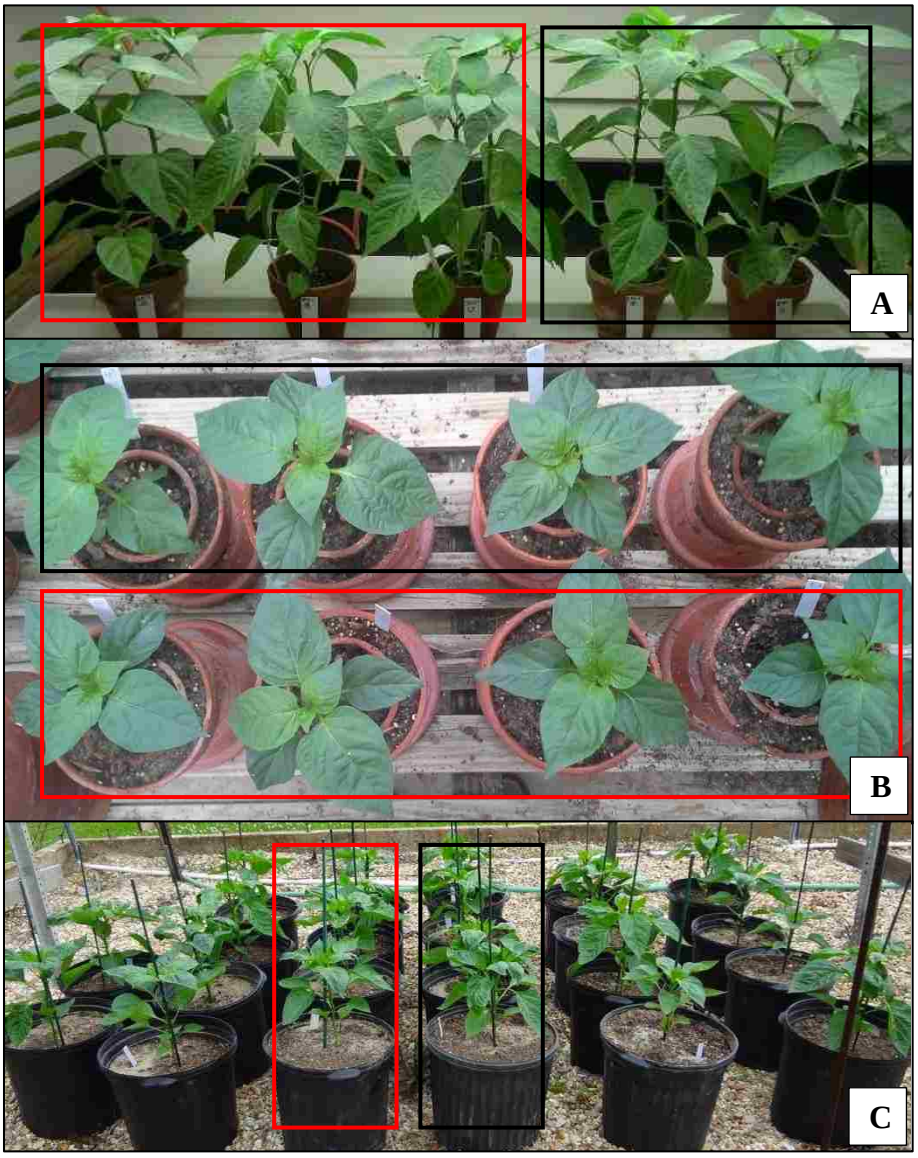

Figure 2.4 Illustration of the phenotype of the two near-isogenic lines throughout the different stages of plant growth, BPEV-positive (red frame), BPEV-negative (black frame). A, Plants growing in the laboratory; B, Plants growing in the greenhouse; C, Plants in open field.

2.4.3 Percentage of Dry Matter

The average weight of dry matter was determined at the end of the experiment and like the variables evaluated above, the BPEV-negative line was greater than the BPEV-positive line (17.4% and 16.9% for BPEV-negative line and BPEV-positive line respectively) (Fig. 2.5D).

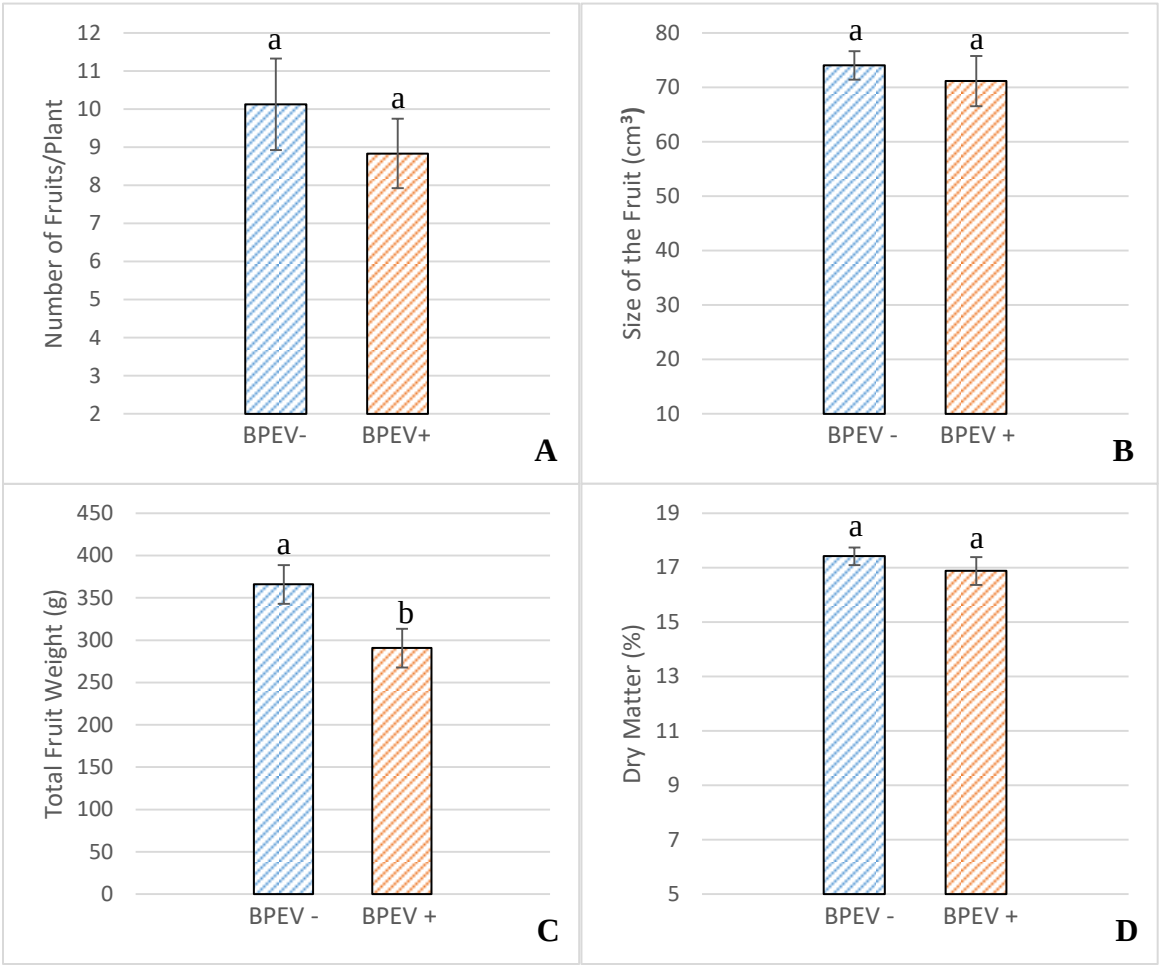

Figure 2.5 Yield evaluation of two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). A, Number of fruits per plant; B, Size of the fruit (cm^3); C, Total fruit weight (g); D, Percentage of dry matter. For A and C, n = 24 from four replicates. For B, n = 17, five sub-sets were randomly selected from each plant. For D, n = 20 (BPEV-) and n = 19 (BPEV+). Different letters indicate statistical difference at $P \leq 0.05$. Bars indicate the standard error.

2.4.4 Seed Germination

The percent seed germination of both NILs was determined (Fig. 2.8A and B). Except for day 3, seeds from the BPEV-negative line showed greater percent germination during the 12-day evaluation period (Fig. 2.8A). Percent of seed germination did not reach 100% in either line.

Figure 2.6 Fruit obtained from two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). Fruit are representative of four independent biological experiments.

Figure 2.7 Illustration of the root length of two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). Figure is representative of three independent experiments.

The BPEV-negative line showed a significantly greater percent seed germination compared to the positive line (Fig. 2.8B). Average of seed germination did not differ among replicates. Like seed germination, the radicle length of the seedling was measured at day 12. The radicle of the BPEV-negative line was significantly longer than the BPEV-positive line (Figs. 2.8C and Fig. 2.7). Similarly, differences were found among all replicates.

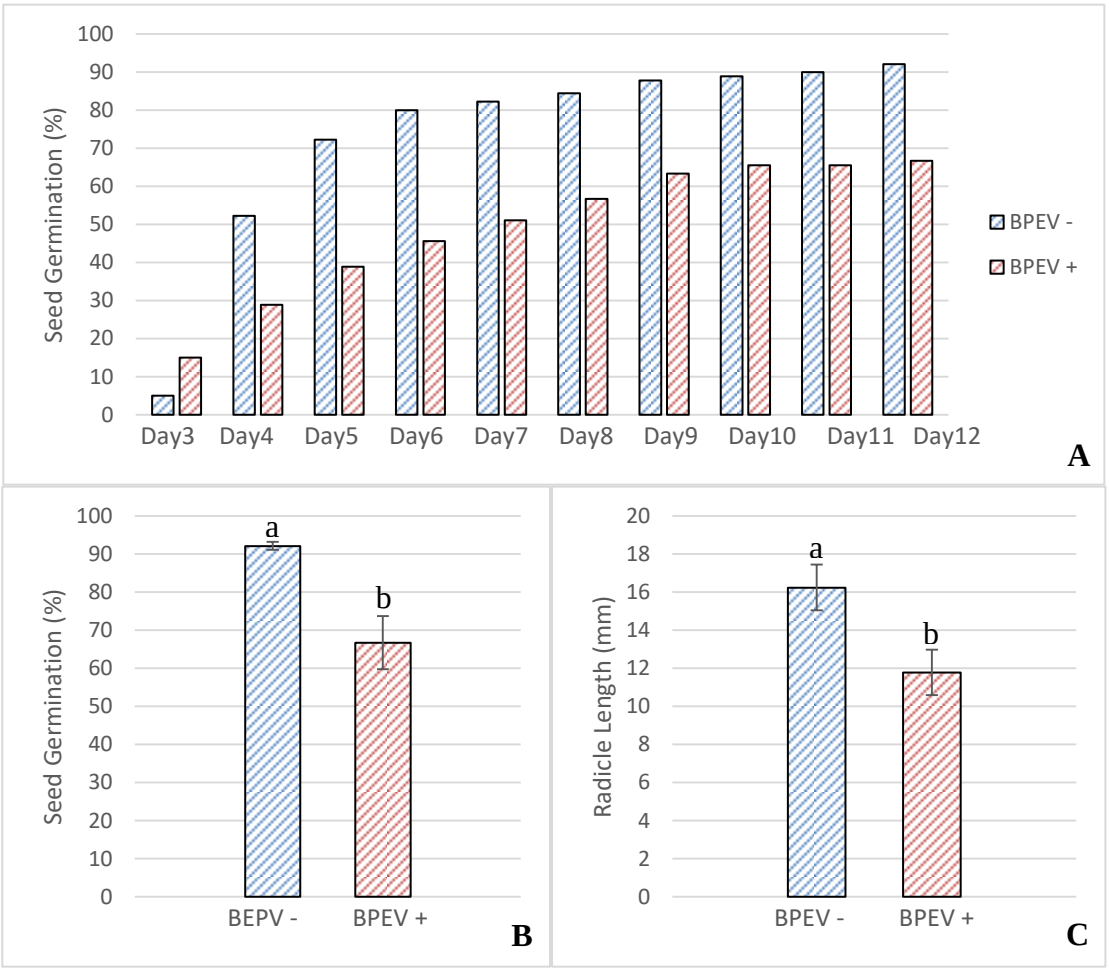

Figure 2.8 Evaluation of seed germination of two near-isogenic lines of bell pepper Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). A, Percent seed germination throughout a period of 12 days; B, Percent seed germination at the end of the experiment; C, Radicle length (mm). For B, n = 29, each replicate consisted of three repetitions, each repetition had 10 seeds. For C, n = 83 (BPEV-) and n = 60 (BPEV+). Different letters indicate statistical difference at $P \leq 0.05$. Bars indicate the standard error.

2.4.5 Height and Stem Thickness

Two vegetative growth variables were evaluated, height of the plant and thickness of the stem. In regard to the height, the BPEV-negative line yielded higher values than the positive line (44.9 cm and 43.3 cm respectively); although the differences were not significant (Fig. 2.9). Similar results were obtained with the thickness of the stem (11.1 mm and 10.9 mm). Significant differences for these variables also occurred among replicates.

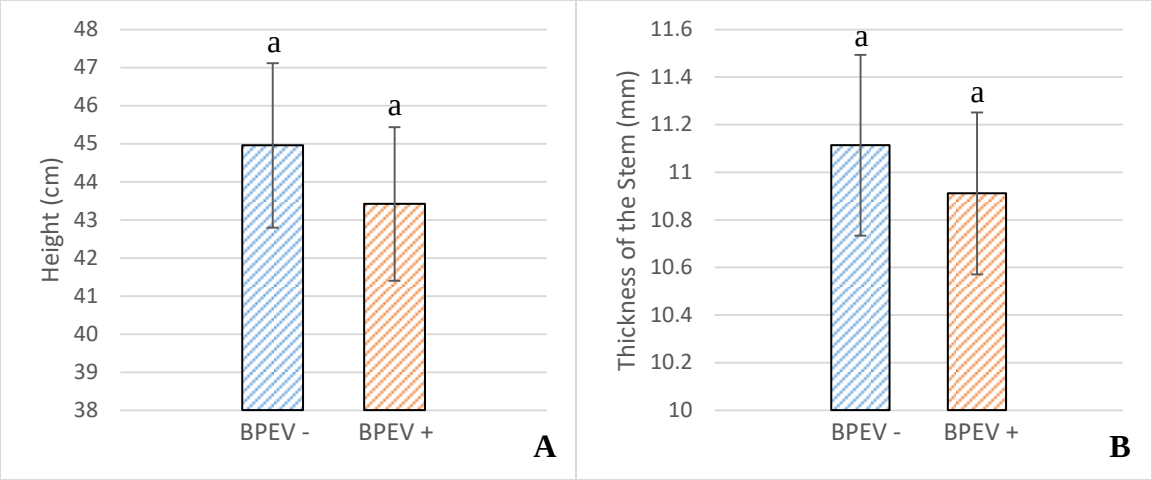

Figure 2.9 Evaluation of the vegetative growth of two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-). A, Height of the plant (cm); B, Thickness of the stem (mm). For A and B, n = 24. Same letters indicate not statistical difference at $P \leq 0.05$. Bars indicate the standard error.

2.5 Discussion

Several papers have been published on the occurrence of persistent plant viruses in crops (Fukuhara *et al*., 1993; Okada *et al*., 2011, 2013; Sabanadzovic *et al*., 2016; Candresse *et al*., 2016) but there is no evidence that persistent viruses cause apparent negative effect in the physiology and phenotypic characteristics of the plant (Roossinck 2010; Fukuhara and Gibbs, 2012, Blanc, 2007). Research on the effect of these viruses to the host physiology and phenotype is lacking.

The results of testing for the presence of endornaviruses in various crop cultivars suggest that endornaviruses have been introduced into some cultivars of common bean, melon, rice, and pepper during modern plant breeding (Okada *et al*., 2011, 2013; Sabanadzovic *et al*., 2016; Valverde *et al*., 2011; Zabalgogeazcoa *et al*., 1993). This is supported by studies conducted with common bean endornaviruses in which the percent of endornaviruses in wild common bean and landraces is lower than in the modern cultivars. Therefore, it appears that during the development of these crops, plant breeders and possibly people involved in earlier domestication of these crops that were unaware of the existence of endornaviruses in the germplasm of these crops, selected endornavirus-infected genotypes. This suggests that the virus may be associated with unknown beneficial traits. Okada *et al*., (2011) tested commercial bell pepper cultivars for BPEV and found all of them infected. In this investigation, 29 commercial bell pepper cultivars were tested by gel electrophoresis and all contained a 15 kbp dsRNA (Appendix 1). This suggests that all bell pepper cultivars grown in the USA may be infected with BPEV or related viruses.

Acute plant viruses can cause severe economic loses in crops. This negative effect is related with interference of the normal physiology of the plant. The reduction of the foliage area and overall biomass of the plant caused by viral infections, leads to lower fruit yield and quality (Boualem *et al.*, 2016; Hull, 2014, Anderson *et al*., 2004). As mentioned before, knowledge on the effect of persistent viruses to plants is lacking. Under the experimental conditions of this investigation, it was found that the BPEV-negative line showed overall higher values in all the variables evaluated when compared with the BPEV-positive line. Except for total weight of the fruit, percent seed germination and root length of the germinated seeds, the results were not statistically significant. Because commercially, bell pepper fruits are classified by size and sold

by weight (Hartz *et al*., 2008; Fonsah, 2009), hence, the weight and size of the fruit are important parameters that need to be evaluated when conducting comparative experiments.

The BPEV-negative line yielded significantly higher percentage of seed germination than the BPEV-positive line. Likewise, the radicle length of the BPEV-negative line was significantly longer than the BPEV-positive line. In common bean (*Phaseolus vulgaris*), the results obtained by Khankhum (2016) were the opposite; the percentage of seed germination and radicle length were higher in seeds infected by endornaviruses. Abiotic factors like temperature critically affect seed germination. Pepper seed germination might be decreased with temperatures lower than 25ºC (Hartz *et al*., 2008). In this experiment, germination was tested at 23 °C and it seems not to affect seed germination in any of the two tested lines in terms of time of initial germination. However the percentage of seed germination did not reach 100 per cent in both lines. This suggests that the combination of low temperature and the presence of the virus, affected the seed germination of the BPEV-positive line which had 25 percent less germination than BPEV-negative line. Plants infected with BPEV were smaller than the virus-free plants, likewise, the diameter of the stem tended to be smaller in the infected plants. This overall reduction in the architecture of the plant may explain the lower fruit weight obtained in the BPEV-infected line. It is well stablished that the smaller the biomass of the plant, the lower the fruit yield it will have (Hartz *et al*., 2008; Jovicich *et al*., 2004).

The presence of an agent in the plant host will cause a hijack of the plant's energy and cell machinery to multiply and spread. For example, infections of the symptomless *Grapevine leafroll-associated virus 3* (GLRaV-3) lead to a decrease in plant biomass (Christov *et al*., 2006).

This indicates that even though there is an absence of symptoms on the plant, there might be a reduction of plant fitness. Photosynthesis is negatively affected in virus-infected plants. It is known that viral-coded proteins, like coat the protein (CP) can inhibit the proper function of the photosystem II rendering the photochemistry pathway into photo-inhibition resulting in chlorosis (Reinero and Beachy, 1986; Hodgson *et al*., 1989). BPEV and other endornaviruses do not cause visible symptoms in the host and it is possible that this might be due to the lack of CP. However, it will not be surprising if protein-encoding genes in BPEV can affect photosynthesis causing an overall reduction of biomass and consequently affecting the yield of the plant. Using two lines of the common bean cv. Black Turtle Soup; one infected with *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2), Khankhum (2016) conducted a comparative study and reported lower chlorophyll in endornavirus-infected plants.

It is known that higher temperatures can enhance some plant mechanisms of pathogen-resistance (Qu *et al*., 2005). Bell pepper is adapted to grow under high temperature conditions and this may trigger mechanisms of the plant that reduce the negative effects caused by BPEV. BPEV is a ubiquitous agent that replicates and spreads during cell division. In that sense, the virus coexists with the host, hence, mechanisms of defense might not be activated (Sela *et al*., 2012). As pointed out earlier, studies on the interaction of endornaviruses with their host are limited (Khankhum, 2016; Khankhum *et al.*, 2016; Moriyama *et al*., 1996). Khankhum (2016) conducted comparative studies using endornavirus-infected and endornavirus-free common bean. In his investigation, it was found that the PvEV1 and PvEV2-infected line had longer pod and higher weight of the seed compared to the endornavirus-negative line. Those results differ from those found in the present study with BPEV. However, the common bean lines were not near-isogenic

and therefore genetic variation may have affected the results. Studies with different crops containing endornaviruses should be carried out to develop a broader perspective of the effect of endornaviruses on plant phenotype and physiology.

Inoculation with microorganisms such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* did not affect seed germination in bell pepper (Herman *et al*., 2008). However, acute viruses can reduce or increase expression of precursors of phytohormones that are involved in seed germination (Dziurka *et al*., 2016). Several phytohormones like salicylic acid, jasmonic acid and ethylene are well known to be involved in the plant immunity system (Alazem and Lin, 2015; Enyedi *et al*., 1992; Singh *et al*., 2004). Since the presence of a virus might accelerate or delay seed germination, further studies should be conducted to compare expression or production of these hormones in response to acute virus and BPEV infection. A virus causing delay in seed germination can affect the viability of the seed. Longer times for germination might expose the seed to the substrate and other metabolic processes can be affected. Furthermore, the seed will undergo prolonged exposure to attack by other pathogens. In this investigation, the radicle length correlated with the percentage of seed germination. Since the non-infected seeds have less inhibition to germinate it is not surprising that the radicle developed more vigorously.

Overall, the BPEV-negative line showed greater fruit yield and vegetative growth per plant than the BPEV-positive line, suggesting that BPEV has a negative effect on bell pepper production. In terms of crop production and market aspects, fruit yield is one of the most important. To evaluate the effect of the virus from the commercial standpoint it is necessary to conduct experiments in a larger scale under open field or greenhouse conditions and evaluate the profits (Fonsah, 2009).

The hypothesis in this study was that BPEV has a mutualistic interaction with the host conferring beneficial effects to the crop. Although this part of the study was limited to some morphological and physiological characters, the mutualistic interaction hypothesis was not supported. However, interactions between BPEV and biotic and abiotic stresses of the host were not evaluated.

CHAPTER III. IDENTIFICATION AND PARTIAL CHARACTERIZATION OF *PEPPER MILD MOTTLE VIRUS*

3.1 Introduction

Peppers are infected by a variety of acute plant viruses that cause economically important diseases. Acute viruses of pepper include: *Cucumber mosaic virus* (CMV) (*Bromoviridae*); *Pepper mottle virus* (PepMoV), *Potato virus Y* (PVY), *Pepper severe mosaic virus*, *Tobacco etch virus* (TEV) and *Pepper yellow mosaic virus*, (*Potyviridae*); *Pepper mild mottle virus* (PMMoV), *Tobacco mosaic virus* (TMV) and *Tobacco mild green mosaic virus* (TMGMV) (*Virgaviridae*, genus *Tobamovirus*); *Pepper golden mosaic virus* and *Tomato yellow leaf curl virus*, (*Geminiviridae*); *Tomato chlorotic spot virus*, *Tomato spotted wilt virus* (TSWV) and *Groundnut ringspot virus* (*Bunyaviridae*) (Gracia *et al*, 1968; Villalon, 1975; Pernezny *et al*., 2003; Black *et al*., 1991). In Louisiana, peppers are a minor crop, but grown throughout the state. The most commonly cultivated types are bell and hot pepper (*Capsicum annuum*) and to a limited extent tabasco pepper (*C. frutescens*). Bell pepper production in Louisiana is mostly for fresh consumption and the hot type is used for sauces (Koske *et al*., 2009). Viruses affecting peppers in Louisiana include TSWV, PVY, TEV, PepMoV and PMMoV (Ariyaratne *et al*., 1996; Hobbs *et al*., 1997; Johnson *et al*., 1995; Valverde *et al*., 2000), the latter has caused significant problems in tabasco pepper production in Louisiana (R. A. Valverde, personal communication).

Among these viruses, PMMoV represents a potential threat to pepper production because of the efficient transmission properties and the long survival in the environment (Rialch *et al*., 2015). Visually, infections of PMMoV are recognized by the development of mild foliar mosaic and

sometimes causing leaf crinkling, the fruits may present symptoms such as distortion, rings and line patterns (Black *et al*., 1991).

Methods to identify and characterize plant viruses include reproduction of the disease after inoculation with the isolated virus, host range tests, symptom expression, mode of transmission, morphology of the virus particle and serological and nucleic acid tests (Hamilton *et al*., 1981). In the last two decades improved methods to identify and characterize plant viruses have been developed. Nucleic acid detection and amplification techniques like analysis of viral dsRNA, polymerase chain reaction (PCR) and its variant reverse transcription PCR (RT-PCR) are powerful nucleic acid tools that have facilitated the identification of plant viruses (Olmos *et al*., 2007). New technologies like next generation sequencing (NGS) allow sequencing billions of bases per day resulting in faster results at relatively low costs (Pop and Salzberg, 2008). The combination of these new methods with bioinformatics has revolutionized the identification and characterization of plant viruses. Bioinformatics includes, genome sequence analyses, statistics, literature analyses (Lysholm, 2012) and several other tools, many of them available online.

During the summer of 2015, while searching for pepper viruses to use in a virus-virus interaction study, samples were collected from bell pepper plants showing fruit and foliar chlorotic mottle symptoms (Fig. 3.1). The plants were growing in experimental plots at LSU Agricultural Center Botanic Gardens (Burden), Baton Rouge, LA.

3.2 Objective

The objective of this investigation was to identify and partially characterize a virus causing mottling symptoms in a bell pepper experimental field. The investigation was conducted using biological and molecular techniques.

3.3 Materials and Methods

3.3.1 Source of the Virus

Bell pepper tissue was collected from the aforementioned experimental plots. The collected tissue was used in mechanical inoculation to bell pepper cv. Marengo. Tissue infected with the unidentified virus was cut finely with a razorblade, placed in folded filter paper and then in plastic bags containing silica gel, and dried for at least 48 h at 4 °C. Dried tissue was used to inoculate bell pepper plants and to conduct other experiments.

Figure 3.1 Bell pepper plant showing fruit and foliar mottle symptoms.

3.3.2 Mechanical Inoculations and Virus Indicator Hosts

Mechanical inoculations of the virus isolate were performed to determine the host range of the virus. Plants were transplanted into 5.6-L clay pots in a soil mix that consisted of 1.5 parts of soil, 1.5 parts of sand and 3 parts of potting mix (Miracle-Gro® Lawn Products, Inc., Marysville, OH). Inoculations were performed to the following plant species: *Phaseolus vulgaris* cv. Black Turtle Soup, *Nicotiana benthamiana*, *C. annuum* cv. Yolo Wonder and *Chenopodium amaranticolor*. Mechanical inoculations were conducted on each plant species by previously dusting the leaves with carborundum. The inoculation was performed using cotton swabs with a dilution of 1:50 in phosphate buffer using sap extracts from 40-day-old virus-infected bell pepper cv. Marengo plants as inoculum. The inoculated leaves were rinsed immediately with distilled water. Purified virus was also used to perform simultaneous inoculations. Plants were kept in a greenhouse (day/night temperatures averaging 25/18 °C respectively) for further symptom evaluation.

3.3.3 DsRNA Extraction and Gel Electrophoresis

The desiccated tissue from bell pepper was ground into a powder with a mortar and pestle and 70 mg was used for dsRNA extractions. DsRNA was extracted using the method of Khankhum *et al*. (2016) briefly described in Charter II. The dsRNA samples were loaded on a 1.2 % agarose gel prepared in 1X TAE (0.04 M tris, 0.02 M sodium acetate (NaAc), 0.001 M EDTA, pH 7.8) buffer. Previously extracted dsRNAs from plants infected with *Bell pepper endornavirus*, CMV, TMV, and TMGMV plus Satellite tobacco mosaic virus and a molecular marker (1 kb DNA ladder (Bio-Rad, Hercules, CA, USA0) were included in the gels. Loads (volume) varied

depending upon the viral dsRNA. The gels were run for 2 h and results recorded with a GelDoc-It2 Imager (UVP, Upland, CA, USA).

3.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Based on the results of dsRNA analyses, a tobamovirus, likely PMMoV was suspected as the causal agent of the foliar mottle of bell pepper. Therefore virus-infected samples were tested by ELISA using PMMoV polyclonal antiserum (AC Diagnostics, Inc., Fayetteville, AR, USA) following the instructions and reagents provided by the company. Leaf tissue was collected from three bell pepper plants infected with the unidentified virus and tissue from one mock-inoculated pepper plant was used in the ELISA testing. At least 0.05 g of leaf tissue was ground in 0.5 ml of extraction buffer (egg albumin grade II (2 g), polyvinylpyrrolidone (10 g), sodium sulfite (1.3 g), sodium azide (0.2 g), tween-20 (10 g), 1X PBST (1000 ml), adjusted to pH 7.3). Purified PMMoV (provided by R. A. Valverde) at a concentration of 0.5 µg/ml was used as a positive control. Alkaline phosphatase was used as substrate for the enzymatic reaction. Wells containing samples that turned yellow were considered positive for PMMoV. Each of the tested plants was duplicated in the reaction (see Fig. 3.4). Plates were read in a microplate auto reader (Model EL311 SX, Bio-Tek^{TM} Instrument Inc.) at 405 nm.

3.3.5 Virus Purification

To increase the virus, mechanical inoculations were performed to 30-day-old bell pepper cv. Marengo plants grown in 5.6-L clay pots with the soil mixture previously described. Two weeks after inoculation, the symptomatic tissue was harvested and used for virus purification. Virus was purified using a method described for the purification of *Sun-hemp mosaic virus* (Dijkstra

and de Jager, 1998). Leaves were ground in sodium phosphate buffer (0.5 M NaH_2PO_4) using a blender. Homogenized tissue extract was clarified with 8% butanol. Four percent polyethylene glycol (PEG Mr 6000) combined with low speed centrifugation (8,000 *g*) was used to concentrate the virus.

3.3.6 Transmission Electron Microscopy

Purified virus samples were negatively stained with 2% phosphotungstic acid pH 7.0, and observed and photographed with a JEOL JSM-1400 transmission electron microscope at the LSU Socolofsky Microscopy Center.

3.3.7 Seed Transmission

Sixty seeds harvested from bell pepper plants cv. Marengo infected with the unidentified virus were dried for 2 days at room temperature and planted in 0.49-L autoclaved clay pots filled with the soil mixture described previously. A thin layer of soil was placed on the seeds to avoid disturbing them during watering. Pots were kept in the greenhouse. To determine virus seed transmission, seedlings were examined daily for virus-like symptoms.

3.3.8 Gel Purification of Viral dsRNA and RNA Sequencing

DsRNA (replicative form of the viruses) was gel purified from 1% agarose gels using QIAEX® II Gel Extraction Kit (Qiagen, Valencia, CA, USA). DsRNAs were placed in RNA stable tubes (Biomatrica, San Diego, CA, USA) and sent to the Roy J. Carver Biotechnology Center, University of Illinois, Urbana, for sequencing. Before making the cDNA library for the

sequencing, dsRNA was heat denatured for 3 min at 95 ^{0}C. After denaturing, the quality of the sample was determined by gel electrophoresis.

Sequencing was conducted by Illumina MySeq (pair-end 2 x 250). The strategies to assemble viral genomes included *de novo* assembly with Spades 3.7.1. 2, mapping and reconstruction with Bowtie2, and elongation and redundancy of contigs with the sequence assembly program CAP3 (Huang and Madan, 1999; Langmead and Salzberg, 2012; Bankevich *et al*., 2012). The numbers of sequences were variable per each dataset; the length of contigs was 6,359 nt. The genome assembly of the virus was performed by Mr. Ricardo Alcala-Briseño (PhD. Student, Department of Plant Pathology, University of Florida).

3.3.9 Sequence analyses

Conserved Protein Domains Analysis: A 6,359 nt long contig which was of a size similar to members of the genus *Tobamovirus* was assembled. To identify regions of similarity between the sequenced virus and other viruses, the Basic Local Alignment Research Tool (BLAST) version 1.2.0 available in the National Center for Biotechnology Information (NCBI) was used (Altschul *et al*., 1997). The entire length of the contig (6,359 nt) was converted into protein using BLASTx and the conserved protein domains were determined using the Conserved Domain Database (CDD) version v3.16 (Marchler *et al*., 2017), available in the NCBI.

Phylogenetic Tree and Conserved Domain Comparisons: The complete nucleotide sequences of several viral accessions (Fig. 3.7) including members of two viral families (*Virgaviridae* and *Potyviridae*) were downloaded from the Genbank. Sequence alignments, including the unidentified virus were performed using the MAFFT online tool (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al*., 2002) using the G-INS-i format. All the sequences alignments were saved as FASTA file format for analysis of gaps in AliView version 1.18 (http://www.ormbunkar.se/aliview/). A phylogenetic tree was created using the MAFFT tool for phylogenetic analysis. A BLAST search was conducted using the conserved domains found in the field viral isolate. These conserved domains were compared with other conserved domains from tobamoviruses found in the GenBank and percentage of amino acid sequence identity was determined.

3.4 Results

3.4.1 Mechanical Inoculations and Virus Indicator Hosts

Except for *P. vulgaris*, necrotic local lesions appeared on leaves of *N. benthamiana* and *C. amaranticolor* four days after inoculation with the sap extract from the unidentified virus (data not shown). Mock-inoculated plants did not show necrotic lesions (data not shown). Initial symptoms of *C. annuum* consisted of mild mottling and leaf distortion (Fig. 3.2A). Infected plants were stunted and their fruits showed color variation patterns (Fig. 3.2B).

3.4.2 DsRNA Extraction and Gel Electrophoresis

DsRNAs were successfully extracted from pepper plants infected with the unidentified virus. Figure 3.3 shows the electrophoretic banding patterns of the analyzed dsRNAs. The dsRNA

corresponding to the unidentified virus isolate was similar in size to dsRNAs of two tobamoviruses (lanes 3 and 4). Although low molecular weight dsRNA (red arrow) detected in TMGMV (lane 4) was not present in the other tobamoviruses.

Figure 3.2 Symptoms caused by the unidentified virus from bell pepper. A, Pepper plant showing mild mottling and leaf distortion (left), the plant in the right was mock-inoculated; B, Pepper fruit showing color variation patterns (left), the fruit in the right is healthy.

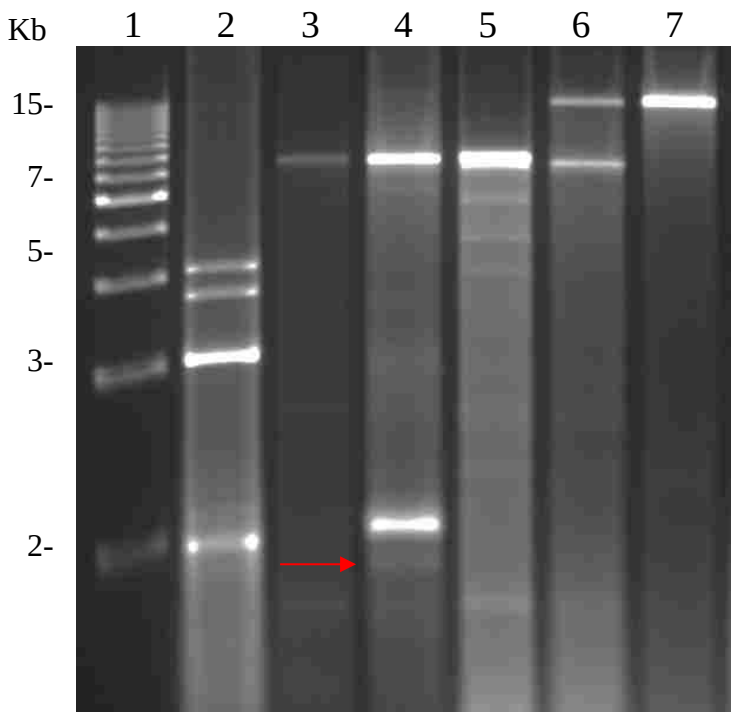

Figure 3.3 Agarose gel electrophoresis of dsRNAs. Lane 1, 1Kb ladder; lane 2, CMV; lane 3, TMV; lane 4, TMGMV; lane 5, the unidentified virus; lane 6, BPEV + PMMoV; lane 7, BPEV. The amount of dsRNA loaded varied according to the sample. Red arrow indicates a dsRNA band unique to TMGMV.

3.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA results indicated that the unidentified virus was PMMoV and it was designated PMMoV-B. As shown in Figure 3.4, peppers inoculated with the unidentified virus, tested positive for PMMoV. Plants with viral symptoms were PMMoV-positive by ELISA while those from mock-inoculated peppers gave lower readings (data no shown). The corresponding samples that tested positive for PMMoV showed symptoms after mechanical inoculations with the unidentified virus.

3.4.4 Transmission Electron Microscopy

Electron microscopy of the purified virus preparation showed the presence of rigid rod shaped virus particles of approximately 300 nm long (Fig. 3.5).

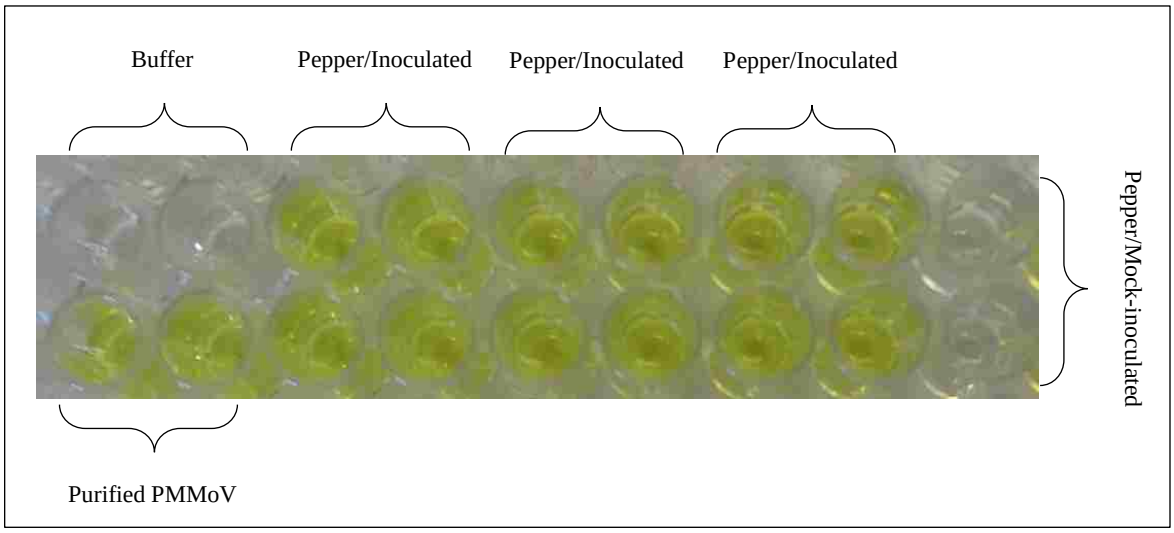

Figure 3.4 ELISA test using PMMoV polyclonal antiserum. Each sample was duplicated.

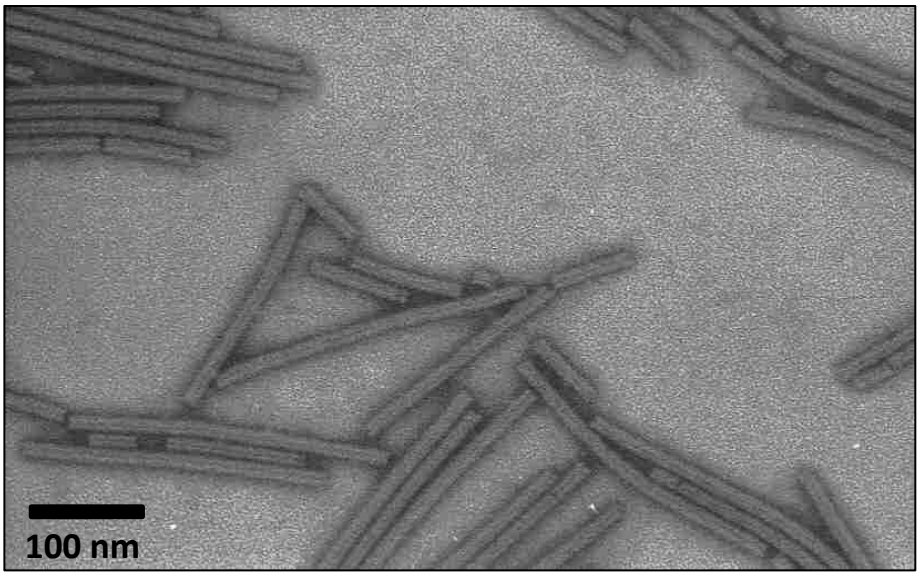

Figure 3.5 Transmission electron microscopy of PMMoV-B. Original photo was taken at a magnification of 40,000X.

3.4.5 Seed Transmission

A total of 52 of 60 seeds germinated. Five of 52 seedlings showed virus-like symptoms that consisted of mild mottling (Fig. 3.6B), representing 9.6% of virus transmission. The presence of the virus was confirmed by dsRNA analysis.

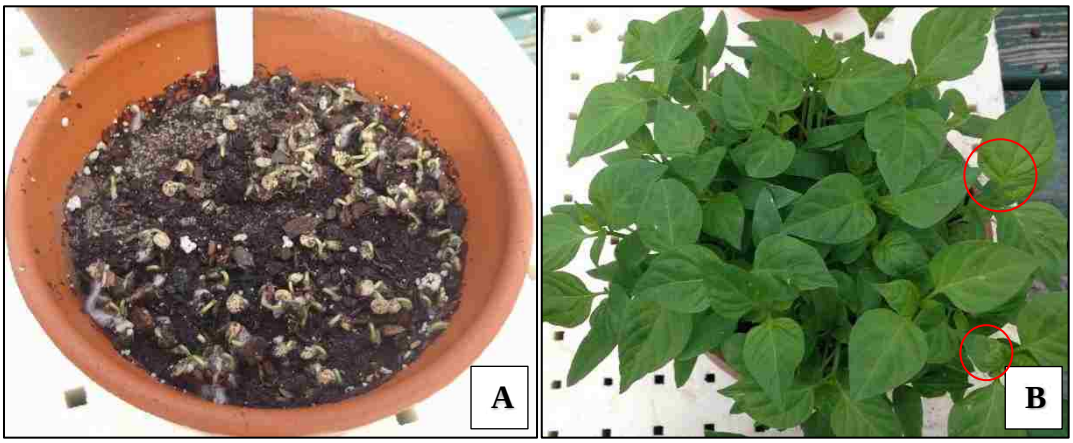

Figure 3.6 Seed transmission of PMMoV-B. A, Pepper seeds germinating 7 days after planting; B, Pepper seedlings 20 days after planting, some showing mild mottling (red circles).

3.4.6 Sequence analyses

The sequence of PMMoV-B was 6,359 nt long. The complete nucleotide sequence of PMMoV-B showed 99.7% identity to the sequence of several PMMoV isolates obtained from GenkBank (Fig. 3.8). Five conserved domains typical of tobamoviruses were found in the nucleotide sequence (Table 3.1).

Table 3.1 List of conserved domains from the nucleotide BLAST.

Protein Domain Name	GenBank Accession	Nucleotide Interval	E-Value
Virus coat protein TMV (CP)	pfam00721	5858-6124	4.54e-05
RNA dependent RNA polymerase (RdRp)	pfam00978	3588-4898	1.52e-93
Movement protein (MP)	pfam01107	4926-5453	1.90e-20
Viral methyltransferase (MTR)	pfam01660	291-1022	1.91e-10
Viral RNA helicase (Hel)	pfam01443	2565-3329	5.89e-18

Figure 3.7 shows the distribution of putative conserved domains found in the CDD. At least five domains were found by the CD-Research of the GenBank.

3.4.7 Phylogenetic Relationships and Conserved Domains Comparisons

Figure 3.8 shows the result of the phylogeny of PMMoV-B. As expected, PMMoV-B clustered with PMMoV isolates and apart from TMV (Fig. 3.8).

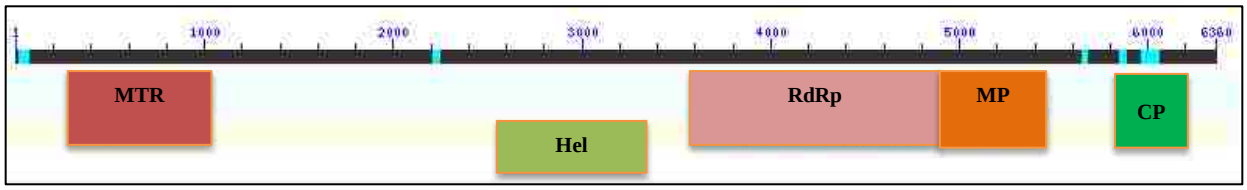

Figure 3.7 Diagram of the genome organization of PMMoV-B showing putative conserved domains. Modified from BLAST 2.6.1 (Altschul *et al*., 1997).

The BLAST search using the full sequence of PMMoV-B detected conserved domains of putative CP, RdRp, MP, MTR and Hel. The amino acid sequence of this domain was highly similar with other PMMoV isolates. The comparison of PMMoV-B with *Tropical soda apple mosaic virus* and TMV produced lower similarity values (Table 3.2).

Table 3.2 Percentage of amino acid sequence identity of PMMoV-B compared to selected tobamoviruses.

Virus Name	Accession No.	CP	RdRp	MP
Pepper mild mottle virus	KX063611.1	99.9	99.9	99.0
Pepper mild mottle virus	LC082100.1	99.9	94.0	94.0
Tropical soda apple mosaic virus	KU659022.1	80.0	80.0	78.0
Tobacco mosaic virus	KR537870.1	67.0	72.0	67.0

CP Coat protein (aa 5858-6124), RdRp RNA dependent RNA polymerase (aa 3588-4898), MP Movement protein (aa 4926-5453).

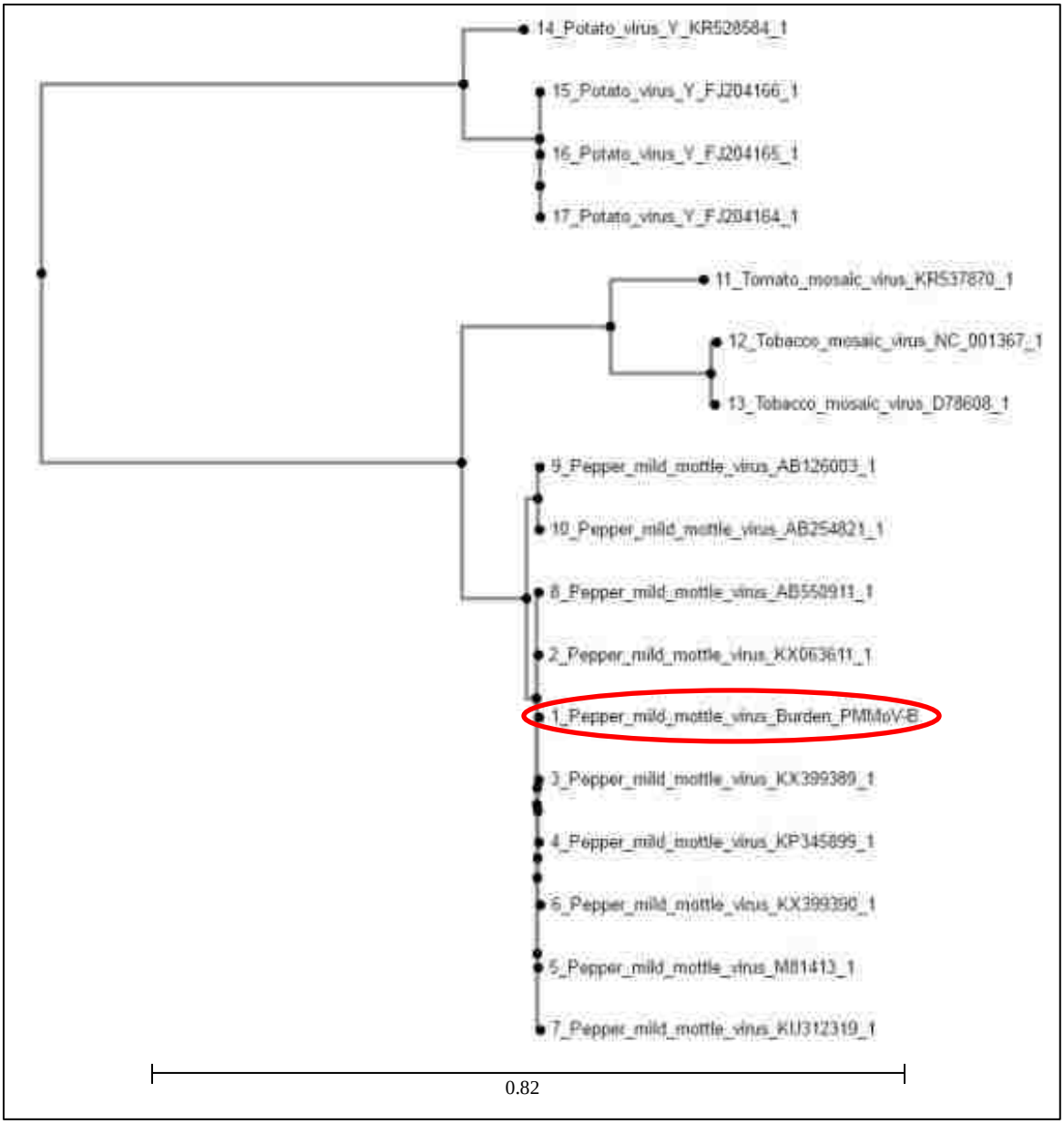

Figure 3.8 Phylogenetic tree constructed using the full nucleotide sequence of PMMoV-B (red oval) and other PMMoV, TMV and PVY isolates obtained from the GenBank. Numbers in the left indicate the order of viral genome alignment in the AliView Software. Code after the virus name is the accession number. The phylogenetic tree was created using MAFFT online tool.

3.5 Discussion

According to the results of host reaction, electrophoretic dsRNA profile, serological properties, particle size and morphology, and nucleotide sequence, the unidentified virus causing foliar and mottle disease in pepper plants was identified as PMMoV. The symptoms in pepper caused by this virus isolate were similar to those described for PMMoV in other reports (Sevik, 2011; Antignus *et al*., 2008; Rialch *et al*., 2015). The identity of the viral isolate was confirmed by ELISA. Electron microscopy revealed the presence of rigid rod-shaped particles typical of tobamoviruses. Although in this investigation, PCR using specific primers for PMMoV was not performed, in experiments conducted in chapter IV; the virus was successfully amplified by qPCR using specific primers for PMMoV viral replicase.

Tobamoviruses are carried in the surface of the seeds and in the case of pepper, the transmission rate ranges from 0-65.3% (McKinney, 1952; Demski, 1981). In this investigation the transmission rate of the virus through seed was 9.6%; which is within the transmission range for tobamoviruses. The embryo of the seed is not normally infected when seed transmission of tobamoviruses occurs (Genda *et al*., 2005); this makes the virus easy to be eliminated through seed treatments (Genda *et al*., 2011). Lack of seed treatment can result in infections in the field by tobamoviruses. In this case, plants found infected in the field could have been due to infection from contaminated seeds or from contaminated soil.

The sequence of PMMoV-B obtained by NGS of the viral dsRNA was *de novo* assembled and analyzed using bioinformatics tools. The genome was 6,359 nt in length. The sequence analysis showed that PMMoV-B had 99.7% identity with other isolates of PMMoV available in

GenBank. PMMoV-B clustered with PMMoV-pMG (KX063611.1), PMMoV strain BR-DF01 (AB550911.1), PMMoV-J47 (KX399389.1) and PMMoV-HN1 (KP345899.1). Putative conserved domains found in NCBI by the BLAST search, revealed that PMMoV-B contains similar conserved domains found in tobamoviruses. The CP gene of tobamoviruses is often used in comparison of conserved domains (Rialch *et al*., 2015). In this investigation the CP of PMMoV-B showed high similarity (99.9%) with other PMMoV isolates found in GenBank. The same similarity was observed for MP, and RdRp but only for one isolate of PMMoV. This high similarity was not observed in the comparison with other tobamovirus species like TMV and *Tropical soda apple mosaic virus*.

During *de novo* assembly and further sequence analyses, the tobamovirus TMGMV was also detected. This is not surprising because NGS is highly sensitive and minor contaminations of the sample are often obtained. Nevertheless, both PMMoV and TMGMV have been found together infecting peppers (Herrera *et al*., 2009). It is possible that these two viruses were infecting the original plants at the experimental plots. However, because TMGMV was being used in the same greenhouse used for PMMoV-B inoculations, a contamination cannot be ruled out. Testing the original field collected sample for TMGMV could determine the source.

Analyses performed by gel electrophoresis profile, revealed that the dsRNA of this isolate has similar size to other tobamoviruses such as TMV and TMGMV. A pattern of fine dsRNA bands below the position of PMMoV-B band was observed, this pattern might be associated with overload of the dsRNA sample and separation of the dsRNA during migration. Furthermore, a low molecular weight dsRNA band was detected in the TMGMV dsRNA profile and was not

present in PMMoV-B. This low molecular weight dsRNA detected in TMGMV which was previously known as TMV-U5 is diagnostic and differentiates TMGMV from the other tobamoviruses (Valverde *et al*., 1986). The absence of the low molecular weight dsRNA band in the dsRNA analysis of PMMoV-B might be an indication that the detection of TMGMV in NGS was likely due to contamination of the sample in the laboratory and not due to a mixed infection in the field.

The information generated in this investigation confirmed the identity of the pepper virus and provided a locally isolated virus to conduct studies on the interaction of PMMoV-B and *Bell pepper endornavirus* which is one of the main objectives of Chapter IV.

CHAPTER IV. MIXED INFECTIONS OF *BELL PEPPER ENDORNAVIRUS* AND *PEPPER MILD MOTTLE VIRUS* IN BELL PEPPER

4.1 Introduction

According to the symptoms caused in their hosts, viruses can be grouped as persistent or acute (Roossinck, 2010). Persistent plant viruses are those that do not cause symptoms on the host, lack cell-to-cell movement, and are transmitted only vertically, via gametes (Blanc, 2007; Roossinck, 2010). The viral families *Amalgaviridae*, *Chrysoviridae*, *Endornaviridae*, and *Partitiviridae* contain some members that are persistent plant viruses. Endornaviruses are persistent viruses, with an RNA genome found infecting plants, fungi and oomycetes. Endornaviruses have been reported in many economically important crops such as pepper (Okada *et al*., 2011) melon (Sabanadzovic *et al*., 2016), avocado (Villanueva *et al.*, 2012), barley (Candresse *et al*., 2016), common bean (Okada *et al.*, 2013) and broad bean (Pfeiffer, 1998). *Bell pepper endornavirus* (BPEV) is a persistent virus reported to infect many bell pepper cultivars grown commercially in the United States (Okada *et al*., 2011; Valverde *et al*., 1990b).

Numerous papers have been published on the interaction of acute viruses co-infecting the same plant (Chávez *et al*., 2016; Kokkinos and Clark, 2006b; Liang *et al*., 2016; Aramburo *et al*., 2015) and most of them resulted in synergistic interactions. A classic example of synergism is the result of a mixed infection between *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV). Infections of sweet potato with SPFMV alone result in a variety of relatively mild foliar symptoms that include vein mottle and ringspot. When infected with SPCSV, sweetpotato shows only mild chlorosis. However when sweetpotato is mixed infected with SPCSV and SPFMV, the symptoms consist of severe mosaic, leaf distortion and

plant stunting (Gibson *et al*., 1998; Karyeija *et al*., 2000; Kokkinos and Clark, 2006b). It has been shown that in sweet potato infected with SPFMV, infection of SPCSV increases the titers of SPFMV (Karyeija *et al*., 2000). Mukasa *et al*. (2006) showed that SPCSV also increased the titer of the ipomovirus *Sweet potato mild mottle virus*. Similarly, soybean plants coinfected with *Soybean mosaic virus* and *Bean pod mottle virus* developed more severe symptoms compared to single infection of these two viruses (Ross, 1968; Anjos and Ghabrial, 1991). In cucumber plants, double infection by *Cucumber mosaic virus* (CMV) and *Zucchini yellow mosaic virus* increased the virus titer of CMV.

The first example of a pre-existing viral strain preventing a secondary infection was demonstrated by McKinney (1926, 1929), using two strains of *Tobacco mosaic virus*. This type of interaction has been used to protect crops against severe viral strains and it is called cross protection (Hull, 2014; Folimonova, 2013; McKinney, 1926). Cross protection is also called superinfection exclusion, and it has been related to different mechanisms at various stages of the viral cycle, which include prevention of the second virus to enter the cell (Lee *et al*., 2005), competition for host factor and intracellular replication sites, translation and replication of the secondary virus (Adams and Brown, 1985; Beachy, 1999; Lee *et al*., 2005), and induction of RNA silencing by the primary strain or protector virus (Ratcliff *et al*., 1999). The best known example of the practical use of cross protection to control a plant disease is the control of citrus tristeza disease of citrus caused by *Citrus tristeza virus* (Folimonova, 2013). The mechanism of cross protection is not fully understood. However, posttranscriptional gene silencing is one the mechanisms that is thought to be involved in cross protection process (Ratcliff *et al*., 1997, 1999).

Bell pepper is affected by several acute viruses, which cause severe loses in fruit yield and quality. Viruses that infect pepper include members of the families *Potyviridae, Bromoviridae, Bunyaviridae, Geminiviridae* and *Virgaviridae. Pepper mild mottle virus* (PMMoV) is one of the most important viruses affecting bell pepper production around the world (Pernezny *et al*., 2003). The virus is seed transmitted and present wherever peppers are grown. Typical symptoms on bell pepper caused by PMMoV are mild foliar mosaic and sometimes leaf crinkling. The fruits may show symptoms that consist of distortion, mottling rings and line patterns (Black *et al*., 1991). Despite the numerous mixed infections of acute viruses in pepper, not much research has been conducted on their effect on the crop. Dufresne *et al*. (1999) reported that *C. annum* genotypes coinfected with a pepper strain of *Andean potato mottle virus* (APMoV) and either *Pepper mottle virus* or *Tobacco etch virus*, showed severe mosaic symptoms in contrast to mild mosaic when infected by APMoV alone.

Okada *et al*., (2011) reported testing seedlings of two bell pepper (*Capsicum annuum*) cultivars, Yolo Wonder and Marengo for BPEV and found that Yolo Wonder seedlings were 100 percent infected while Marengo had 136 seedlings infected and one virus-free. The BPEV-negative plant was selected and together with a BPEV-positive plant, self-pollinated to generate two Marengo bell pepper lines. In preliminary experiments, both the BPEV-positive and the BPEV-negative Marengo lines were mechanically inoculated with several acute viruses but the results did not reveal differences in their reaction to the virus (Escalante and Valverde, 2016). Similarly, in a study with endornaviruses of common bean, Khankhum (2016) did not find differences in virus

symptoms after mechanical inoculation of two common bean lines (one endornavirus-free and the other endornavirus-infected) with *Sunn hemp mosaic virus*. Other than the aforementioned investigations, there have not been reports on the interaction of endornaviruses and acute viruses or other plant pathogens.

4.2 Objective

The purpose of this investigation was to evaluate the interactions between, BPEV and PMMoV, using two bell pepper cv. Marengo near-isogenic lines (NIL); one infected with BPEV and the other BPEV-free.

4.3 Material and Methods

4.3.1 Source of Plant Materials and PMMoV

The bell pepper cv. Marengo NIL described in Chapter II was used in all experiments conducted in this investigation. Similarly, the isolate of PMMoV (PMMoV-B) partially characterized in Chapter III was used to conduct mechanical inoculations to the NILs.

4.3.2 PMMoV-B Increase

Bell pepper plants infected with PMMoV-B were kept in the greenhouse. The greenhouse day/night temperatures averaged 25/18 °C respectively. The virus was also kept in the laboratory (4 ºC) as desiccated tissue in silica gel. Mechanical inoculations of PMMoV-B were performed using 30-day-old Marengo bell pepper plants grown in a greenhouse in 5.6-L clay pots in a soil mix described in Chapter II. Symptomatic tissue was harvested two weeks after inoculation and used for virus purification as described in Chapter III. Based on preliminary PMMoV-B

infectivity assays (Ishibashi *et al*., 2009), purified virus was diluted to 50 µg/ml, using 0.05 M sodium phosphate buffer (pH 7.2), placed in 1.5-ml micro-centrifuge tubes and stored at -20 ºC.

4.3.3 Host-Virus and Virus-Virus Interactions

To evaluate interactions between, BPEV and PMMoV-B, two NILs were mechanically inoculated with purified PMMoV-B in the laboratory. Four plants of each line (three plants per line were randomly selected to be analyzed), were planted in autoclaved clay pots (0.49-L) in the soil mixture described in Chapter II. Plants were placed under artificial light (54W/120V 60Hz/4.0A Lamps) in the laboratory with an average temperature of 23 ºC and a 15 h photoperiod.

Twenty five-day-old plants were used for mechanical inoculations. Virus inoculations were conducted on four plants of each line using aliquots of virus dilutions stored at -20 ºC. Three leaves of each test plant previously dusted with carborundum were inoculated with 1.0 ml of the purified virus using cotton swabs. The inoculated leaves were rinsed immediately with distilled water. Four plants of each line were also mock inoculated using phosphate buffer. Inoculated and mock inoculated plants were kept in the dark overnight before placing them under the lights. Symptoms were recorded daily for 16 days. Similar experiments were conducted in the greenhouse using 1:50 dilutions of sap extracts from 40-day-old PMMoV-B infected plants as inoculum.

4.3.4 BPEV and PMMoV Detection

Electrophoretic analysis of viral dsRNA (replicative form of the viruses) was used to monitor virus infections. DsRNA was extracted from fresh or desiccated plant tissues using the method of Khankhum *et al*. (2016) which is described in Charter II. Plants were tested for BPEV and PMMoV-B seven days after inoculation (DAI).

4.3.5 Virus and RNA Quantification

Enzyme-Linked Immunosorbent Assay (ELISA): To determine the success of mechanical inoculations with PMMoV-B, in addition to dsRNA analyses, plants were tested by ELISA using PMMoV polyclonal antiserum (AC Diagnostics, Inc., Fayetteville, AR) following instructions and reagents provided by the company. Leaf tissue was collected and 0.05 g ground in 0.5 ml of extraction buffer (egg albumin grade II (2 g), polyvinylpyrrolidone (10 g), sodium sulfite (1.3 g), sodium azide (0.2 g), tween-20 (10 g), 1X PBST (1000 ml), adjusted to pH 7.3). Alkaline phosphatase was used as a substrate for the enzymatic reaction and the absorbance representing relative virus titer was measured using a microplate at 405 nm using an ELISA reader (Model EL311 SX, Bio-Tek^{TM} Instrument Inc., Winooski, VT, USA). The ELISA test was also used to estimate the relative titter of PMMoV-B in the inoculated plants. Approximately 1.0 g of leaf tissue from leaves located at nodes six and eight (counting from the base of the stem to the top) was harvested from inoculated plants seven and 14 days after inoculation (DAI) and 0.05 g used for ELISA testing. Four independent experiments were performed. Four biological repetitions (two technical repetitions each) were used to perform the reading. The ELISA experiments were independent from RNA quantification experiments.

BPEV and PMMoV RNA Quantification: Approximately 1.0 g of leaf tissue from leaves located at nodes six and eight (counting from the base of the stem to the top) was harvested 7 and 14 DAI. Tissue was ground into a powder with liquid nitrogen using sterile mortars and pestles. After grinding, 100 mg of powdered tissue was placed in a 2-ml microcentrifuge tube and stored at -70 ºC. Frozen tissue was used for RNA extraction using the Plant Total RNA Kit (Spectrum™, Sigma-Aldrich Co., St. Louis, MO). The RNA was treated with DNase for 30 min using the On-Column DNase I Digestion Set (Sigma-Aldrich Co.). Total RNA was eluted using 50 µl of RNase-free water (Ambion®, Life Technologies™, Carlsbad, CA). To determine the RNA concentration of the samples (ng/µl), 2 µl of total RNA were measured in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The quality and integrity of the extracted RNA was evaluated by running samples in a 1% agarose gel electrophoresis. Extracted RNA samples were stored at -70 ºC for quantitative polymerase chain reaction (qPCR) analyses.

4.3.6 Quantitative PCR (qPCR)

Primer and Probe Design: Forward and reverse primers (TaqMan® FAM) and probes (Roche Diagnostics, Penzberg, Germany) were designed using the nucleotide sequence of the viral replicase (RdRp) of BPEV and PMMoV available in GenBank. An evaluation of hairpin and self-complementation of the primers was conducted using the BLAST sequence alignment search tool, from the National Center for Biotechnology Information (NCBI). For the development of suitable probes, the Universal ProbeLibrary of Roche was used. Ubiquitin-conjugating enzyme (UBI-3) has been suggested by Wan *et. al*. (2011) as a stable gene under abiotic stress conditions of bell pepper; therefore, primers for this gene were designed (Table

4.1) to be used as a reference gene for normalization of gene expression. Specific primers for the BPEV and PMMoV RdRp were also designed using the nucleotide sequence available in GenBank (Table 4.1).

Table 4.1 Probes, forward and reverse primers designed for each of the selected gene sequence target of BPEV and PMMoV.

Virus/Target Gene	Catalog No. (Roche Probes)	Primer and Probe
BPEV/RdRp	#91 (04692080001)	F = GCACAACAGTCATTTTAACTGGA R = CCAGTCAATCTCATGGCATC Probe = GAGGAGAG
PMMoV/RdRp	#151 (04694376001)	F = ATACGCTGTCGCTTTGCAC R = AGTGCTGCCCCAAATTCAT Probe = ATTCCAGC
UBI-3	#86 (04689119001)	F = TGGAAGTATTTGCCTTGATATTCTC R = GCAGGACCTTCGATATGGTT Probe = GCAGTGGA

qPCR Reactions: To homogenize the extracted total RNA, samples were diluted to 50 ng/µl using nuclease-free water. The volume of each qPCR reaction consisted of 11 µl, distributed as follows: 2 µl of RNA template, 5 µl of iTaq universal probes reaction mix (2x), 0.25 µl of iScrip reverse transcriptase, 0.5 µl of both forward and reverse primers, 0.2 µl of fluorogenic probe and 2.55 µl of nuclease-free water. All components were added in the order provided by the manufacturer (iTaq™ Universal Probes One-Step Kit, Hercules, CA, USA). The reaction mix was place in Hard-Shell Low-Profile 96-Well Semi-Skirted PCR plates and sealed with an optically transparent film (Microseal 'B' Adhesive Seals, Bio-Rad Laboratories, Inc., Hercules, CA). Plates were gently vortex to ensure thorough mixing of the reaction components. The reaction was performed on a CFX96 Touch™ Real-Time PCR Sequence Detection System (Bio-Rad Inc., Hercules, CA, USA). Three biological repetitions (two technical repetitions each) were used per replicate. Forty cycles of the following PCR thermal cycler were conducted for each sample: reverse

transcription reaction (10 min at 50 ºC), polymerase activation and DNA denaturation (1 min at 95 ºC), amplification reactions consisted of: denaturation (2 min at 95 ºC), annealing/extension + plate read (30 sec at 54 ºC.

4.3.7 Experimental Design and Statistical Analysis

To perform the data analysis, the Cq values for each NIL were compared side by side. To determine the gene expression for each virus, the data was transformed by using the algorithm $2^{-\Delta\Delta Cq}$ described by the Real Time Guide of Bio-Rad (2006). The average of fold change titer was determined by normalization of the data to a reference gene. A completely randomized design was used for the performed experiments. The averaged data obtained from each pepper line was analyzed by One-Way ANOVA using SPSS (IBM© SPSS© Statistics Version 24) through the General Desktop Virtual Lab of Louisiana State University. Post Hoc analysis (Tukey $P \leq 0.05$) could not be performed between lines because there were fewer than three treatments. The comparisons were considered statistically significant at $P \leq 0.05$. Three biological repetitions and two technical repetitions were used.

4.4 Results

4.4.1 Virus Detection and Symptom Evaluation

Both BPEV and PMMoV-B were readily detected by electrophoretic analysis of viral dsRNA (Fig. 4.1). PMMoV-B was also consistently detected by ELISA. A representative result of the electrophoretic analyses of dsRNAs extracted from Marengo bell pepper with single (PMMoV-B) and mixed (BPEV + PMMoV-B) viral infections is shown in Figure 4.1. Judging by the intensity of the Gel-Red-stained dsRNA bands, the relative amount of dsRNA of PMMoV-B

extracted from the BPEV-negative plants is slightly greater than the one extracted from the BPEV-positive line. This was confirmed by measuring the absorbance of the bands using the GelDoc-It2 Imager (UVP, Upland, CA, USA).

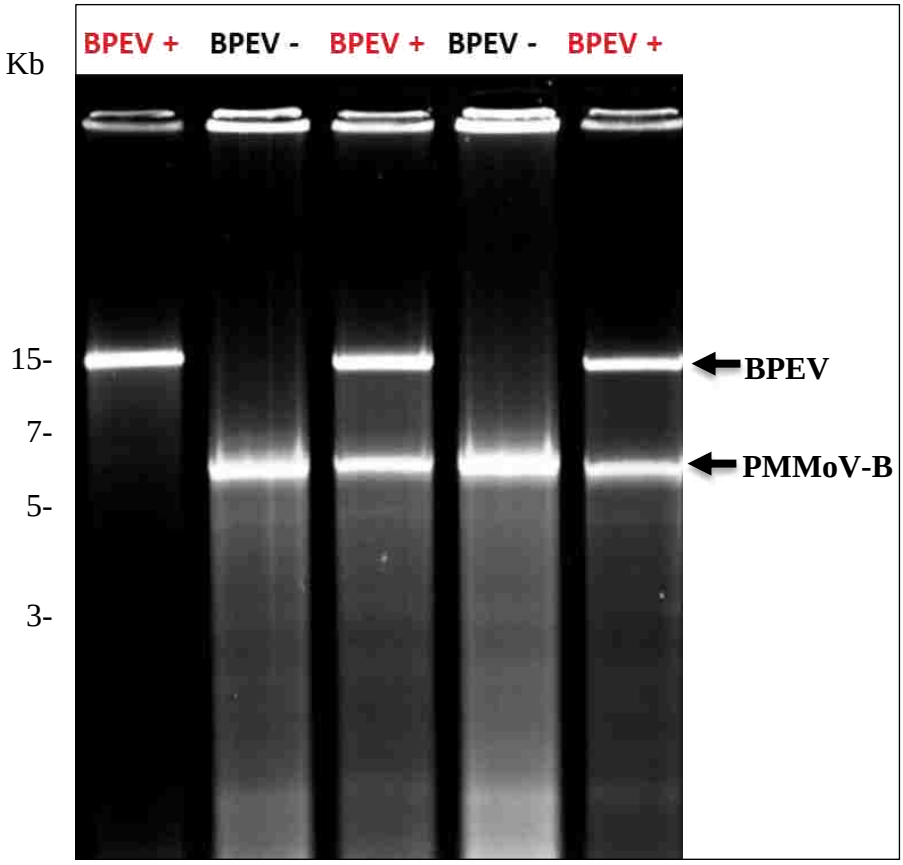

Figure 4.1 Agarose (1.2%) gel electrophoresis of dsRNA extracted from two bell pepper cv. Marengo near-isogenic lines infected with PMMoV-B. The gel was run for 2 h. at 70 V. BPEV+ = BPEV-positive; BPEV- = BPEV-negative.

Inoculated plants of both lines began to show similar mild mottle symptoms on the inoculated leaves 3 days after inoculation (DAI). At 7 DAI the BPEV-negative line showed mild mottle and systemic necrosis while the BPEV-positive line showed only mild mottle (Fig. 4.2). At 9-14 DAI

the BPEV-negative line showed mottle and necrosis on the inoculated leaves and systemic necrosis and mottle while the BPEV-positive showed mottle on the inoculated leaves. Nevertheless, necrosis was observed 13 DAI on inoculated leaves of some plants of the BPEV-positive line (Table 4.2).

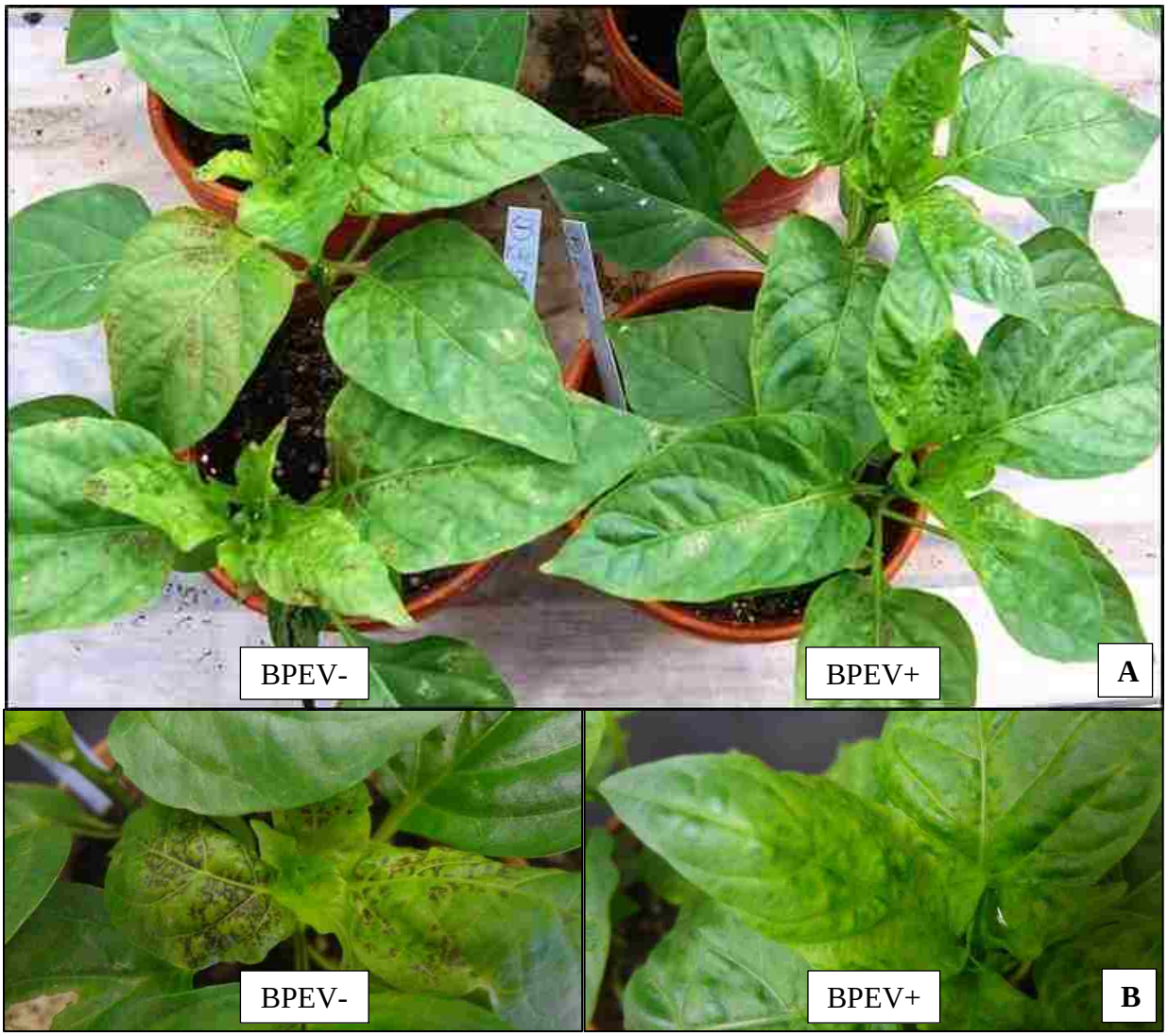

Figure 4.2 Symptoms on bell pepper caused by PMMoV-B. A, Symptoms on two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of PBEV (BPEV-), 7 DAI with PMMoV-B; B, Close up of the symptoms. Figure is representative of symptoms recorded in three independent biological experiments.

4.4.2 Relative Titer of PMMoV-B

At 7 DAI the relative amount of PMMoV-B determined by ELISA was higher in the BPEV-negative line than in plants of the BPEV-positive line; however, the differences were not statistically significant (Fig. 4.3). At 14 DAI, both lines showed similar ELISA reactions of PMMoV-B (1.55 (BPEV-negative line) and 1.57 (BPEV-positive line)) (Fig. 4.3).

Table 4.2 Description of symptoms caused by PMMoV-B in two near-isogenic lines of bell pepper cv. Marengo; one infected with BPEV (BPEV+) and the other free of BPEV (BPEV-) after mechanical inoculation. Ns = no symptoms; M = mottle; Mm = mild mottle; Sn = severe necrosis.

Days After Inoculation	Bell Pepper Near-Isogenic Line	
	BPEV-	BPEV+
01	Ns	Ns
03	Mm	Mm
05	Mm	Mm
07	Sn/M	Mm
09	Sn/M	M
11	Sn/M	M
13	Sn/M	Sn/M
14	Sn/M	Sn/M
16	Sn/M	Sn/M

4.4.3 Quantitative PCR and Gene Expression

The RdRp gene was used as target sequence for both BPEV and PMMoV-B. The Cq value of both viruses was determined. High Cq values of qPCR indicate a lower nucleic acid target (Real Time Guide of Bio-Rad, 2006). Plants of the BPEV-positive line inoculated with PMMoV-B

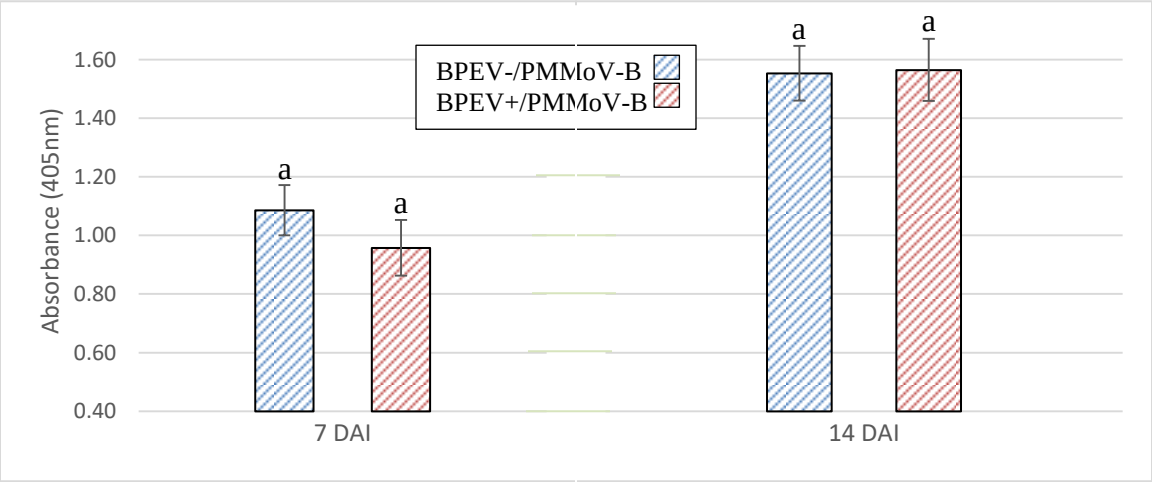

Figure 4.3 Relative quantification by ELISA of the PMMoV-B titer in two bell pepper cv. Marengo near-isogenic lines. For both BPEV-negative (BPEV-) and BPEV-positive (BPEB+) n = 16. Biological repetitions were duplicated to technical repetitions. Values with the same letters indicate no statistical difference between treatments at P≤0.05. Bars indicate the standard error. DAI = days after inoculation.

yielded lower Cq value of the target sequence (BPEV-RdRp), at 7 and 14 DAI compared to the mock inoculated plants of the BPEV-positive line (Table 4.3), indicating lower amounts of BPEV RNA in the latter.

Table 4.3 Cq values of BPEV and PMMoV-B in two bell pepper cv. Marengo near-isogenic lines. 7 and 14 DAI quantified by real-time PCR. For BPEV+/Mock *n = 4*, for the rest inoculation type *n = 6*, and each biological repetition had two technical repetitions. BPEV+ = BPEV-positive; BPEV- = BPEV-negative.

Target	Bell Pepper Line/Inoculation	Days After Inoculation	Average Cq Value of the Target
BPEV	BPEV+/PMMoV-B	7	24.24
		14	26.30
BPEV	BPEV+/Mock	7	23.41
		14	23.12
PMMoV-B	BPEV+/PMMoV-B	7	20.25
		14	12.44
PMMoV-B	BPEV-/PMMoV-B	7	16.04
		14	11.67

Table 4.4 shows the relative fold change of BPEV target at 7 and 14 DAI. BPEV-positive plants infected with PMMoV-B yielded higher Cq at 7 and 14 DAI compared to plants of the BPEV-negative, suggesting less nucleic acid of the target sequence in the BPEV-positive plants.

Table 4.4 Relative RNA titer of BPEV and PMMoV-B 7 and 14 DAI in two bell pepper cv. Marengo near-isogenic lines, quantified by real-time PCR. For both BPEV and PMMoV-B *n = 6*, and each biological repetition had two technical repetitions. Common letters indicate no statistical difference at $P \leq 0.05$. Statistical analysis was performed among the same target. BPEV+ = BPEV-positive; BPEV- = BPEV-negative.

Target	Bell pepper NIL/Inoculation	Days after inoculation (DAI)	Average relative titer of the target
BPEV	BPEV+/PMMoV-B	7	0.70±0.1[a]
BPEV	BPEV+/PMMoV-B	14	0.75±0.3[a]
PMMoV-B	BPEV+/PMMoV-B	7	3.23±2.3[a]
PMMoV-B	BPEV+/PMMoV-B	14	13.97±8.1[a]

4.5 Discussion

There has been extensive research conducted in the area of mixed viral infections in plants; however, most of this work has been conducted with acute viruses which cause a variety of plant diseases (Ross, 1968; Anjos and Ghabrial, 1991; Dufresne *et al*. in 1999; Havelda and Maule, 2000; Khankhum, 2016; Murota *et al*., 2017; Kokkinos and Clark, 2006). Nevertheless, limited research has been conducted on the interaction between persistent viruses, the host and acute viruses.

Symptoms of PMMoV-B in mechanically inoculated plants were more severe in the BPEV-negative line compared to the BPEV-positive line. Moreover, the relative virus titer of PMMoV-B measured by ELISA and qPCR was also greater in plants of the BPEV-negative line than in plants of the BPEV-positive line. This might suggest that interference or competition occurred between BPEV and PMMoV-B for host resources used for replication. In this investigation it

was found that plants with mixed infection of BPEV and PMMoV-B yielded greater PMMoV-B Cq values than those infected with PMMoV-B alone, although these differences were not statistically significant. Both BPEV and PMMoV-B showed an increase of virus titer at 14 DAI compared to 7 DAI.

In plant virus cross protection, tolerance to a virus is conferred to a plant by previous inoculation of the plant with a mild strain of the same virus, (Neofytou, 2016; Folimonova, 2013). However, this partial activation of the plant immune system has been conducted with success only with closely related viruses. BPEV is ubiquitously present in the host since the time of seed formation and one will expect that the plant will show tolerance or even immunity to related endornaviruses such as Hot pepper endornavirus (Lim *et al*., 2015) and other recently discovered endornaviruses of pepper (R.A. Valverde, personal communication). It is possible that the presence of BPEV could cause partial activation of the plant immune system, and when infection by another virus occurs, symptoms are ameliorated. This may be associated with the activation of gene silencing through the production of small RNAs which play an important role in plant defense against viral diseases (Huang *et al*., 2016; Gouveia *et al*., 2017; Neofytou, 2016). The only other report of endornavirus-host interaction is the production of small RNAs in bell pepper infected with BPEV (Sela *et al*., 2012). This indicates the activation of host gene silencing and supports the hypothesis that endornaviruses have an active role in the infected plant.

At 13 DAI, some BPEV-positive plants inoculated with PMMoV-B showed symptoms similar to those observed in the BPEV-negative plants at 7 DAI. This reaction may be related to the amount of PMMoV-B accumulated, which may have reached a point in which the plant cannot

interfere with virus replication. In this investigation, relatively large amounts of PMMoV-B inoculum were used compared to the amount of inoculum that naturally infects a pepper plant. Pepper plants become naturally infected by PMMoV mainly by mechanical plant contact with infested materials that include tools, equipment, seed coat or other plants.

The lack of a synergetic interaction between BPEV and PMMoV-B could be due to the suppression of protein expression such as the coat protein which has been associated with virus symptoms in TMV (Dawson *et al*., 1988). It is not known if the BPEV-negative line is more susceptible to symptoms caused by PMMoV-B due to the lack of this endornavirus. It is possible that the absence of BPEV might allow PMMoV-B to suppress the mechanisms of plant defense by suppressing RNA silencing as suggested in other studies (Kreuze *et al*., 2005; Ahlquist, 2002; Mukasa *et al*., 2006). It has been shown that co-infection of two acute viruses resulted in synergistic interactions (Mukasa *et al*., 2006; Karyeija *et al*., 2000; Kokkinos and Clark, 2006b; Univeros *et al*., 2007; Vance, 1991; Valverde *et al*., 2007). In these reports, one of the co-infecting viruses served as enhancer, allowing enhanced accumulation of the other virus in the host. The synergistic interactions of these viruses have been associated with the suppression of the host defense mechanisms by viral protein associated with RNA-silencing suppression (Kreuze *et al*., 2005). In this investigation, co-infection of BPEV and PMMoV-B appears to result in an antagonistic interaction; however, it is not known if the interaction is a response to a pre-activation of the plant defense by BPEV or a competition of both viruses for the host resources for replication.

It is possible that BPEV helps the host to express proteins like catalases which are known to be involved in the decomposition of reactive oxygen species (ROS) (Murota *et. al*., 2017). If this is the case, BPEV might not necessarily suppress the replication of PMMoV-B, but help the plant to have less accumulation of ROS, rendering less severe symptoms. It is well known that phytohormones like jasmonic acid (JA), salicylic acid and ethylene play an important role in plant immunity (Alazem and Lin, 2015). However, there are viruses like *Rice ragged stunt virus* that can suppress JA-mediated defense in order to facilitate virus infection (Zhang *et al*., 2016). BPEV might have an effect on PMMoV-B whereby the acute virus is partially disabled of its abilities to inhibit metabolic pathways of phytohormones like JA; hence, that replication and virus symptoms expression is reduced.

BPEV might not only play a role in activating the defense mechanisms to biotic agents, but also might have an adaptive effect on the host by reducing stress caused by abiotic factors. For example, *Arabidopsis halleri* contains one persistent virus, *Arabidopsis halleri partitivirus 1*, (Kamitani *et al*., 2016), and this plant often inhabits soils contaminated by heavy metals (Kubota and Takenaka, 2003). The ability of the plant to survive these conditions might be conferred by the persistent virus, as it has been demonstrated that under particular conditions of abiotic stress, viruses may have beneficial effects on their hosts (Xu *et al.,* 2008). They showed that infections of *Cucumber mosaic virus* (CMV) improve drought tolerance in several plant species and also enhanced freezing tolerance of beets. In the same study, *Nicotiana benthamiana* plants inoculated with CMV, TMV, or *Brome mosaic virus* were significantly more resistant to drought

stress than non-inoculated plants. Although having two NILs is helpful to study the role endornaviruses plant in the host reaction to biotic and abiotic agents. The development of an inoculation method for persistent viruses is necessary to confirm the interactions and effects of these viruses on the host.

The results of this investigation suggest that the continuous infection of BPEV may have triggered the plant immune response and therefore, it is active when the plant is infected by PMMoV-B. Further experiments that involve other endornavirus-infected plants and other acute viruses need to be conducted to have a better understanding of the acute-endornavirus virus interactions.

CONCLUSIONS

The aim of this study was to obtain information on the interactions of *Bell pepper endornavirus* (BPEV) with bell pepper and *Pepper mild mottle virus* (PMMoV). This was accomplished with a comparative study using two near-isogenic lines (NIL) of bell pepper cv. Marengo, one infected with BPEV and the other free of BPEV. By using these NILs, the variation of the parameters evaluated in this study were likely due to the presence/absence of BPEV and not to genetic variability between the lines.

The results on the evaluation of the two NILs showed that there were not statistically significant differences in the overall phenotypic characteristics between the two bell pepper lines. This was confirmed with data of the vegetative plant growth which included visual observations throughout the different phenological stages of the plants, fruit shape, plant height, stem thickness and percentage of the dry matter. The BPEV-negative line showed greater percent seed germination and radicle length.

Furthermore, fruit from the two lines did not differ in size. However, the total fruit weight of the BPEV-negative line was significantly greater than the BPEV-positive line. This suggests that infections of bell pepper with BPEV have a negative effect on fruit production. Such studies should also be conducted under commercial pepper production conditions to confirm this negative effect.

A field virus isolate from bell pepper was successfully identified and partially characterized. Biological and molecular techniques were used to determine the identity of the virus. The virus was identified as an isolate of PMMoV and was designated PMMoV-B. The identification of the virus was aided by Next Generation Sequencing (NGS) using viral dsRNA. Bioinformatics tools available in the web from the National Center for Biotechnology Information (NCBI) were used to analyze sequence data and to compare PMMoV-B with related viruses.

The isolation and identification of PMMoV-B enable a two-way interaction study; a persistent virus (BPEV) and an acute virus (PMMoV-B). In the BPEV-PMMoV interactions, the BPEV-positive line exhibited less severe PMMoV-B induced symptoms, initial lower virus titer and less viral RNA accumulation than the BPEV-negative line. Although the data analyses did not result in statistical differences, the negative effect of BPEV on PMMoV-B was consistent in the various tests, suggesting that BPEV has an antagonistic effect on PMMoV-B.

The hypothesis of this investigation was that BPEV is in a mutualistic interaction with the host bell pepper. However, the results of the study do not completely support the hypothesis. Nevertheless, the antagonistic effect of BPEV on PMMoV-B by reducing the early symptom expression suggests a positive effect of BPEV on the host.

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APPENDIX

Appendix 1 Seed source, fruit color, and BPEV infection status of commercial bell pepper cultivars tested by dsRNA analysis and agarose gel electrophoresis (1.2%) for the presence of endornavirus-like.

Cultivar	Fruit Color	Seed Source	BPEV +/-
Bianca F1	Red	BGHS[1]	+[2]
Bullnose	Red	JS[3]	+
Canary Bell	Yellow	JS	+
Cupid F1 OG	Red	BGHS	+
Emerald Giant	Red	JS	+
Eros F1 OG	Yellow	JS	+
Etuida	Orange	JS	+
Flavorburst F1	Yellow	BGHS	+
Georgescu Chocolate	Chocolate-brown	JS	+
Gourmet F1	Orange	BGHS	+
Horizon	Orange	JS	+
Intruder F1	Red	BGHS	+
Islander F1	Red	BGHS	+
King of the North	Red	JS	+
Lilac Bell	Yellow/Purple	JS	+
Marengo BPEV-negative NIL	Yellow	RV[4]	-[5]
Marengo BPEV-positive NIL	Yellow	RV	+
Marta Polka	Yellow	BGHS	+
Midnight Dreams	Dark purple	JS	+
Olympus F1 OG	Red	BGHS	+
Orange Bell	Orange	JS	+
Ozark Giant	Red	JS	+
Purple Beauty	Dark purple	JS	+
Quadrado D'Asti Giallo	Yellow	JS	+
Snapper F1	Red	BGHS	+
Sprinter F1	Red	BGHS	+
Sweet Sunrise F1 OG	Yellow	BGHS	+
White Cloud	Light yellow	JS	+
X3R® Red Knight F1	Red	BGHS	+
Yankee Bell OG	Red	BGHS	+
Yellow Monster	Yellow	JS	+

[1] Baker Greek Heirloom Seed Co. Mansfield, MO, USA
[2] Positive for endornavirus-like dsRNA
[3] Johnny's Seeds Co. Winslow, ME, USA
[4] R. A. Valverde, Louisiana State University Agricultural Center
[5] Negative for endornavirus-like dsRNA

VITA

César Escalante Guardado was born in Lempira, Honduras. He went to an agricultural high school named "Instituto Técnico Industrial Froylan Turcios" from 2007 to 2009. He started his bachelor's degree in Agronomy at the National University of Agriculture (UNA) in Catacamas, Olancho, Honduras in January 2010. During his time at UNA, he was elected General Secretary of the UNA Student Association (AEUNA) and together with the AEUNA members he participated in the creation of the new Student Government Constitution. César did his undergraduate research project at "Universidad Autónoma del Estado de México (UAEMex)," Toluca, México in 2013. His research focused on the application of secondary metabolites that inhibit the activation of the enzyme pectin-methylesterase in ornamental crops. At UAEMex, he also conducted experiments using silicon to evaluate the effect of this element on the post-harvest life of *Lilium* spp. After that he went back to Honduras and graduated with his B.S. in Agronomy in 2013. In 2014, he came to LSU and worked with Dr. Daniel Fromme at the Dean Lee Research & Extension Center in Alexandria, LA. Then he joined Dr. Rodrigo A. Valverde's lab as an intern. During this time he learned many laboratory techniques. He became a graduate student under the mentoring of Dr. Valverde in 2015 and started working in a research project to determine the effects of *Bell pepper endornavirus* (BPEV) on two near-isogenic lines of bell pepper cv. Marengo, as well as the interaction of BPEV with *Pepper mild mottle virus*. He anticipates graduating with his master's degree in May 2017.