2016

Persistent RNA Viruses of Common Bean (Phaseolus vulgaris): Distribution and Interaction with the Host and Acute Plant Viruses

Surasak Khankhum
Louisiana State University and Agricultural and Mechanical College, surakhankhum@yahoo.com

PERSISTENT RNA VIRUSES OF COMMON BEAN (*PHASEOLUS VULGARIS*): DISTRIBUTION AND INTERACTION WITH THE HOST AND ACUTE PLANT VIRUSES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by
Surasak Khankhum
B.S., Mahasarakham University, Thailand, 2001
M.S., Kasetsart University, Thailand, 2007
May 2016

This dissertation is dedicated to my parents, Sou and Nimnual Khankhum, for their forever love and efforts to provide me the best education, as well as, to my sisters, Kochamol Kruawan, Sukumarl Khankhum, and Yupadee Kaewviset, for their support, encouragement, and motivation to live in another part of the world. I also dedicate this work to my extended family for their unwavering support to get through the tough times.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my major professor Dr. Rodrigo A. Valverde for his patient guidance, understanding, advice, assistance, and encouragement throughout my doctoral study. I am also thankful to my graduate committee members Dr. Charles Overstreet, Dr. Jeffrey W. Hoy, and Dr. Jong H. Ham for their advice and helpful suggestions. A special thanks to Dr. Gregg Henderson for being my graduate school representative.

I wish to express my appreciation to the faculty, the staff, and the graduate students of the department of Plant Pathology and Crop Physiology at LSU for their friendship, support, and motivation during my graduate studies.

I would like to thank The Ministry of Science and Technology of Thailand, Royal Thai Government, and Mahasarakham University for their financial support that allowed the opportunity to pursue the study in a doctoral degree in the U.S.A. I also would like to thank the Office of Educational Affairs (OEA) staff at the Royal Thai Embassy in Washington DC for their help and care throughout my study. The partial support for the research from the USDA National Institute of Food and Agriculture (NIFA) and the US-Israel Binational Agricultural Research and Development Fund (BARD) is appreciated**.**

Finally, I wish to express my appreciation to my former advisor Dr. Pissawan Chiemsombat for introducing me to plant virology. I am also thankful to all of my former teachers for their affection, patience, support, and encouragement. Your efforts are greatly appreciated.

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ABSTRACT

Common bean (*Phaseolus vulgaris*) is the most important legume for direct human consumption. Common bean originated and was domesticated in the Americas but now is grown worldwide. As in the case of other crops, common bean can be infected with acute and persistent plant viruses.

A modified dsRNA extraction method was developed and used in this study. The method was fast, economic, versatile, and required relatively small amounts of desiccated plant tissue. The method was successfully used to extract dsRNAs from plants infected with RNA plant viruses and to investigate the occurrence of two endornaviruses, *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2), in breeding lines, cultivars, landraces, and wild genotypes of common bean from the two centers of common bean domestication: Mesoamerica and the Andes. The two endornaviruses were detected in many common bean genotypes of Mesoamerican origin but rarely in genotypes of Andean origin.

A comparative study of morphological and physiological characteristics between two common bean lines of the cultivar Black Turtle Soup (BTS); one infected with PvEV1 and PvEV2 (BTS+) and the other endornavirus-free (BTS-) was conducted. Morphological differences between the two lines were not observed. However, the study revealed that common bean endornaviruses may promote seed germination, pod length, and carotenoid content. Nevertheless, endornaviruses were associated with lower chlorophyll content. When interactions studies were conducted between PvEV1 and PvEV2 and three acute viruses, synergistic effects were obtained. Quantitative RT-PCR results supported a synergism between PvEV1 and *Sunn-hemp mosaic virus*.

More research should be conducted to determine the type of symbiotic interaction that exists between common bean and endornaviruses.

CHAPTER 1.
LITERATURE REVIEW

1.1 Common Bean

Common bean (*Phaseolus vulgaris*) is a legume in the family Fabaceae. It is a self-pollinated plant with an outcrossing rate of less than 1%. The common bean genome (587 Mb) is comprised of 11 chromosomes. The genome is made of about 27,000 genes with a high proportion of transposon insertions (Schmutz et al., 2014). Common bean is the main grain legume for direct human consumption. It represents a rich source of protein, vitamins, minerals, and fiber, especially for poor populations of Africa and Latin America (Broughton et al., 2003). Common bean originated and was domesticated in the new world and is now grown worldwide. Based on the edible parts, common bean is divided in two groups, dry beans and snap beans (e.g., green, string, or French beans) (Schwartz et al., 2005). Based on the type of plant growth, common bean can be separated in determinate and indeterminate types (Kelly, 2010).

The domestication of common bean took place in two geographical locations, Mesoamerica and the Andes (Singh et al., 1991). These two divergent gene pools also had several local domestication events (secondary centers of domestication). Based on DNA analysis, there appears to have been limited domestication events in the Andean gene pool resulting in less genetic diversity. In contrast, multiple domestication events are recorded in the Mesoamerican gene pool. These multiple domestication events resulted in a greater genetic diversity in this pool and suggest that Mesoamerica is likely the origin of common bean (Bitocchi et al., 2012; Kwak and Gepts, 2009). The major classes of common bean are primarily recognized by seed morphology which includes navy, small white, small red, pink, red kidney, great northern, pinto, black, cranberry, white kidney, flat small white, soldier, snap, and yellow

eye (Bliss, 1980; Kelly, 2010). These commercially available market classes of common bean are grouped in either the Andean or the Mesoamerican gene pool.

Biochemical, morphological, and stress resistant characters of common bean are usually regarded as the main selection parameters in a breeding program. Common bean breeders are also interested in improving specific characteristics of a common bean variety, quality, and yield. Nutritional quality components have been used as important criteria in common bean breeding. Polyphenols in bean including tannin, anthocyanins, and flavonoids are critical in developing practical strategies to improve common bean quality. Polyphenols, such as anthocyanins, exhibit strong antioxidant activity, while other polyphenol types exhibit antimutagenic and antigenotoxic activities (Azevedo et al., 2003; Wong et al., 2003). Common bean genotypes with high content of polyphenols can increase market opportunities for bean production in the functional food and nutraceutical industry (Akond et al., 2011). Breeding for better morphological characters, including increasing seed and leaf sizes and changing growth habit, is also targeted in common bean breeding (Bitocchi et al., 2013; Gaut, 2014; Larson et al., 2014). Breeding common bean for resistance to abiotic and biotic stresses is another important aspect of bean improvement.

1.2 Common Bean Pathogens

The common bean growing system is under the influence of climatic, edaphic, biotic and abiotic parameters (Broughton et al., 2003). In spite of the advances in plant disease control, common bean diseases continue to be an important limiting factor to production. According to the report of Schwartz et al. (2005) on common bean diseases, fungi are the major disease causing agents followed by viruses and bacteria. The major diseases of common bean include rust, anthracnose, mosaic virus, white mold, root rot, and bacterial blight (Kelly, 2010; Schwartz et al., 2005). Among fungal pathogens that infect common bean, *Phaeoisariopsis griseola*

causing angular leaf spot and rust *Uromyces appendiculatus* are the most disease causing agents reported in the Americas. Other fungal pathogens causing problems in common bean production include *Thanatephorus cucumeris* causing web blight, *Sclerotinia sclerotiorum* causing white mold, *Phoma exigua* var. *diversispora* causing Ascochyta blight, *Fusarium solani* f. sp. *phaseoli* casing wilt and yellows, *Macrophomina phaseolina* casing stem blight, *Sclerotium rolfsii* casing southern blight and *F. solani* f. sp. *phaseoli* , *Rhizoctonia solani* , and *Aphanomyces euteiches* casing root rot (Schwartz et al., 2005; Singh, 1992; Singh and Schwartz, 2010). Among bacterial pathogens infecting common bean, *Xanthomonas campestris* pv. *phaseoli* causing common bacterial blight is the major disease of common bean growing. In the USA, *Pseudomonas syringae* pv. *phaseolicola* causing halo blight, *P. syringae*, and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* are reported to cause yield losses (Schwartz et al., 2005; Singh, 1992; Singh and Schwartz, 2010). New information on genetic diversity and the use of a broad-based parental germplasm will improve disease resistance and agronomic characteristics (Schwartz et al., 2005).

1.3 Common Bean Viruses

Based on host reaction, plant viruses can be divided into acute and persistent (Roossinck, 2010). Acute viruses are the most studied and cause a variety effects on the phenotype and physiology of the host. In contrast, persistent viruses have been reported with less frequency and in general, have not been shown to have detectable effects on the host.

Common bean viruses have been reported to cause significant problem for bean production (Schwartz et al., 2005). The seed transmitted and aphid-vectored potyviruses *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are the most prevalent viruses (Morales and Bos, 1988). BCMV-infected common bean usually expresses

mosaic, necrosis, and leaf malformation symptoms depending on viral strains. Differences in degree of severity depend on the bean genotype (Drijfhout et al., 1978; Morales, 2006; Morales and Bos, 1988). Both BCMV and BCMNV occur worldwide and in some areas can limit production (Morales, 2006). *Bean yellow mosaic virus* (BYMV) is another potyvirus causing problems in common growing areas around the world. BYMV can cause various degrees of disease in common bean genotypes. Symptoms in infected common bean can consist of mosaic, epinasty, dwarfing, vein and top necrosis, plant malformation, and plant death depending on viral strains and common bean cultivars (Morales, 2006; Schwartz et al., 2005). Other economically important common bean viruses include *Bean golden mosaic virus* (BGMV) and *Bean golden yellow mosaic* (BGYMV) which are whitefly (*Bemisia tabaci*) transmitted begomoviruses in the family *Geminiviridae* (Blair et al., 1995, Morales and Anderson, 2001). BGMV-infected common bean shows systemic foliar mosaic, yellowing, and flower abortion (Morales, 2006; Schwartz et al., 2005). Pod malformation caused by BGYMV can result in a lower number of pods per plant and number of seeds per pod (Román et al., 2004). High levels of resistance in common bean cultivars to BGYMV were reported in red-seeded common bean genotypes (Román et al., 2004; Velez et al., 1998). The cucumovirus *Cucumber mosaic virus* (CMV) infects many plant species, including common bean (Morales, 2006; Schwartz et al., 2005). Foliar symptoms caused by CMV infection include mosaic, curling, chlorotic mottle, and dark green vein-banding, while pods are mostly curled, mottled, and reduced in size (Davis and Hampton, 1986). Some strains of CMV have been shown to be seed-borne in common bean (Hampton and Francki, 1992).

1.4 Endornaviruses

Five viral families contain RNA viruses that infect plants and fungi. They include *Partitiviridae, Chrysoviridae, Amalgaviridae, Reoviridiidae,* and *Endornaviridae* (Fukuhara and Gibbs, 2012). In the case of fungal viruses, it has been well documented that they often affect the fungi-plant interactions by altering the physiology of the fungus. This has been studied in detail for viruses of plant pathogenic fungi (Ghabrial and Suzuki, 2009). With the exception of a few acute reoviruses, all other members of these five families known to infect plants are persistent viruses. This group of viruses has not been shown to have detectable effects on the host plants. There is very little information on persistent plant viruses, primarily due to the apparent lack of effect on the host phenotype.

Endornaviruses are persistent RNA viruses with a genome that ranges from 9.8 to 17.6 kb in length; infect plants, fungi, and oomycetes; lack cell-to-cell movement; are present in every cell; are transmitted only via gametes; and do not cause apparent symptoms (Fukuhara and Moriyama, 2008). They infect economically important crops, such as avocado, barley, broad bean, common bean, cucurbits, pepper, some plant pathogenic fungi, and the oomycete *Phytophthora* (Fukuhara and Moriyama, 2008; Hacker et al., 2005; Okada et al., 2011; Villanueva et al., 2012). Currently, all described endornaviruses are included in a single genus, the *Endornavirus,* and a single family the *Endornaviridae* (Fukuhara and Gibbs, 2012).

With the exception of *Vicia faba endornavirus* (VfEV), which is associated with male sterility, endornaviruses do not appear to affect the phenotype of the host and are generally found at constant concentrations per cell in every tissue and at every developmental stage (Moriyama et al., 1999; Valverde et al., 1990b). Although VfEV dsRNA has been associated with membranous vesicles in the cytoplasm, endornaviruses are not associated with virus-like particles.

Endornaviruses encode a single polypeptide which is presumed to be processed by virus-encoded proteases. Based on conserved domain database comparisons, the genome of all completely sequenced endornaviruses contains conserved motifs of an RNA-dependent RNA polymerase (RdRp) similar to the alpha-like virus superfamily of positive-stranded RNA viruses (Roossinck et al., 2011a, 2011b).

In plants, endornaviruses are transmitted exclusively vertically with high efficiency to the progeny (Moriyama et al., 1996; Valverde and Gutierrez, 2007). In the case of BPEV infecting bell pepper, transmission through pollen ranged between 35% and 60%, whereas maternal transmission ranged between 70% and 90%, while in self-pollinated plants, the transmission rate is often near 100% (Valverde and Gutierrez, 2007). Although maternal transmission likely occurs from a virus-infected egg cell, the mechanism for pollen transmission has not been elucidated. Transmission through pollen probably occurs during the double fertilization, more specifically, during the formation of the zygote. Before the formation of the zygote, the virus may be carried into the egg cell by one of the pollen sperm cells. As mentioned earlier, the transmission rate of an infected/self-pollinated plant is not always 100%, and virus-free lines of the same plant cultivar have been obtained (Okada et al., 2011, 2013). This suggests that the virus is not always able to move into the egg cell or the pollen.

1.5 Endornaviruses of Common Bean

In common bean, two endornaviruses, *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2), have been identified in the bean cultivar Black Turtle Soup (BTS) (Okada et al., 2013) and in many other common bean cultivars (Khankhum et al., 2015). Their molecular characterization, including genome organization and the full sequence has been completed (Okada et al., 2013). The genome organization of these two viruses

is shown in Figure 1.1. Phylogenetic analyses of the RNA-dependent RNA polymerase (RdRp) gene indicate that PvEV1 is closely related to Bell pepper endornavirus whereas PvEV2 is closely related to *Persea Americana endornavirus* (PaEV) (Okada et al., 2013).

As in the case of endornaviruses of bell pepper and melon, endornaviruses of common bean have been reported to occur with high frequency (Okada et al., 2013, 2011; Sabanadzovic and Valverde, 2011; Segundo et al., 2008). In a virus incidence study conducted in Spain, all 664 plants from commercial greenhouse farms tested positive for endornaviruses (Segundo et al., 2008).

These two endornaviruses are often present in common bean germplasm of Mesoamerican origin but not in germplasm of Andean origin (Khankhum et al., 2015). The role of these endornaviruses in the plant and their interaction with pathogens or other biotic or abiotic agents is not known. Because it is possible that common bean endornaviruses were "introduced" in this crop before or during domestication, their use as molecular markers to determine the origin of common bean genotypes could be useful. These viruses may also play a role in the diversity of the Mesoamerican germplasm.

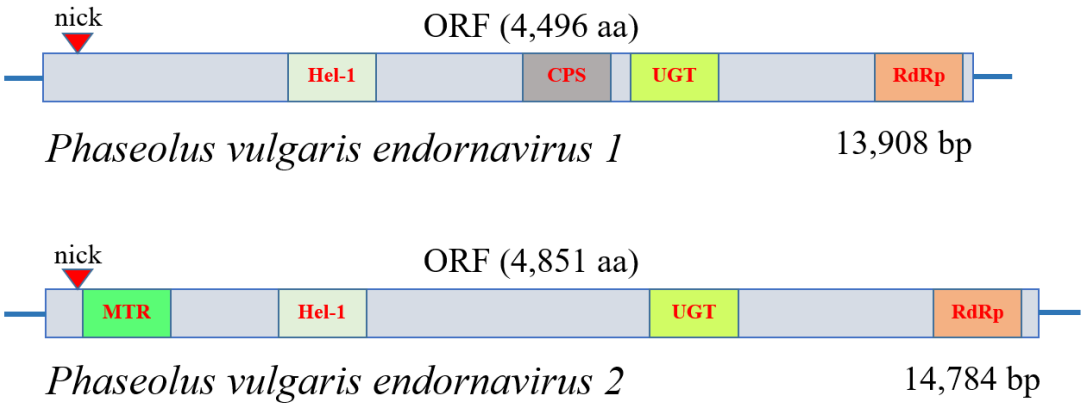

Figure 1.1. Genome organization of PvEV1 and PvEV2 (modified from Okada et al., 2013).

1.6 Detection of Plant Endornaviruses

Detection and transmission of plant viruses can be accomplished by several methods, such as host range, vectors, light and electron microscopes, serology, molecular hybridization, PCR-based techniques, and electrophoresis of viral dsRNA. Horizontal viral transmission techniques, including grafting, mechanical inoculation, dodder, and vectors, are generally used to evaluate transmissibility of plant viruses. However, these techniques cannot be used to transmit endornaviruses. Vertical transmission through gametes is the only known transmission of endornaviruses (Fukuhara and Moriyama, 2008; Okada et al., 2011; Valverde and Gutierrez, 2007).

Many dsRNA extraction and electrophoretic analyses methods have been reported as a tool to detect RNA virus infection (Akin et al., 1998; Balijja et al., 2008; Castillo et al., 2011; DePaulo and Powell, 1995; Franklin, 1966; Morris and Dodds, 1979, Morris et al., 1983; Okada et al., 2015; Rao et al., 2008; Tzanetakis and Martin, 2008; Valverde *et al*., 1990a). This technique is simple and relatively inexpensive and can overcome the problem of instability of viral ssRNA (Valverde et al., 1990a; 1990b). This technique is non-specific and can detect mixed viral infections (Rao et al., 2008; Valverde et al., 1990b). One of the limitations of most published dsRNA methods is the need for a relatively large amount of tissue. This can present a problem when only limited amounts are available. The amounts of reagents required by most dsRNA methods can be a limiting factor as well because some reagents can be expensive, and waste disposal can be a problem when large volumes of them are used. Furthermore, most methods use liquid nitrogen to grind tissues which is not readily available in many laboratories. Therefore, an extraction method that is rapid, economic, versatile, and requires small amounts of desiccated tissue is needed.

Another virus detection method is the enzyme-linked immunosorbent assay (ELISA). Relative virus titer in infected plants can be evaluated by using ELISA. This technique is based on the measurement of antigen-antibody interaction. Specific antibodies against the virus can be produced in laboratory animals, such as rats and mice. The antigen-antibody interaction can be detected using an enzyme label antibody yielding a colored product that can be easily visualized or be read in a microplate reader. Endornaviruses do not have coat protein and therefore serological detection is not an option. Monoclonal antibodies have been used successfully against purified cytoplasmic vesicles from plants infected with the endornavirus *Vicia fava endornavirus* (VfEV). However, it is possible that those antibodies possibly recognized a glycoprotein of the vesicles and were not specific for the endornavirus detection (Fukuhara and Gibbs, 2012).

Various molecular techniques have been used to detect and identify plant viruses. PCR-based techniques, such as reverse transcriptase PCR (RT-PCR), can be used to amplify a specific region of the viral RNA genome. The amplified PCR product can be subsequently cloned and sequenced. RT-PCR has been used to detect double infections of PvEV1 and PvEV2 in common bean (Khankhum et al., 2015; Okada et al., 2013). RT-PCR was used successfully used to obtain the complete nucleotide sequence of the first endornavirus in rice, *Oryza sativa endornavirus* (OsEV) (Moriyama et al., 1995). Many others endornaviruses have been detected and sequenced using this technique as an initial step (Khalifa and Pearson, 2014; Okada et al., 2011, 2013; Sabanadzovic and Valverde, 2011; Valverde and Sabanadzovic, 2009). However, although RT-PCR is a very sensitive and specific technique for the detection and identification of RNA plant viruses, the amount of RNA template can be a limiting factor. One solution to this limitation is real-time PCR or quantitative PCR.

Real-time or quantitative PCR (qPCR) is a technique that enhances detection, amplification, and quantification of a specific nucleic acid sequence. The amplified product can be quantified during the cycles due to the detection of fluorescence signal of a fluorogenic probe in amplification reaction. There are two common methods used for the detection of the PCR products. The first is using a non-specific fluorescent dye, such as SYBR green dye. This dye intercalates with any double-stranded DNA that is amplified in the reaction. Although SYBR green dye is less expensive, it is less sensitive compared to TaqMan probes. Because of the non-specificity problem of the SYBR green dye, it is not recommended for the quantification of a target. The second is a sequence-specific probe, such as the TaqMan fluorogenic probe. This probe is a specific oligonucleotide to the gene target that is labelled with a fluorescent reporter to the probe. The probe is hydrolyzed by the 5´ nuclease activity of Taq DNA polymerase when the primer is extended resulting in a fluorescence signal. TaqMan probes have been used extensively to investigate RNA titers in virus-infected plants. Quantitative RT-PCR has been used to determine differential gene expression of sweet potato plants mixed infected with potyvirus *Sweet potato feathery mottle virus* (SPFMV) and crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) (Kokkinos et al., 2006). Real time PCR was more effective than conventional PCR for the detection of five sweet potato viruses [SPFMV, SPCSV, *Sweet potato virus G* (SPVG), *Ipomoea vein mosaic virus* (IVMV), and *Sweet potato leaf curl virus* (SPLCV)] (Kokkinos and Clark, 2006). TaqMan probes were shown to be useful for the rapid and efficient detection of *Raspberry bushy dwarf virus* and *Raspberry leaf mottle virus* (Quito-Avila and Martin, 2012). These probes were used to detect viruses in single aphids. In addition, the results showed a synergistic interaction between the two viruses by comparing the viral RNA concentrations. The

TaqMan probe was shown to be fast, simple, sensitive, and less expensive procedure for detection and quantification of *Wheat yellow mosaic virus* (WYMV) (Liu et al., 2013).

Quantitative PCR assays have been developed for the detection and relative quantification, in singleplex, reactions of the potyviruses SPFMV, SPVG, IVMV, the crinivirus SPCSV, and the begomovirus SPLCV directly from infected sweet potato plants. There was no significant effect of potential inhibitors in total nucleic acid extracts from sweet potato leaves on the performance of the qPCR assays. Virus titers of SPFMV, IVMV, and SPVG were quantified and found to be lower in singly infected sweet potato plants compared with singly infected Brazilian morning-glory (*Ipomoea setosa*) and Scarlet O'Hara (*I. nil*) plants. qPCR was a more efficient detection method for SPLCV than conventional PCR assays (Kokkinos and Clark, 2006).

High-throughput sequencing or next-generation sequencing (NGS) has been successfully used for virus identification and whole viral genome studies (Espach et al., 2012; Jo et al., 2015; Jo et al., 2016; Sela et al., 2012). This technique coupled with bioinformatics has been used to analyze the transcriptome of bell pepper infected with *Bell pepper endornavirus* (BPEV). Results showed that BPEV is present in the host transcriptome with low copy numbers ranging from 0.01% to 0.18% (Jo et al., 2016). This tool was successful to identify BPEV in various pepper cultivars and provided sequence data for phylogenetic and recombination analyses of pepper endornaviruses using pepper transcriptome data.

1.7 Interactions Between Plant Viruses and Their Hosts

During virus infection, the host plant usually responds to an infection by activating general or specific defense pathways (Whitham et al., 2003). At the same time, once the virus enters the host cell, it needs the cell machinery for virus replication and assembly. At gene level,

effects of virus infection to the host plant can be determined through selected genes that are differentially expressed in the host during the infection process. Sweet potato plants infected with sweet potato virus disease (SPVD) caused by mixed infection of SPFMV and SPCSV had a reduction of expression levels of genes directly or indirectly involved in the photosynthetic pathway (Kokkinos et al., 2006). In general, symptoms of virus-infected plants are local and/or systemic symptoms. Localized lesions are developed near the site of virus entry which often leads to necrotic, chlorotic, and ring spot lesions. Systemic symptoms can consist of mosaic, yellows, chlorosis, necrosis, ring spots, wilting, leaf rolling, growth reduction, and malformations among others. Chlorosis occurs when infected cells lose chlorophyll and other pigments (Hull, 2014). Reductions in chlorophyll content in viral-infected plants affect photosynthetic capacity and chloroplast structure (Funayama-Noguchi and Terashima, 2006; Guo et al., 2005). The reduction in photosynthesis in virus-infected plants is also correlated with the reduction of rubisco and proteins associated with the photosynthetic pathway (Naidu et al, 1986; van Kooten et al., 1990). Reduction of morphological and growth characters of banana infected with *Banana bunchy top virus* (BBTV) is another example of virus infection that affects chlorophyll. Effects of BBTV infection include reduction of petiole length and distance between petioles, pseudostem diameter, plant height and canopy, leaf area, and also significantly decreases chlorophyll a and b and total chlorophyll contents (Hooks et al., 2008).

1.8 Interactions Between Endornaviruses and Plant Pathogens

Interactions between endornaviruses and plant pathogens, such as acute viruses, fungi, or bacteria, have not been studied. It is possible that like acute viruses, endornaviruses could affect the host response to infection by any of these plant pathogens. One common result of mixed infections of plants by two acute viruses is synergism. The synergism results from one virus

being able to block the host immune system for the other virus. As a result, the host expresses more severe symptoms than when infected by one virus alone (Pruss et al., 1997). Because persistent viruses are common but in most cases undetected, it would not be surprising if they could interact with acute viruses and result in disease more severe than that caused by the acute virus alone. Nevertheless, it is also possible that the activation of the plant immune system by persistent viruses could result in less severe diseases, such as in the case of cross protection. With the exception of VfEV, which is associated with male sterility, most of endornaviruses do not appear to affect the host phenotype (Pfeiffer, 1998).

It is noteworthy to mention that it appears that during the development of cultivars of crops, such as bell pepper and melon, plant breeders, unaware of the existence of endornaviruses in the germplasm of these crops, selected endornavirus-infected breeding lines (Okada et al 2011; Sabanadzovic et al 2016. This could be an indication that the presence of endornaviruses in these crops is beneficial. Whether this is the case for the Mesoamerican bean cultivars remains to be determined.

1.9 Hypothesis

The hypothesis of this investigation is that endornaviruses of common bean are in a symbiotic interaction of the mutualistic type with the host plant. The host allows the virus to replicate, and in return, the virus provides the plant a beneficial effect.

1.10 Objectives

1. To develop a practical and quick dsRNA extraction method that can be used to obtain viral dsRNA from a large number of plant samples infected with RNA viruses.
2. To investigate differential infection patterns of PvEV1 and PvEV2 infecting common bean genotypes between common bean gene pools.

3. To investigate the association of PvEV1 and PvEV2 with increases or decreases in seed germination, plant growth, pigment content, and grain yield of Black Turtle Soup common bean.
4. To investigate interactions of PvEV1 and PvEV2 with *Tobacco ringspot virus*, *Tobacco mosaic virus* and *Sunn-hemp mosaic virus*.

CHAPTER 2.
DEVELOPMENT OF A PRACTICAL DSRNA EXTRACTION METHOD FOR DETECTION OF RNA VIRUSES

2.1 Introduction

In plants and fungi infected with RNA viruses, large (~1.0-20.0 kb) double-stranded RNAs (dsRNAs) are found in the form of genomic segments of dsRNA viruses and replicative forms of single-stranded RNA viruses (Buck, 1999; Nuss and Koltin, 1990), satellite viruses, and satellite RNAs (Hillman et al., 2000; Valverde and Dodds, 1986). Large dsRNAs have been extracted from plants infected with acute and persistent viruses (Roossinck, 2010) and fungi infected with mycoviruses, and these extracts have been used for viral disease diagnosis and virus identification, and to clone and sequence plant and fungal RNA viruses (Bar-Joseph et al., 1983; Enebak et al,. 1994; Herrero et al., 2009; Jelkman et al., 1989; Khalifa and Pearson, 2014; Morris and Dodds, 1979; Okada et al., 2011; Rott and Jelkmann, 2001; Sabanadzovic and Valverde, 2011; Valverde and Sabanadzovic, 2009; Valverde et al., 1986, 1990b; Zhang and Rowhani, 2000). Recently, using deep sequencing, viral dsRNAs has been used to obtain the complete sequence of virus genomes from plants and fungi and to identify virus-like elements in aquatic microbial populations (Al Rwahnih et al., 2011; Candresse et al., 2013; Coetzee et al., 2010; Deker and Parker, 2014; Espach et al., 2012; Nerva et al., 2015; Quito-Avila et al., 2011).

Over the past 50 years, many methods for large dsRNA extraction from virus-infected plant, animal, fungal, and bacterial tissues have been reported in the literature (Akin et al., 1998; Balijja et al., 2008; Castillo et al., 2011; DePaulo and Powell, 1995; Franklin, 1966; Morris and Dodds, 1979, Morris et al., 1983; Okada et al., 2015; Tzanetakis and Martin, 2008). Grinding tissue in liquid nitrogen is the first step in most of these methods; however, liquid nitrogen is not readily available in many laboratories. Moreover, the majority of these methods requires a

relatively large amount of tissue which can present a problem when only limited amounts are available. The amount of reagents required by these methods can be a limiting factor as well because some reagents can be expensive, and waste disposal can be a problem when large volumes of some reagents are used. None of these methods have addressed the use of desiccated plant tissues, including tissues infected with fungi, for dsRNA extraction. This is particularly important because virus-infected desiccated tissues have been the conventional approach for the long-term storage of many plant viruses and, in the case of virus testing, samples can be readily available from any laboratory.

2.2 Objective

To develop a practical and quick dsRNA extraction method that can be used to obtain viral dsRNA from a large number of plant samples infected with RNA viruses.

This Chapter reports and validates a modification of a method described by Morris et al. (1983) for the extraction and electrophoretic analyses of viral dsRNAs from plants. The modified method is fast, economic, versatile, and requires small amounts of tissue. The modified dsRNA extraction method described in this chapter, together with the method reported by Valverde et al. (1990a) were used in Chapter 3 of this dissertation to test many *Phaseolus* spp. genotypes for the presence or absence of common bean endornaviruses.

2.3 Materials and Methods

2.3.1 Source of plant tissues for dsRNA extractions

For dsRNA extractions, foliar tissues from plants inoculated with Louisiana isolates of acute viruses or naturally infected with persistent viruses (Table 2.1) were used. The amount of fresh tissue collected depended upon availability, but in general, ranged from 1.0-5.0 g. Seeds from plants infected with persistent viruses and previously desiccated foliar tissues from plants

infected with acute viruses stored at 4°C in silica gel for at least 20 years were also used for dsRNA extraction in this investigation (Table 2.1). With the exception of previously desiccated foliar tissues and tissues from virus-free plants were also collected and used in the extractions.

Table 2.1. Plants infected with acute and persistent viruses and type of tissue tested.

Acute viruses			
Plant species	Common name	Virus	Tissue
Avena sativa cv California Red	Oat	*Brome mosaic virus* (BMV)	FD[a]
Capsicum annuum cv Marengo	Bell pepper	*Pepper mild mottle virus* (PMMoV)	FD
Citrus x limon cv Meyer	Lemon	*Citrus tristeza virus* (CTV)	FPD[b]
Cyrtomium falcatum	Japanese holly fern	Japanese holly fern mottle virus (JHFMoV)	FD
Glycine max cv AG 4934	Soybean	*Soybean mosaic virus* (SMV)	FD
Nicotiana tabacum cv NC95	Tobacco	*Cucumber mosaic virus* (CMV)	FD
N. tabacum cv Havana	Tobacco	*Tobacco necrosis virus* (TNV)	FPD
N. tabacum cv Havana	Tobacco	*Tobacco mild green mosaic virus* (TMGMV)	FPD
N. tabacum cv Havana	Tobacco	*Tobacco mosaic virus* (TMV)	FPD
Phaseolus vulgaris cv Top Crop	Common bean	*Tobacco ringspot virus* (TRSV)	FD
Stenotaphrum secundatum	St. Augustine grass	*Panicum mosaic virus* (PMV)	FD
Persistent viruses			
Plant species	Common name	Virus	Tissue
Basella alba cv Eclipse	Malabar spinach	Basella alba endornavirus (BaEV)	FD
C. chinense PI 159236	Habanero	*Bell pepper endornavirus* (BPEV)	FSD[c]
C.annuum cv Marengo	Bell pepper	*Bell pepper endornavirus*	FSD
C. annuum cv Jalapeño M	Jalapeño pepper	*Pepper cryptic virus 1* (PCV1)	SD[d]
C. annuum cv Hungarian	Yellow wax pepper	*Pepper cryptic virus 2* (PCV2)	SD
P. vulgaris cv Black Turtle Soup	Common bean	*Phaseolus vulgaris endornavirus 1* and *2* (PvEV1 and PvEV2)	FSD
P. vulgaris cv Majesty	Common bean	*Phaseolus vulgaris endornavirus 1* (PvEV1)	FSD
Solanum lycopersicum cv UC82	Tomato	*Southern tomato virus* (STV)	FSD

[a]Foliar desiccated; [b]Foliar previously desiccated; [c]Foliar and seed desiccated; FSD; [d]Seed desiccated

2.3.2 Desiccation of plant tissues

Tissues were cut finely with a razor blade, placed in folded coffee filter and then in glass jar (plastic bag or plastic jar can also be used) containing silica gel, and covered the jar with the cap and then stored for at least 48 h at 4°C (Figure 2.1). In the case of seeds, 0.5 g of crushed seed was desiccated as described above.

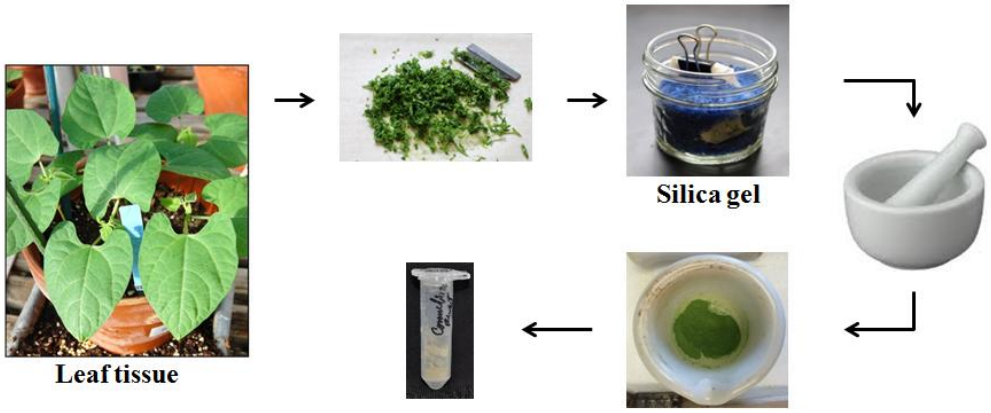

Figure 2.1. A schematic diagram of tissue preparation using silica gel.

2.3.3 Description of the dsRNA extraction and gel electrophoretic analysis

All desiccated tissues from plants listed in Table 2.1 were ground into a powder with a mortar and pestle and 50-70 mg was used for dsRNA extractions. Fresh tissues (0.5 g) of plants infected with *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) were also ground using liquid nitrogen and in STE (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 6.8) buffer. Ground tissue was placed in a 2 ml micro centrifuge tube and 500 µl of STE saturated phenol, 500 µl of STE buffer, 100 µl of 10% SDS, and 100 µl of a 2 % bentonite suspension were added. The sample was vortex-mixed for 1 min and then centrifuged for 3 min at 8,000 g. Four hundred microliters of the supernatant was collected, placed in a new 2 ml tube, and 440 µl of STE added, followed by 160 µl of 100 % ethanol (EtOH). After mixing

briefly, 100 mg of cellulose fibers (medium) (Catalog No. C6288, Sigma-Aldrich, St. Louis, MO, USA) were added and sample vortex-mixed for 10 sec, centrifuged at 8,000 g for 3 min, and the upper phase discarded. One milliliter of STE containing ethanol (16 %) was added to the tube containing the cellulose, vortex mixed for 10 sec, centrifuged at 8,000 g for 3 min, and the upper phase discarded. The later step was repeated one more time. After centrifugation, the upper phase was discarded and 500 µl of STE added, vortex-mixed for 5 sec, and centrifuged at 8,000 g for 3 min. Four hundred microliters of the upper phase were collected and placed in a 2 ml tube containing 30 µl of 3 M sodium acetate, pH 5.5 and 1.2 ml of frozen 100 % ethanol. The tube was vortex-mixed for 5 sec, centrifuged at 13,000 g for 10 min at room temperature, and then dried for 15 min at 37°C to eliminate ethanol residues. The dsRNA pellet was suspended in 35 µl of RNase-free water. To eliminate host DNA, 3.5 of 10 X DNase buffer and 1 unit of RNase-free DNase 1 (Fisher Scientific, Waltham, MA, USA) were added and incubated at 37°C for 15 min. Four microliters of 4x electrophoresis buffer (TAE) (0.04 M Tris, 0.02 M sodium acetate (NaAc), 0.001 M EDTA, pH 7.8) containing 20 % glycerol, 0.01 % bromophenol blue, and 1 µl of 10,000 X GelRed^{TM} (Biotium, Hayward, CA, USA) was added to the sample. On average, 15 µl of the DNase-treated sample were loaded on a 1.2 % agarose gel prepared in 1x TAE buffer. However, sample loads varied depending upon the plant or fungal host due to variation in dsRNA yields. Gels were run for 2 h and results recorded with a GelDoc-It2 Imager (UVP, Upland, CA, USA). Molecular marker consisted of 1 kb DNA ladder (Bio-Rad, Hercules, CA). At least two independent dsRNA extractions were conducted for all the samples tested. When dsRNAs were obtained, their dsRNA nature was confirmed by treatment of the gels with RNase under high salt conditions as described by Morris and Dodds (1979). A schematic diagram of the dsRNA extraction method is shown in Figure 2.2.

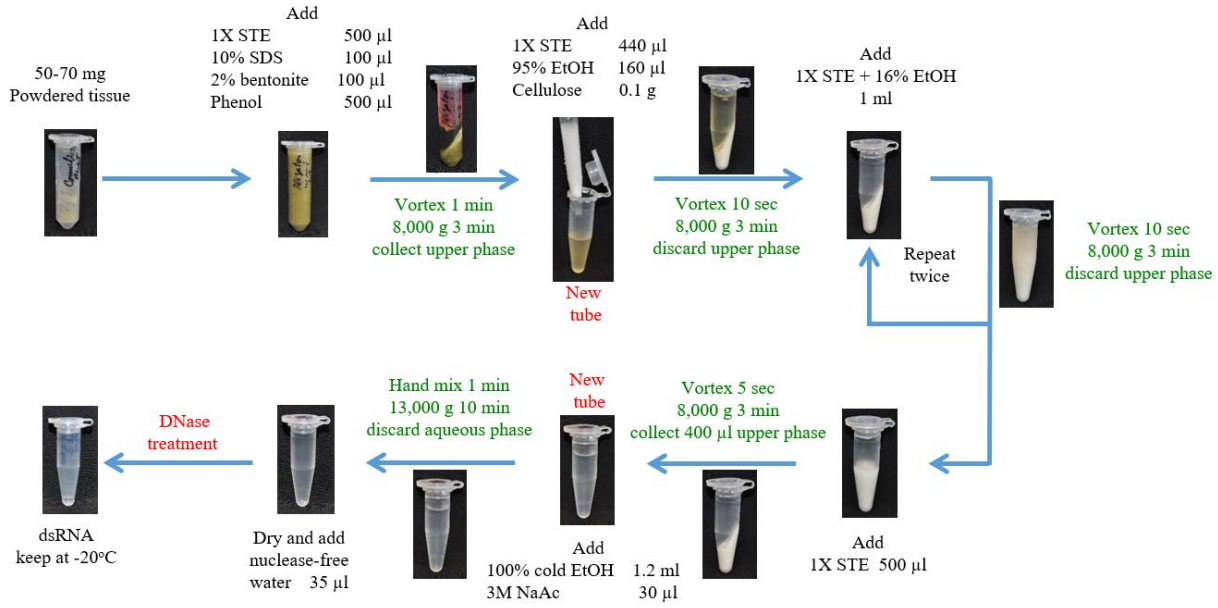

Figure 2.2. A schematic diagram of the modified dsRNA extraction method.

2.3.4 RT-PCR

To confirm their viral nature, extracted dsRNAs from plants infected with *Tobacco ringspot virus* (TRSV), *Soybean mosaic virus* (SMV), PvEV1 and PvEV2 were heat denatured (95°C, 3 min) and used in RT-PCR reactions using reported virus-specific primers and PCR conditions (Colinet et al., 1998; Khankhum et al., 2015; Sabanadzovic et al., 2010).

2.4 Results

DsRNAs were successfully extracted from most virus-infected plants. Figures 2.3, 2.4, and 2.5 show electrophoretic banding pattern profiles of viral dsRNAs obtained from plants infected with acute and persistent viruses respectively. With the exception of TRSV and SMV, viral dsRNA was detected by gel electrophoresis from all other virus-infected plants, including virus-infected seeds. These profiles were consistently obtained and were similar to those reported in previous investigations (Valverde et al., 1986, 1990a). DsRNAs were obtained with all

samples when this method was used to extract dsRNA from virus-infected plant samples stored as desiccated tissue in silica gels, including some stored for up to 20 years. DsRNA extractions from virus-free plants did not yield large dsRNAs.

In general, dsRNA yields extracted from 50-70 mg of desiccated tissue ranged from 50-500 ng depending on the plant virus species. When compared with liquid nitrogen and STE buffer, grinding desiccated tissues yielded slightly higher amounts of dsRNA after gel electrophoresis (Figure 2.3).

TRSV and SMV were detected by RT-PCR using the extracts that were negative by gel electrophoresis as templates. Similarly, using purified dsRNAs as templates, we were able to amplify DNA fragments of PvEV1 and UmV-H1

TRSV and SMV were detected by RT-PCR using the extracts that were negative by gel electrophoresis as templates (Figure 2.6). Obtained DNA amplicons from TRSV were 348 and 766 bp. Similarly, DNA fragments of PvEV1 (303 bp) and PvEV2 (519 bp) were amplified using purified dsRNAs as templates.

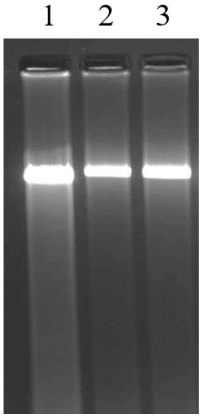

Figure 2.3. Agarose gel electrophoresis of dsRNAs extracted from common bean cv Black Turtle Soup double-infected with PvEV1 and PvEV2 using three different tissue grinding methods. Lane 1, ground dried tissue; lane 2, fresh tissue ground with liquid nitrogen; and lane 3, fresh tissue ground with STE buffer.

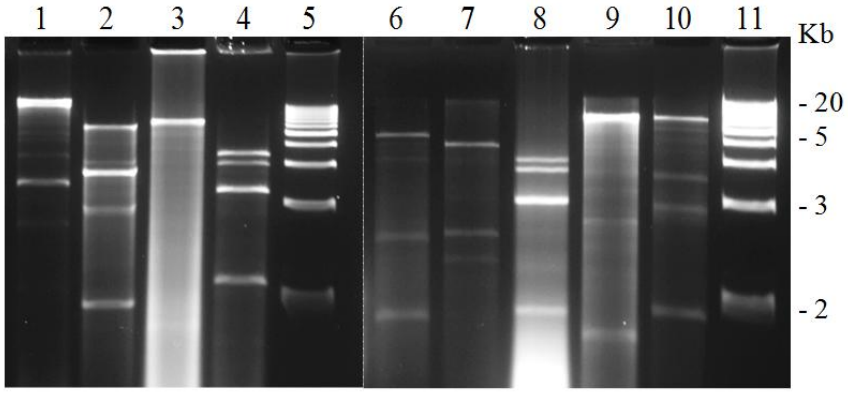

Figure 2.4. Agarose gel electrophoresis of dsRNAs extracted from plants infected with acute viruses. Lane 1, CTV; lane 2, JHFMoV; lane 3, PMMoV; lane 4, CMV; lanes 5 and 11, 1 kb ladder (Bio-Rad); lane 6, PMV; lane 7, TNV; lane 8, BMV; lane 9, TMV; lane 10, TMGMV.

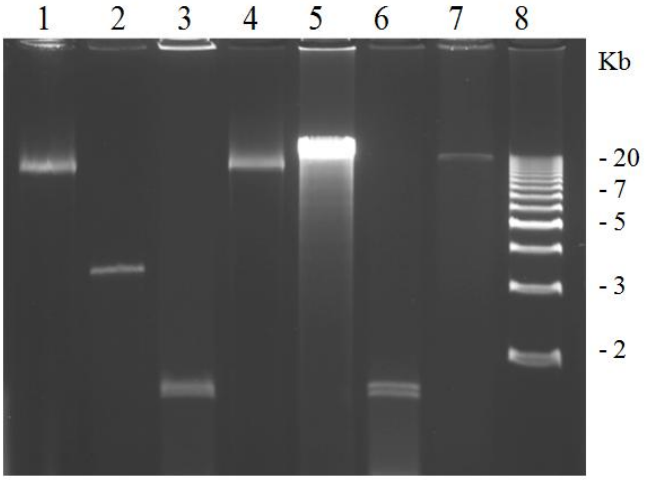

Figure 2.5. Agarose gel electrophoresis of dsRNAs extracted from plants infected with persistent viruses. Lane 1, BaEV; lane 2, STV; lane 3, PCV2; lane 4, PvEV1; lane 5, BPEV (Marengo); lane 6, PCV1; lane 7, BPEV (PI 159236); lane 8, 1 kb ladder (Bio-Rad).

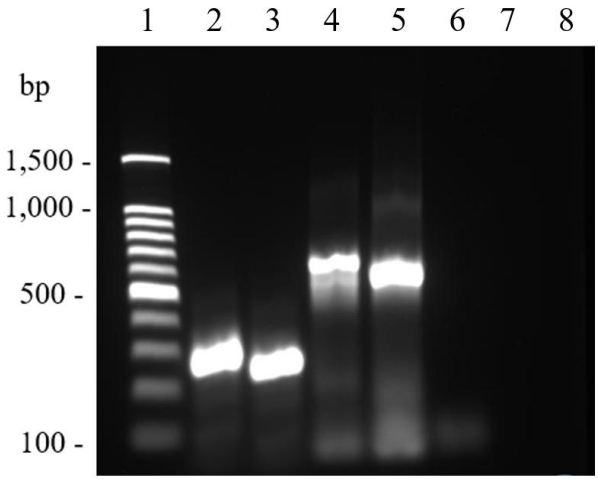

Figure 2.6. Agarose gel electrophoresis of RT-PCR products amplified from dsRNA extracted from plant infected with acute viruses. Lane 1, 100 bp DNA ladder (Promega); lane 2, TRSV (348 bp); lane 3, PvEV1 (303 bp); lane 4, TRSV (766 bp); lane 5, PvEV2 (519 bp); lane 6-7, SHMV; lane 8, water control.

2.5 Discussion

It has been well established that extraction and purification of large dsRNAs from virus-infected plant and fungal tissues are powerful tools for virus research. These viral dsRNAs have been extracted and used as reagent to identify viruses and to study viromes and ecogenomics in natural ecosystems (Coetzee et al., 2010; Deker and Parker, 2014; Nerva et al., 2015; Okada et al., 2011; Roossinck et al., 2010; Tzanetakis et al., 2004; Valverde and Sabanadzovic, 2009). Therefore, it is clear that the extraction and purification of viral dsRNA from plants and fungi plays an important role in plant and fungal virus research, surveys, identification, and diagnosis and there is a need for dsRNA extraction methods from plant tissues that are simple, fast, and economical.

The dsRNA extraction method presented here was successfully used to obtain dsRNAs from plants infected with RNA viruses. It is based on phenol extraction combined with cellulose-

binding of dsRNA. The method consists of a modification of the "non-phenol batch procedure" described by Morris et al. (1983). The addition of phenol and DNase treatments were essential for successful dsRNA extractions. This method provides the user several improvements from previously described methods. These include short processing time, small tissue sample size, relatively high dsRNA yields, and most important low cost and low amounts of toxic waste. Furthermore, this method allows for a large number of samples to be processed in a short period of time using low amounts of reagents. For dsRNA binding, we used cellulose fibers (medium) manufactured by Sigma-Aldrich, which is readily available worldwide. In contrast, the cellulose (Whatman CF-11) used in other methods is no longer produced by the manufacturer. During the development of this method, we optimized the amounts of each reagent to maximize dsRNA yields. The level of detection was improved by staining dsRNA with GelRed™ instead of ethidium bromide. It has been demonstrated that for DNA staining, GelRed™ is a safe alternative to ethidium bromide and increases the sensitivity of detection (Huang et al., 2010). The electrophoretic dsRNA profiles reported here for plant viruses were similar to those reported in previous investigations (Sabanadzovic and Valverde, 2011; Valverde et al., 1986, 1990a; Valverde and Sabanadzovic, 2009). Extraction of viral dsRNA from seeds infected with persistent viruses BPEV, PvEV1 (*Endornaviridae*), PCV1, PCV2 (*Partitiviridae*), and *Southern tomato virus* (*Amalgaviridae*), yielded expected dsRNA profiles after gel electrophoresis (Okada et al., 2011; Sabanadzovic and Valverde, 2011; Sabanadzovic et al., 2009). This suggests that this method will be practical to test seeds for the presence of persistent viruses.

Replicative forms of viral dsRNAs have been used successfully as templates for RT-PCR reactions (Herrero et al., 2009; Khalifa and Pearson, 2014; Okada et al., 2011, 2013; Rott and Jelkmann, 2001; Sabanadzovic and Valverde, 2011; Valverde and Sabanadzovic, 2009). This

was confirmed using dsRNAs extracted by the developed method, including extracts from TRSV-infected tobacco and SMV-infected soybean. The latter suggests that the low amounts of RNA (dsRNA, ssRNA, or both) purified by the method reported here are not a limitation for successfully RT-PCR reactions. As in the case of TRSV, we were not able to detect by gel electrophoresis dsRNA extracted from plants infected with the potyvirus SMV. It has been reported that potyviruses yield lower amounts of dsRNA when compared with other plant viruses and larger amounts of tissue need to be used to obtain detectable quantities by gel electrophoresis (Valverde et al., 1986). When using the method presented here, in these cases, it is recommended to conduct individual extractions of several samples and then pool them during the last dsRNA extraction step.

During the process of evaluating the dsRNA yield from various plant species, it was found that the degree of sample grinding was critical. Samples ground to a fine powder yielded higher amounts of dsRNA than those that were coarser. This effect was particularly noticeable with virus-infected plant samples that were difficult to grind such as species of the Graminaceae and Poaceae. These results suggest that grinding tissues in liquid nitrogen does not increase dsRNA yields when compared with silica gel desiccated tissues. Therefore, desiccated tissue presents a practical alternative to many laboratories that do not have access to liquid nitrogen.

Because host DNA is often co-purified with viral dsRNA, DNase treatments were conducted with all samples tested. This is highly recommended, particularly when testing plants infected with viruses that yield dsRNAs in agarose gel electrophoresis similar in size to plant DNA such as endornaviruses and some acute viruses. With some plant/virus combination, smaller (0.5-1.0 kb) unidentified host nucleic acids presumably to be ribosomal RNA were co-purified with the large dsRNAs.

The dsRNA extraction method presented here, was used to extract dsRNA from desiccated tissues of plant viruses stored for up to 30 years. Although the modified method was not used with herbaria specimens, it was used to test plant tissues infected with biotrofic fungi that were pressed and dried following the same procedures used in herbaria and fungaria and successfully detected putative mycoviruses (data not shown). These results illustrate the suitability of the method to test samples for dsRNA from herbaria, fungaria, and samples from old virus collections that consist of desiccated tissues. The results suggest that this method could be used as an initial step in studies on the discovery, characterization, distribution, and evolution of plant viruses and mycoviruses.

The modified method presented here is similar to other previously published dsRNA extraction methods, however, it contains several improvements that increase the overall extraction efficiency including the number of samples that can be processed. This method also increases the practicality of using dsRNA as reagent for plant and fungal virus diagnosis, identification, and sequencing. Furthermore, this method could be very helpful to researchers interested in virome analyses of phytobiomes.

CHAPTER 3.
PHASEOLUS VULGARIS ENDORNAVIRUS 1 AND *PHASEOLUS VULGARIS ENDORNAVIRUS 2* INFECTING COMMON BEAN (*PHASEOLUS VULGARIS*) GENOTYPES SHOW DIFFERENTIAL INFECTION PATTERNS BETWEEN COMMON BEAN GENE POOLS*

3.1 Introduction

Common bean (*Phaseolus vulgaris* L.), which includes dry and snap beans, is the most important legume for direct human consumption. Common bean originated and was domesticated in the Americas but now is grown worldwide (Broughton et al., 2003). There is evidence that the domestication of common bean took place in two geographical regions, Mesoamerica and the Andes region of South America (Bitocchi et al., 2013; Singh et al., 1991). The two gene-divergent gene pools of common bean can be differentiated by morphological and molecular characteristics (Becerra-Velásquez and Gepts, 1994; Gepts et al., 1986; Koenig and Gepts, 1989; Kwak and Gepts, 2009; Singh et al., 1991). Using three 'omics' platforms, Mensack et al. (2010) have shown that common bean cultivars from the two major centers of domestication differed in their transcriptome, proteome, and metabolome profiles. Recent studies by Bitocchi et al. (2013) provide more evidence for these two independent but parallel centers of domestication for common bean. Sequence data of genotypes from the Mesoamerican and Andean gene pools confirmed the two independent domestication events and also revealed that less than 10% of the 74 Mb of sequence putatively involved in domestication is shared by the

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*Most of the content of this chapter was previously published as: Khankhum, S., R. A. Valverde, M. A. Pastor-Corrales, J. M. Osorno, and S. Sabanadzovic. 2015. Two endornaviruses show differential infection patterns between gene pools of *Phaseolus vulgaris*. *Archives of Virology*. 160(4): 1131-1137. It is reprinted by permission of Springer. Permission letter is in the Appendix.

two gene pools (Schmutz et al., 2014). Several methods have been developed to determine the genetic diversity in common bean, and these methods have been useful in breeding programs (Kwak and Gepts, 2009; Papa and Gepts, 2003). It has been shown that the genetic diversity of the Mesoamerican gene pool is greater than the Andean one suggesting a Mesoamerican origin of common bean (Bitocchi et al., 2012; Kwak and Gepts, 2009).

Based on the type of relationship with the host, plant viruses can be grouped as acute or persistent (Roossinck, 2010). Acute viruses are well studied and cause disease in plants. In contrast, persistent viruses do not appear to affect the phenotype of the plant host. Persistent viruses include members of the family *Amalgaviridae, Chrysoviridae, Endornaviridae*, and *Partitiviridae* (Adams et al., 2014; Roossinck, 2010). Endornaviruses are RNA viruses with a genome that ranges from 9.8 to 17.6 kb and infect plants, fungi, and oomycetes. Endornaviruses lack cell-to-cell movement, are transmitted only via gametes, and do not cause apparent symptoms (Fukuhara, 1999; Fukuhara and Gibbs, 2012). They infect economically important crops, such as avocado (*Persea Americana* Mill.) (Villanueva et al., 2012), barley (*Hordeum vulgare* L.) (Candresse et al., 2016), bell pepper (*Capsicum annuum* L.) (Okada et al., 2011; Valverde et al., 1990b), broad bean (*Vicia faba* L.) (Pfeiffer, 1998), common bean (Okada et al., 2013; Wakarchuk and Hamilton, 1990), melon (*Cucumis melo* L.) (Sabanadzovic et al., 2016), rice (*Oryza sativa* L.) (Fukuhara and Moriyama, 2008), some plant pathogenic fungi (Fukuhara and Gibbs, 2012; Osaki et al., 2006), and the oomycete *Phytophthora* sp (Hacker et al., 2005). Currently, all described endornaviruses are included in a single family, the *Endornaviridae* (Fukuhara, 1999). The role of endornaviruses in the plant and their origin are not known. One possible origin is fungal by inter-kingdom host jumping as suggested by Liu et al. (2012) and Roossinck (2013) for plant partitiviruses. Nevertheless, it has been shown that several

endornaviruses may have acquired genes from bacteria through horizontal gene transfer and may have originated from bacteria (Song et al., 2013).

Phaseolus vulgaris endornavirus was first reported in the common bean cultivar Black Turtle Soup by Wakarchuck and Hamilton (1990). However, recently, it was reported that Black Turtle Soup and other common bean cultivars of various market classes are infected by two distantly related endornavirus species which were designated *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) (Adams et al., 2014; Okada et al., 2013). These results were obtained from testing a limited number of common bean cultivars for these two endornaviruses and suggest that these viruses may be present in other cultivars (Okada et al., 2013).

3.2 Objectives

1. To identify the endornaviruses infecting cultivated and wild *P. vulgaris* from the two major centers of domestication.
2. To determine the geographical distribution of endornaviruses in *P. vulgaris* genotypes.

This was accomplished by testing common bean cultivars, breeding-lines, landraces, and wild *P. vulgaris* genotypes as well as other *Phaseolus* species from both centers of common bean domestication, Mesoamerica and the Andes, for the presence of PvEV1 and PvEV2. Furthermore, DNA sequences from RT-PCR products obtained from selected endornavirus-infected genotypes were analyzed.

3.3 Materials and Methods

3.3.1 Source of plant materials

Most modern common bean cultivars and breeding-lines of various market classes tested in this investigation originated from a collection of the USDA-ARS Soybean Genomics and

Improvement Laboratory, Beltsville, MD and the Dry Bean Breeding and Genetics Program, Department of Plant Sciences, North Dakota State University, Fargo, ND. Many black seeded cultivars were provided by Donald Halseth, Cornell University, NY. Some Andean genotypes were obtained from two commercial seed companies, Burpee (Warminster, PA) and Heirloom Seeds (West Finley, PA). Plant introduction (PI) lines of landraces of common bean, wild *P. vulgaris* and other *Phaseolus* species were provided by the USDA-ARS, National Plant Germplasm System, Western Regional Plant Introduction Station, Pullman, WA. Figure 3.1 illustrates the seed morphology of the tested *Phaseolus* germplasm. *Phaseolus vulgaris* genotypes tested included 69 and 42 common bean cultivars and breeding-lines of Mesoamerican and Andean origin respectively (Table 3.1); 36 and 42 landraces collected in the Mesoamerican and the Andean regions respectively (Table 3.2); 62 and 26 wild *P. vulgaris* collected in the Mesoamerican and the Andean regions respectively (Table 3.3). Eighteen other *Phaseolus* species, including four domesticated species (*P. acutifolius* A. Gray, *P. coccineus* L., *P. dumosus* Macfad., and *P. lunatus* L.) were also tested (Table 3.4). Positive and negative controls consisted of two lines of the common bean cultivar Black Turtle Soup obtained in previous investigations (Okada et al., 2013): one mixed-infected with PvEV1 and PvEV2 and the other endornavirus-free. All plants were grown in a greenhouse located on the Baton Rouge campus of Louisiana State University. Day/night temperatures averaged 25/18°C respectively. Seeds were planted in steam sterilized soil mix (two parts soil, one part sand, three parts sphagnum moss).

3.3.2 Sample desiccation and dsRNA extraction

Three grams of foliar tissues from 3- to 4-week-old plants were collected. The tissue was rolled and cut with a razorblade in pieces of approximately 2 mm wide, dried overnight with silica gel at 4°C, and used for dsRNA extractions. At least two independent dsRNA extractions

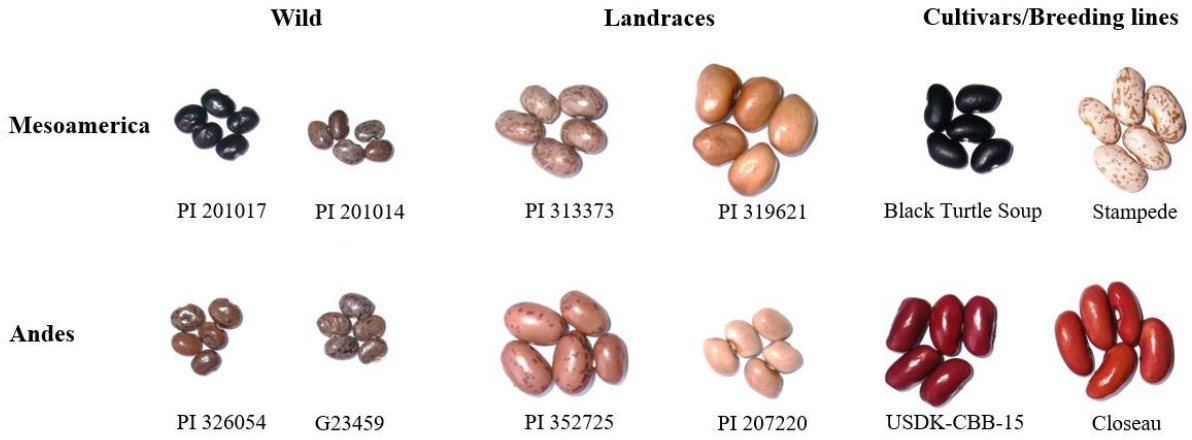

Figure 3.1. Illustration of the size, color, and morphology of seeds from wild, landraces, and cultivars/breeding lines of *Phaseolus vulgaris*.

from single plants from each genotype were conducted. The dsRNA was extracted using phenol and purified using CF-11 chromatography as described by Valverde et al. (1990a) or by the modified dsRNA extraction method described in chapter 2. DsRNA samples were treated with DNase (1 unit of DNase/1 μg of dsRNA) for 15 min at 37°C. Aliquots of dsRNA samples were first electrophoresed in 1.2% agarose gel (TAE buffer) for 2 h at 70 V. Samples with the presence of dsRNA were second electrophoresed in 0.75% agarose gels for 16-24 h at 30 V with at least one buffer change. The presence or absence of dsRNAs was determined by ethidium bromide or GelRed™ (Biotium, Hayward, CA, USA staining and visualization under UV light. The molecular size of dsRNAs was estimated by comparison with dsRNAs extracted from Black Turtle Soup infected by PvEV1 (14 kb) and PvEV2 (15 kb) (Figure 3.3 B). Other dsRNA aliquots were used in reverse transcription-PCR (RT-PCR).

3.3.3 Reverse-transcription polymerase chain reaction and DNA sequence

A duplex, single tube RT-PCR for the simultaneous and discriminatory detection of both PvEV1 and PvEV2 developed in a previous investigation (Okada et al., 2013) was used to confirm results of gel electrophoresis. Primers were designed to amplify a genomic segment of 303 bp of the RNA-dependent RNA polymerase gene (RdRp) in the case of PvEV1 (PvEV-1For, GTAAACCAGGGAATTGGTGG and PvEV-1Rev, GATTGATTGGGCTGTATAGTG) and a 519 bp segment of the same genomic region in the case of PvEV2 (PvEV-2 F, TGTTAGGCGTGTGTCCCCA and PvEV-2R, GTTGCTGTATTGCTCGTGTC) without cross-interference. After denaturation, dsRNA was mixed with 12.5 µl of the 2x reaction buffer, 0.5 µl of 5 mM $MgSO_4$, 1 µl of each of the four primers (100 ng/µl), 0.75 µl RT/Taq mix, and 2 µl of RNase-free water for a total volume of 25 µl. This mix was subjected to the following conditions: reverse transcription for 20 min at 53°C, denaturation for 2 min 30 s at 94°C, and 35 cycles of PCR (94°C for 20 s, 56°C for 35 s, and 68°C for 45 s) followed by final extension step at 68°C for 5 min. The presence and size of virus-specific PCR products were determined by comparisons with a PCR Marker (Promega, Madison, WI) in 1.2% agarose gel electrophoresis (Figure 3.3 A). PCR products representative of the various *P. vulgaris* genotypes (cultivars and breeding-lines, landraces, and wild) were separated by agarose gel electrophoresis, gel purified, cloned using the pGEM-T kit (Promega), and sequenced (Macrogen, Rockville, MD). Sequences of the PCR products were aligned with reported sequences for PvEV1 and PvEV2 infecting Black Turtle Soup common bean (GenBank/EMBL/DDBJ accessions numbers AB719397 and AB719398 respectively) and percentage of identity determined. Bean genotypes were called positive for each virus if both dsRNA and RT-PCR detected the presence of virus. Phylogenetic analyses comparing nucleotide sequences of the RdRp gene of PvEV1 and PvEV2 were carried

out using ClustalW version 2 (Larkin et al., 2007) and Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6) (Tamura et al., 2013) with 1000 bootstrap replications.

3.4 Results

An example of a typical endornavirus-screening gel is shown in Figure 3.2 and 3.3. The results indicate that the endornaviruses PvEV1 and PvEV2 are present in many *P. vulgaris* genotypes. Representative results of electrophoresis and RT-PCR detection of PvEV1 and PvEV2 from common bean genotypes are illustrated in Figure 3.3. Indeed, 93 of 247 (38%) tested genotypes contained these endornaviruses (Tables 3.1, 3.2, and 3.3). Nevertheless, there was a clear distinction between the genotypes tested from the two main centers of common bean domestication: although the endornaviruses were almost universally present in *P. vulgaris* genotypes of Mesoamerican origin, the Andean *P. vulgaris* were virtually endornavirus-free (Table 3.4). Endornavirus infections were detected in 63 of 68 (93%) common bean genotypes of Mesoamerican origin. In contrast, endornaviruses were detected only in 4 of 42 (9%) genotypes common bean of Andean origin. With the exception of the cultivar Jackpot, NW-63, NW-410, Othello, and Victor which were endornavirus-free, all other 63 Mesoamerican common bean cultivars and breeding-lines were double-infected with PvEV1 and PvEV2. The four infected genotypes of Andean origin were the breeding-lines BD-1002 and CPC 99814 infected with PvEV1; USDK-CBB-15 infected with PvEV1 and PvEV2; and the cultivars Closeau and Red Rover infected with PvEV2 (Tables 3.1).

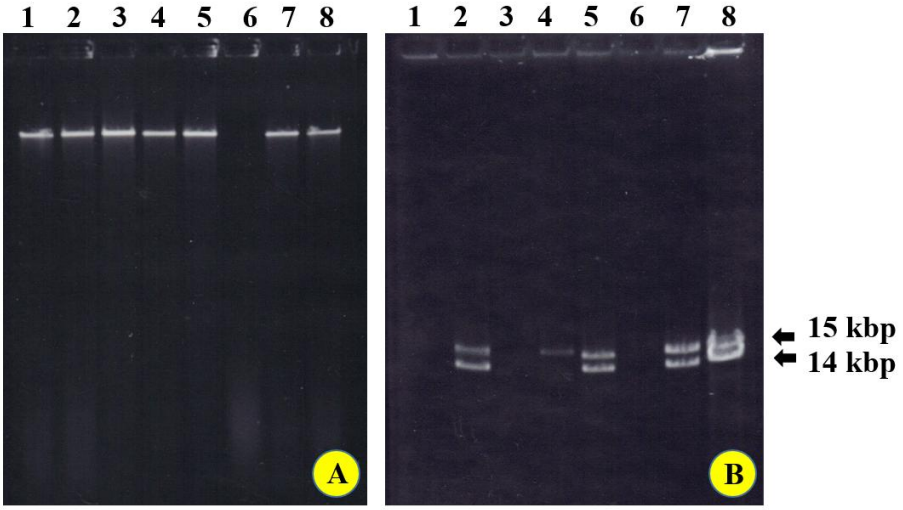

Figure 3.2. Typical endornavirus-screening gel. (A) dsRNAs extracted from *P. vulgaris*: 1, PI 201017; 2, Loreto 3, Black Turtle Soup (BTS+); 4, T-39; 5, Buster ; 6, BTS-; 7, PI 201019; 8, Majesty and run in a 1.2 % agarose for 2 h. (B) dsRNAs extracted from *P. vulgaris* cultivars: 1, BTS-; 2, North Star; 3, Mountcalm, 4, Closeau; 5, Buster; 6 Pink Panther; 7, Loreto; 8, T-39 and run in 0.75 % agarose for 24 h.

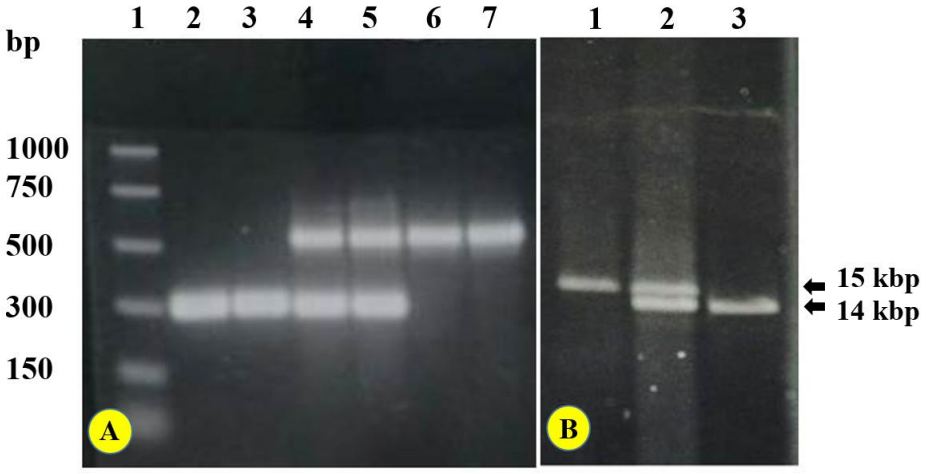

Figure 3.3. Agarose gel electrophoresis. (A) Results of RT-PCR testing of common bean cultivars infected with PvEV1 and/or PvEV2. 1, PCR Marker; 2, Bellagio (PvEV1); 3, Majesty (PvEV1); 4, Jaguar (PvEV1 and PvEV2); 5, Stampede (PvEV1 and PvEV2); 6, Closeau (PvEV2); and 7, Red Rover (PvEV2). (B) Viral dsRNAs extracted from three common bean cultivars and ran in 0.75% agarose at 30 V for 16 h. 1, Closeau infected with PvEV2; 2, Black Turtle Soup infected with PvEV1 and PvEV2; and 3, BD 1002 infected with PvEV1.

Table 3.1. Common bean genotypes of various market classes from breeding programs in the United States tested by gel electrophoresis and PCR for *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2).

Cultivar/Breeding Line/PI[a] Line	Market Class	Seed Source	PvEV1	PvEV2
Mesoamerican Origin				
W6 28059	Black	NPGS-WRPIS[b]	+[c]	+
PI 599021	Black	NPGS-WRPIS	+	+
B 210237	Black	USDA-ARS SGIL[d]	+	+
Black Velvet	Black	CU[e]	+	+
96-148	Black	CU	+	+
Midnight	Black	CU	+	+
Jolly Roger	Black	CU	+	+
ISBTR-13	Black	USDA-ARS SGIL	+	+
115 M	Black	USDA-ARS SGIL	+	+
B201240	Black	USDA-ARS SGIL	+	+
SW-B2010240	Black	USDA-ARS SGIL	+	+
Jet Black	Black	USDA-ARS SGIL	+	+
Shania	Black	USDA-ARS SGIL	+	+
Zorro	Black	USDA-ARS SGIL	+	+
Eclipse	Black	USDA-ARS SGIL	+	+
Loreto	Black	NDSU[f]	+	+
Jaguar	Black	NDSU	+	+
Hungerford	Great Northern	USDA-ARS SGIL	+	+
Sawtooth	Great Northern	USDA-ARS SGIL	+	+
Coyne	Great Northern	USDA-ARS SGIL	+	+
ISB-97-471	Great Northern	USDA-ARS SGIL	+	+
Big Horn	Great Northern	USDA-ARS SGIL	+	+
93:208G	Great Northern	USDA-ARS SGIL	+	+
PK 7-4	Pink	USDA-ARS SGIL	+	+
PK 7-5	Pink	USDA-ARS SGIL	+	+
USPK7-5	Pink	USDA-ARS SGIL	+	+
UC Pink 9634	Pink	USDA-ARS SGIL	+	+
Roza	Pink	USDA-ARS SGIL	+	+
PI 578261 (Victor)	Pink	WPRS	-[g]	-
Jackpot	Pinto	USDA-ARS SGIL	-	-
Santa Fe	Pinto	USDA-ARS SGIL	+	+
Othello	Pinto	USDA-ARS SGIL	-	-
PI 550013 (NW-410)	Pinto	WPRS	-	-
Stampede	Pinto	USDA-ARS SGIL	+	+
Buster	Pinto	NDSU	+	+
La Paz	Pinto	NDSU	+	+
Medicine Hat	Pinto	NDSU	+	+
Maverick	Pinto	NDSU	+	+
ND-307	Pinto	NDSU	+	+
Windbreaker	Pinto	NDSU	+	+

(Table 3.1. continued)

Cultivar/Breeding Line/PI[a] Line	Market Class	Seed Source	PvEV1	PvEV2
Mesoamerican Origin				
N33210	Navy	USDA-ARS SGIL	+	+
Seahawk	Navy	USDA-ARS SGIL	+	+
Dublin	Navy	USDA-ARS SGIL	+	+
Lightening	Navy	USDA-ARS SGIL	+	+
Norstar	Navy	NDSU	+	+
Navigator	Navy	NDSU	+	+
T-9905	Navy	NDSU	+	+
Ensign	Navy	NDSU	+	+
Medalist	Navy	NDSU	+	+
Avalanche	Navy	NDSU	+	+
Vista	Navy	NDSU	+	+
N97774	Navy	USDA-ARS SGIL	+	+
Seahawk	Navy	USDA-ARS SGIL	+	+
ISB 99-1815-2	Navy	USDA-ARS SGIL	+	+
ISB 1816	Navy	USDA-ARS SGIL	+	+
N128420	Navy	USDA-ARS SGIL	+	+
OAC Rex	Navy	USDA-ARS SGIL	+	+
PI 608450	Navy	NPGS-WRPIS	+	+
Merlot	Small red	USDA-ARS SGIL	+	+
PI 633423	Small red	WIPRS	+	+
R930365	Small red	USDA-ARS SGIL	+	+
R02002	Small red	USDA-ARS SGIL	+	+
R97003	Small red	USDA-ARS SGIL	+	+
R02189	Small red	USDA-ARS SGIL	+	+
NW-63	Small red	USDA-ARS SGIL	-	-
CPC 00250	Yellow	USDA-ARS SGIL	+	+
Flor 9623	Flor de Mayo	USDA-ARS SGIL	+	+
Desert Rose	Flor de Mayo	USDA-ARS SGIL	+	+
Andean Origin				
USWA 33	Light Red Kidney	USDA-ARS SGIL	-	-
1120-V96	Light Red Kidney	USDA-ARS SGIL	-	-
UCD 9830	Light Red Kidney	USDA-ARS SGIL	-	-
773-V98	Light Red Kidney	USDA-ARS SGIL	-	-
1062-V98	Light Red Kidney	USDA-ARS SGIL	-	-
Blush	Light Red Kidney	USDA-ARS SGIL	-	-
SW LRK 7	Light Red Kidney	USDA-ARS SGIL	-	-
OAC Rosario	Light Red Kidney	USDA-ARS SGIL	-	-
CELRK	Light Red Kidney	USDA-ARS SGIL	-	-
Pink Panther	Light Red Kidney	NDSU	-	-
Foxfire	Light Red Kidney	NDSU	-	-
Closeau	Light Red Kidney	NDSU	-	+
Red Rover	Light Red Kidney	NDSU	-	+

(Table 3.1. continued)

Cultivar/Breeding Line/PI[a] Line	Market Class	Seed Source	PvEV1	PvEV2
H 9659-37-2	Light Red Kidney	USDA-ARS SGIL	-	-
Mogul	Light Red Kidney	USDA-ARS SGIL	-	-
CPC 00247	White Kidney	USDA-ARS SGIL	-	-
USWK-70	White Kidney	USDA-ARS SGIL	-	-
Silver Cloud	White Kidney	USDA-ARS SGIL	-	-
Beluga	White Kidney	USDA-ARS SGIL	-	-
PI 550265	White Kidney	NPGS-WRPIS	-	-
D000264	Dark Red Kidney	USDA-ARS SGIL	-	-
Montcalm	Dark Red Kidney	NDSU	-	-
Red Hawk	Dark Red Kidney	NDSU	-	-
PI 639867 (USDK-CBB-15)	Dark Red Kidney	NPGS-WRPIS	+	+
BD 1002	Cranberry	USDA-ARS SGIL	+	-
BD 1004	Cranberry	USDA-ARS SGIL	-	-
BD 1003	Cranberry	USDA-ARS SGIL	-	-
Capri	Cranberry	USDA-ARS SGIL	-	-
CPC 99814	Cranberry	USDA-ARS SGIL	+	-
Hooter	Cranberry	USDA-ARS SGIL	-	-
Cardinal	Cranberry	USDA-ARS SGIL	-	-
Hooter	Cranberry	USDA-ARS SGIL	-	-
Cardinal	Cranberry	USDA-ARS SGIL	-	-
UCD 0801	Cranberry	USDA-ARS SGIL	-	-
Blue Lake	Snap Bean	Heirloom	-	-
PI 549537	Snap Bean	NPGS-WRPIS	-	-
PI 550379	Snap Bean	NPGS-WRPIS	-	-
PI 550299	Snap Bean	NPGS-WRPIS	-	-
Cherokee Wax	Snap Bean	Heirloom	-	-
Kentucky Wonder	Snap Bean	Heirloom	-	-
Dragon Tongue	Snap Bean	Burpee	-	-
Manitoba Black	Snap Bean	NPGS-WRPIS	-	-
Royalty Purple	Snap Bean	Heirloom	-	-
Top Crop	Snap Bean	Heirloom	-	-

[a] Plant introduction
[b] National Plant Germplasm System, Western Regional Plant Introduction Station
[c] Positive
[d] USDA-ARS Soybean Genomics and Improvement Laboratory
[e] Cornell University
[f] North Dakota State University
[g] Negative

Table 3.2. Landraces of common bean tested by gel electrophoresis and PCR for *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2).

Plant Identification	PI[a] Line	Location/Country of Origin	PvEV1	PvEV2
Mesoamerican Origin				
Papago red bean	476861	Arizona, USA	-[b]	-
Negro	203958	Veracruz, México	-	-
G18795	209467	Chiapas, México	+[c]	-
Rosado	224728	Chiapas, México	+	-
Frijol garbancillo	311903	Jalisco, México	+	-
Colorado criollo	311978	Oaxaca, México	-	-
Bayo rata	313313	Durango, México	+	-
Burro bola	313373	Jalisco, México	+	-
Frijol bayo	319621	Aguascalientes, México	+	-
Frijol bolito	319677	Nayarit, México	+	-
Frijol de ratón	325676	Oaxaca, México	+	+
Poroto amarillo	417723	Veracruz, México	-	-
Coyote	417697	Jalisco, México	-	-
Criollo mateado	417725	Puebla, México	-	-
Negro Sahuatoba	614096	México, México	+	+
Pinto Bayacora	614098	México, México	+	+
G147	164896	Guatemala	-	-
Co. No. 20794	311164	Guatemala	-	-
No. (8-48)	164897	Guatemala	-	-
G2218	311834	Alta Verapaz ,Guatemala	-	-
Frijol de gato	311843	Quiché, Guatemala	+	-
G18756	200967	Jutiapa, Guatemala	-	-
G1856	310514	Copán, Honduras	-	-
Frijol blanco	326106	Copán, Honduras	-	-
Chontaleño	310866	Chontales, Nicaragua	-	-
Frijol pardo tineco	150413	El salvador	-	-
G1739	307820	El salvador	-	-
Frijol tineco color	311786	El salvador	-	-
Frijol tineco negro	311787	El salvador	-	-
Frijol tineco negro	311790	El salvador	-	-
G1362	209488	San José, Costa Rica	-	+
G1637	209482	San José, Costa Rica	-	-
Criollo blanco No. 1	308907	San José, Costa Rica	-	-
Col. No. 23 sel. No. 5	309825	San José, Costa Rica	-	-
Chimbolos	309885	Costa Rica	+	+
CR-93-05	661723	Puntarenas, Costa Rica	+	+
Andean Origin				
DGD 3042	661821	Chuquisaca, Bolivia	-	-
Coscorrón blanco	282104	Chile	-	-
Amarillos	151014	Chile	-	-
Caraota negra	207141	Antioquia, Colombia	-	-

(Table 3.2. continued)

Plant Identification	PI[a] Line	Location/Country of Origin	PvEV1	PvEV2
Querétaro 7-5	207389	Colombia	-	-
Matahambre	207443	Colombia	-	-
Uribe de Arbol	151412	Colombia	-	-
Estrada Rosado	207148	Colombia	-	-
Venadito	207218	Colombia	+	-
Cachaco	207220	Colombia	-	
Guarzo Rojo	207420	Colombia	+	+
Blanco Torta	152311	Ecuador	-	-
Amarillo	299019	Ecuador	-	-
Blanco	415886	Ecuador, Carchi	-	-
Frijol Blanco Grande	415909	Ecuador, Imbabura	-	-
Canario LM 57	269207	Lima, Perú	-	-
Plomo LM 57	269210	Lima, Perú	-	-
Frijol negro	290998	Lima, Perú	-	-
Poroto blanco grande	415954	Lima	-	-
Poroto blanco chico	415955	Lima	-	-
PV-3	260418	Bolivia	-	-
G107	152208	Bolivia	-	-
G108	152215	Bolivia		
W6 17492	661806	Bolivia	-	-
Pardo escuro	306157	Brazil, Minas Gerais	-	-
Tres-Cores	306168	Brazil, Minas Gerais	-	-
Roxtaho	337025	Brazil, Sao Paulo	-	-
Feijao Rajodo Parana	337030	Brazil, Sao Paulo	-	-
Frijao Creme	337091	Brazil, Sao Paulo	-	-
G19250	337501	Brazil, Rio Grande do Sul	-	-
Faveta	352725	Brazil, Paraiba	-	-
G13982	638859	Argentina	-	-
Poroto	638886	Argentina	-	-
Poroto overo	638888	Argentina		
Amarillos	151014	Chile	-	-
Azulillo	151015	Chile	-	-
Cristal Bayo	151026	Chile	-	-
Flageolet Amarillo	151029	Chile	-	-
Mantecoso	282022	Chile	-	-
No. 242	193006	Venezuela	+	-
No. 245	193007	Venezuela	-	-
Preto	306163	Venezuela	-	-

[a] Plant introduction
[b] Negative
[c] Positive

Table 3.3. Wild *Phaseolus vulgaris* tested for *Phaseolus vulgaris* endornavirus 1 (PvEV1) and *Phaseolus vulgaris* endornavirus 2 (PvEV2) by gel electrophoresis and RT-PCR.

Plant Identification	PI[a] Line	Location/Country of Origin	PvEV1	PvEV2
Mesoamerican Origin				
Silvestre 3-5	535421	México	-[b]	-
Silvestre 11	535424	México	-	-
TRAS 308	535425	México	-	-
G12867	318698	Nayarit, México	-	-
G11027E	Unknown	Durango, México	-	-
G10022	329441	Durango, México	-	-
TARS 280	535420	Durango, México	-	-
G23432	Unknown	Ocuilan, México	-	-
G10018A	Unknown	Michoacán, México	-	-
Frijol de coyote	318700	Michoacán, México	-	-
TARS 199	535416	Colima, México	-	-
G12870	318703	Sinaloa, México	-	-
NI 1103	535449	Morelos, México	-	-
NI 1052	535426	Morelos, México	-	-
NI 404	535405	Morelos, México	-	-
NI 1085	535441	Morelos, México	-	-
NI 1088	535444	Morelos, México	-	-
NI 1100	535447	Morelos, México	-	-
NI 1102	535448	Morelos, México	-	-
G12872	325677	Morelos, México	-	-
NI 1052	535426	Morelos, México	-	-
TARS 202	535419	Jalisco, México	-	-
Coyote	417697	Jalisco, México	-	-
G13566	417669	Jalisco, México	-	-
G1255	417782	Jalisco, México	-	-
Frijol de ratón	317349	Jalisco, México	-	-
TARS 134	535413	Jalisco, México	-	-
G12910	417653	Guanajuato, México	-	-
NI 4068	535406	Guerrero, México	-	+[c]
NI 578	535408	Oaxaca, México	-	-
Frijol de ratón	325676	Oaxaca, México	+	+
Frijol Chaneca	325680	Oaxaca, México	-	-
G12876	325682	Oaxaca, México	-	-
G12851	201011	Santa Rosa, Guatemala	-	-
G23439	DGD-2440	Santa Rosa, Guatemala	-	-
G23440	DGD-2459	Guatemala, Guatemala	-	+
3075	638915	Guatemala	-	-
3083	638916	Guatemala	-	-
3074	661845	Guatemala	+	-
3081	661846	Guatemala	-	-
3057	W20507	Guatemala	-	-

(Table 3.3. continued)

Plant Identification	PI[a] Line	Location/Country of Origin	PvEV1	PvEV2
Col. No. 20794	311164	Guatemala	-	-
G50506	Unknown	Solola, Guatemala	-	-
Unknown	343950	Huehuetenango, Guatemala	-	-
G50384	Unknown	Huehuetenango, Guatemala	+	+
G12584	201017	Jutiapa, Guatemala	-	+
G12855	201019	Jutiapa, Guatemala	-	+
G18756	200967	Jutiapa, Guatemala	-	-
G12853	201016	Jutiapa, Guatemala	-	
No.3338	201010	Quetzaltenango, Guatemala	-	+
G23434C	Unknown	El Progreso, Guatemala	-	-
CO 78-G-3A	535414	Jalapa, Guatemala	-	-
Frijol Seco	661847	Sacatepequez, Guatemala	-	-
G13504	201014	Ahuachapán, El Salvador	-	+
G12852	201013	Ahuachapán, El Salvador	-	-
G50722	SB-6	El Paraiso, Honduras	-	-
G1856	310514	Copán, Honduras	-	-
TARS 148	535398	Puerto Rico	-	-
TARS 258	535401	Puerto Rico	-	-
Andean Origin				
G24688	OT-276	Boyacá, Colombia	-	-
G24615	OT-161	Cundinamarca, Colombia	-	-
W-C1111	390770	Apurimac, Perú	-	-
NI 622	535411	Apurimac, Perú	-	-
G23455	DGD-2581	Cuzco, Perú	-	-
G23459	DGD-2600	Cuzco, Perú	-	-
G23421	DGD-2152	Junín, Perú	-	-
G23442	DGD-2484	Cochabamba, Bolivia	-	-
G23443	DGD-2491	Chuquisaca, Bolivia	-	-
DGD 3012	661818	Chuquisaca, Bolivia	-	-
G23445	DGD-2501	Tarija, Bolivia	-	-
DGD 2501	661822	Tarija, Bolivia	-	-
DGD 3020	661819	Tarija, Bolivia	-	-
TARS 283	535423	Brazil	-	-
G21057	642122	Argentina	-	-
G19890	DGD-626	Salta, Argentina	-	-
Poroto del campo	638880	Argentina	-	-
Poroto del Zorro	W6 16998	Argentina	-	-
W6 17499	661807	Salta, Argentina	-	-
A2-007-1	638881	Argentina	-	-
A2-017-1	642128	Argentina	-	-
VAVILOV 6369	661801	Argentina	-	-
Poroto	642125	Argentina	-	-

(Table 3.3. continued)

Plant Identification	PI[a] Line	Location/Country of Origin	PvEV1	PvEV2
G10021	266910	Argentina	-	-
A2-006-2	640970	Argentina	-	-
Poroto del Zorro	642124	Argentina	-	-

[a] Plant introduction
[b] Negative
[c] Positive

Testing 36 common bean landraces of Mesoamerican origin for PvEV1 and PvEV2 yielded 14 (39%) that were endornavirus-infected; but only 3 of 42 (7%) tested landraces of Andean origin were infected (Table 3.2 and 3.4). Testing 59 wild *P. vulgaris* genotypes of Mesoamerican origin resulted in nine infected (15%): six with PvEV2, two with PvEV1 and PvEV2, and one with PvEV1. In contrast, these endornaviruses were not detected in 26 wild *P. vulgaris* of Andean origin (Table 3.3 and 3.4). Furthermore, these two endornaviruses were not detected in 18 other *Phaseolus* species. The summary of percentages of common bean endornaviruses infection is shown in Table 3.4. PvEV1 or PvEV2 was not detected in 18 other *Phaseolus* species which included four other domesticated species: *P. acutifolius*, *P. coccineus*, *P. dumosus*, and *P. lunatus* (Table 3.5).

Analyses of the nucleotide sequences of the RT-PCR products of PvE1 and PvEV2 obtained from selected *P. vulgaris* genotypes indicate that similar or closely related endornaviruses infecting wild *P. vulgaris* were also infecting the common bean landraces, cultivars, and breeding-lines (Table 3.6). Sequences of both viruses from cultivars and breeding-lines (Mesoamerican and Andean) were 98-100% identical to the corresponding sequence of PvEV1 and PvEV2 infecting Black Turtle Soup (AB719398). Most PCR products of PvEV2 from cultivars, breeding-lines, landraces, and wild *P. vulgaris* that were selected for sequencing were 98-99% identical to PvEV2 of Black Turtle Soup (Table 3.6). However, there were four

common bean landraces and two wild *P. vulgaris* infected with PvEV1 that yielded PCR products with sequences that ranged from 95-96% identity to the corresponding sequence of PvEV1 of Black Turtle Soup (AB719397).

Phylogenetic analysis of PvEV1 showed that wild *P. vulgaris* genotypes were grouped separately from most of landraces and cultivars and breeding lines (Figure 3.4). This separated clade included two wild genotypes from Guatemala and two landraces from Mexico (Table 3.2 and 3.3, Figure 3.4). Other landraces and cultivars and breeding lines clustered in the same clade. The analysis also found that one landrace, PI 309855, from Costa Rica was completely out of the group (Table 3.2, Figure 3.4). Figure 3.5 shows the phylogenetic analysis of PvEV2. The tree shows that *P. vulgaris* genotypes separated into two major clades that included a mixture of wild, landraces, cultivars, and breeding lines from both centers of domestication. However, three genotypes, PI 207420 from Colombia, and Flor 9623 and Maverick from USA, clustered into an individual branch of the tree (Figure 3.5).

Table 3.4. Summary of the occurrence of *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) in cultivars, breeding lines, landraces, and wild *P. vulgaris* of Mesoamerican and Andean origins.

Origin	Genotype	Endornavirus			Total virus-infected	Total virus-free	Total tested	% infected
		PvEV1	PvEV2	PvEV1 and PvEV2				
Meso-america	Cultivars and breeding lines	0	0	63	63	5	68	93
	Landraces	8	1	5	14	22	36	39
	Wild	1	6	2	9	50	59	15
Andes	Cultivars and breeding lines	2	2	1	5	37	42	12
	Landraces	2	0	1	3	39	42	7
	Wild	0	0	0	0	26	26	0

Table 3.5. *Phaseolus* species tested for *Phaseolus vulgaris endornavirus1* and *Phaseolus vulgaris endornavirus 2* by gel electrophoresis and found to be negative.

Species	Plant Introduction Line	Location/Country of Origin
acutifolius var. *acutifolius*	535208	Durango, México
acutifolius var. *acutifolius*	535214	Arizona, USA
acutifolius var. *acutifolius*	256424	El Salvador
acutifolius var. *tenuifolius*	535382	Durango, México
angustissimus	535273	New México, USA
angustissimus	535272	New México, USA
augusti	653237	Argentina
augusti	632862	Argentina
augusti	W6 17480	Argentina
carteri	653247	Baja California, México
coccineus	317572	Chimaltenango, Guatemala
coccineus	203931	Hidalgo, México
dumosus	195388	Totonicapán, Guatemala
dumosus	201340	Puebla, México
dumosus	311196	Jalapa, Guatemala
dumosus	194585	Solola, Guatemala
filiformis	535307	Sonora, México
filiformis	535296	Baja California, México
filiformis	535293	Baja California, México
filiformis	632353	Durango, México
filiformis	535300	Arizona, USA
filiformis	535310	Texas, USA
filiformis	535306	Arizona, USA
filiformis	535292	Puerto Rico
glabellus	535311	San Luis Potosí, México
glabellus	653231	México
glabellus	638836	México
hintonii	535378	Coahuila, México
hintonii	535379	Coahuila, México
leptostachyus	535320	Durango, México
leptostachyus	535328	Oaxaca, México
leptostachyus	535314	Oaxaca, México
leptostachyus	535318	Federal District, México
leptostachyus	535317	Jalisco, México
leptostachyus	535323	Nayarit, México
leptostachyus	535329	Jalapa, Guatemala
leptostachyus	535330	Jalapa, Guatemala
leptostachyus	494131	San Luis Potosí, México
lunatus	256820	Ecuador
lunatus	535344	Chaco, Argentina
lunatus	535346	Venezuela
lunatus	256423	Santa Ana, Salvador

(Table 3.5. continued)

Species	Plant Introduction Line	Location/Country of Origin
lunatus	256809	Cauca, Colombia
maculatus subsp. *ritensis*	494138	Jalisco, México
maculatus subsp. *ritensis*	535372	Arizona, USA
maculatus subsp. *ritensis*	661844	México
microcarpus	430196	México
microcarpus	535362	Jalisco, México
microcarpus	535361	Oaxaca, México
microcarpus	535359	Chiapas, México
microcarpus	430197	México
microcarpus	535363	Durango, México
microcarpus	W6 15700	México
microcarpus	535358	Oaxaca, México
microcarpus	535353	Jalisco, México
oligospermus	535365	Guatemala
parvifolius	535376	Nuevo León, México
parvifolius	653250	Jalisco, México
polystachios subsp. *sinuatus*	642133	Texas, USA
xanthotrichus	640978	Guatemala
zimapanensis	535385	San Luis Potosí, México
zimapanensis	535381	Jalisco, México
zimapanensis	535388	Querétaro, México

Table 3.6. Percentage of nucleotide sequence identity (segment of the RdRp) of *Phaseolus vulgaris endornavirus1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) isolated from different *P. vulgaris* genotypes with the corresponding sequences of PvEV1 and PvEV2 from the cultivar Black Turtle Soup.

Genotype/Plant Identification	PI[a] Line/Market Class	Nt Identity (%)	
		PvEV1	PvEV2
Wild *Phaseolus vulgaris*			
Mesoamerican Origin			
G50384	Unknown	95	NS[b]
3074	661845	95	-[c]
Frijol de ratón	325676	99	99
NI 4068	535406	-	99
Landraces			
Mesoamerican Origin			
G18795	209467	95	-
Rosado	224728	95	-
Bayo rata	313313	97	-
Frijol bayo	319621	95	-
Frijol bolito	319677	96	-

(Table 3.6. continued)

Genotype/Plant Identification	PI[a] Line/Market Class	Nt Identity (%)	
		PvEV1	PvEV2
Andean Origin			
Negro Sahuatoba	614096	99	99
Pinto Bayacora	614098	100	99
G2218	311834	98	-
CR-93-05	661723	99	99
Venadito	207218	99	-
Guarzo Rojo	207420	99	98
No. 242	193006	99	-
Unknown	306133	98	NS
Merlot	Small red	99	99
Cultivars/Breeding Lines			
Mesoamerican Origin			
Midnight	Black	99	99
Eclipse	Black	100	99
T-39	Black	100	99
Hungerford	Great Northern	99	99
93:208G	Great Northern	99	NS
Matterhorn	Great Northern	99	99
USPK7-5	Pink	99	99
UC Pink 9634	Pink	99	99
Santa Fe	Pinto	100	99
Stampede	Pinto	99	99
Maverick	Pinto	99	98
Dublin	Navy	100	99
Navigator	Navy	100	99
Aurora	Navy	99	99
R02002	Small red	100	99
R97003	Small red	100	NS
Flor 9623	Flor de Mayo	99	98
Andean Origin			
Bellagio	Dark Red Kidney	99	-
USDK-CBB-15	Dark Red Kidney	99	99
Majesty	Cranberry	98	-
BD 1002	Cranberry	99	-
CPC99814	Cranberry	99	-
Fuji	Navy	99	-
Red Rover	Light Red Kidney	-	99

[a] Plant introduction, [b] Not sequenced, [c] Virus negative

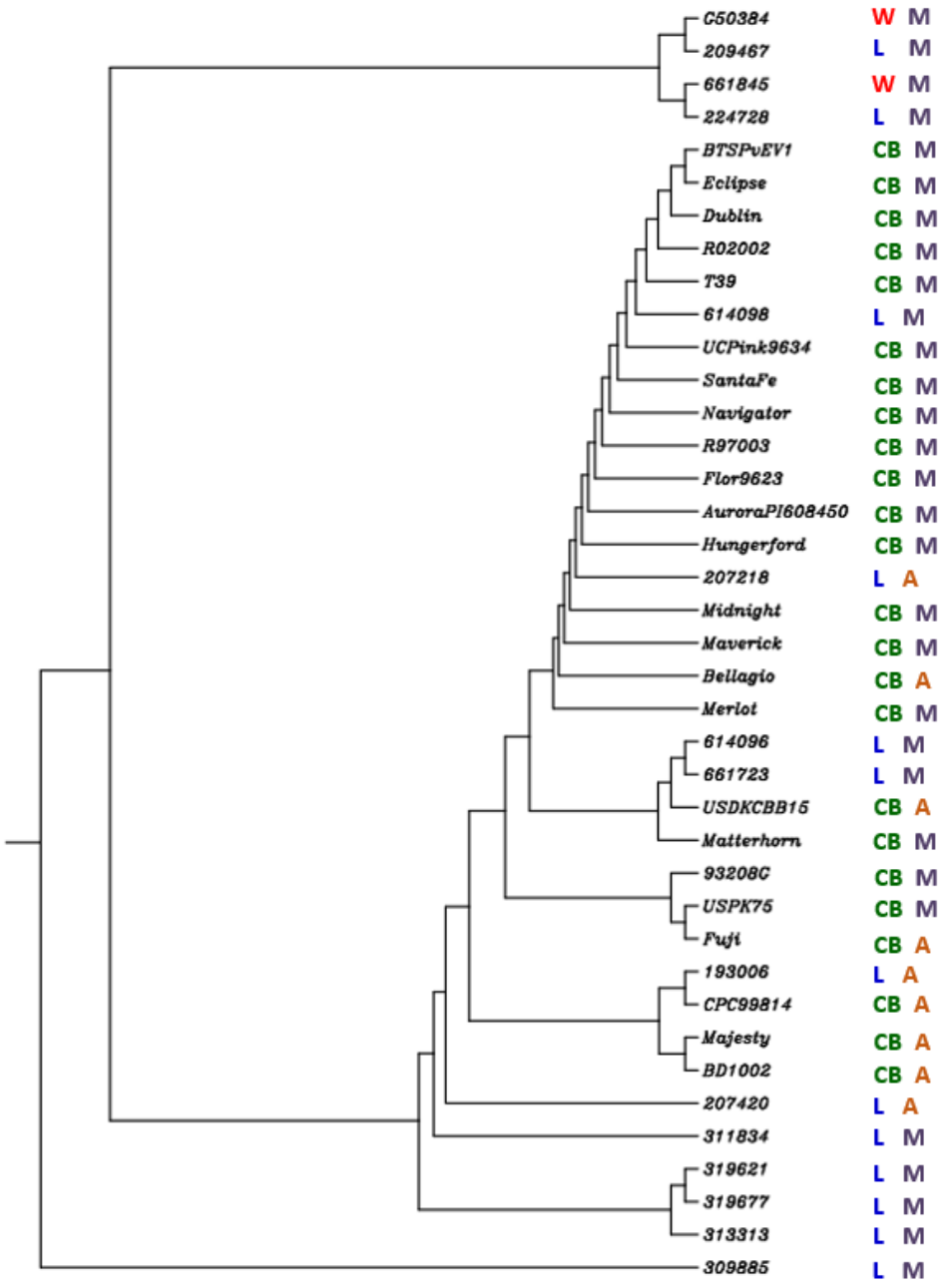

Figure 3.4. Phylogenetic tree using partial RdRp nucleotide sequences of *Phaseolus vulgaris endornavirus 1*. The tree was constructed using ClustalW and MEGA with 1000 bootstrap replications. Abbreviations represent: W, wild; L, landraces; CB, cultivars and breeding lines; M, Mesoamerica; A, Andes.

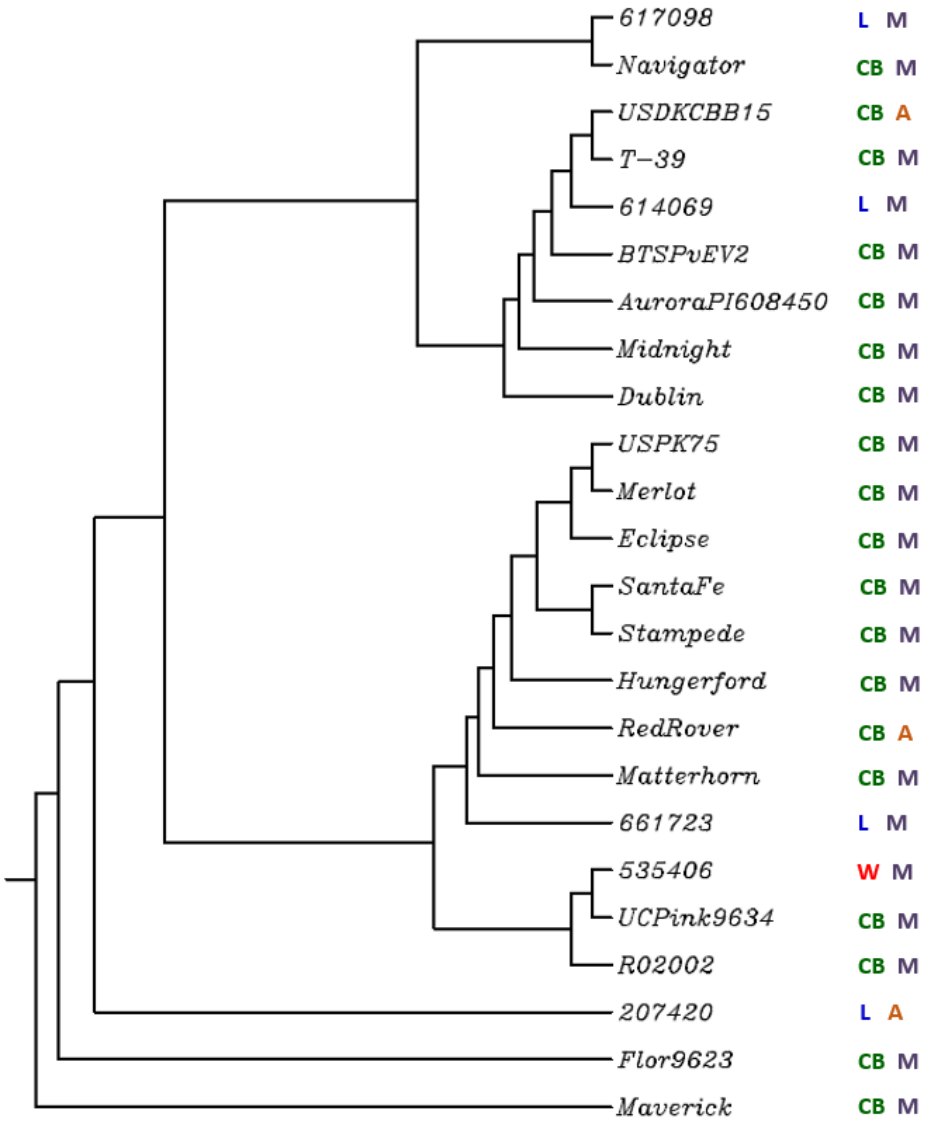

Figure 3.5. Phylogenetic tree using partial RdRp nucleotide sequences of *Phaseolus vulgaris endornavirus 2*. The tree was constructed using ClustalW and MEGA with 1000 bootstrap replications. Abbreviations represent: W, wild; L, landraces; CB, cultivars and breeding lines; M, Mesoamerica; A, Andes.

3.5 Discussion

The results from this investigation show that Mesoamerican-domesticated common bean genotypes are often double-infected with PvEV1 and PvEV2. Moreover, these endornaviruses were detected in some wild *P. vulgaris* from this region. In contrast, these viruses were not detected in wild *P. vulgaris* from the Andean region and were present in a low percentage in Andean-domesticated common bean genotypes. Although PvEV1 and PvEV2 double-infections were present in nearly all common bean cultivars and breeding-lines of Mesoamerican origin, three cultivars and one breeding line were endornavirus-free. Pedigree examinations of two endornavirus-free cultivars of Mesoamerican origin, Othello and Jackpot, revealed that some of their progenitors were endornavirus-free Andean genotypes. Therefore, it is likely that during the development of these two cultivars, virus-free lines were selected. Okada and collaborators (Okada et al., 2013) reported that two Andean cultivars, Bellagio and Majesty, were infected by PvEV1 alone. After examining the pedigree of these two cultivars, we determined that some of the progenitors were of Mesoamerican origin infected with PvEV1 and, therefore, the likely source of the virus. Similarly, in the case of two Andean cultivars, Closeau and Red Rover, infected with PvEV2, pedigree examinations revealed that a Mesoamerican landrace from Costa Rica (PI 209488, Table 3.2) was a common ancestor of these cultivars and likely the source of PvEV2. This landrace was tested and found to be infected with PvEV2. It is likely that the infection of Andean genotypes with endornaviruses is the product of introgressions among gene pools by modern plant breeding. This is not surprising because endornaviruses are transmitted at relatively high percentages through both gametes (Fukuhara and Moriyama, 2008).

The sources of endornaviruses in some crop cultivars, such as rice and barley, have been determined to be infected cultivars or breeding-lines (Valverde et al., 2011; Zabalgogeazcoa et

al., 1993). Therefore, endornaviruses in crops most likely originated from infected wild species that were selected during crop domestication and introduced to some cultivars during the breeding process. The low (15%) occurrence of PvEV1 and PvEV2 in wild species, medium (39%) in land races, and high (93%) in cultivars and breeding-lines supports this idea.

Reports of mutualistic-symbiotic interactions between plants and viruses are very limited (Marquez et al., 2007; Roossinck, 2013; Zabalgogeazcoa and Gildow, 1992). Endornaviruses do not seem to have an adverse effect on common bean domesticated in Mesoamerica. All tested endornavirus-infected common bean cultivars and breeding-lines of Mesoamerican origin were infected simultaneously with both PvEV1 and PvEV2. These two endornaviruses seem to coexist in the plant host as double-infections, and at the present time we do not know if they interact with each other. We hypothesize that PvEV1 and PvEV2 are in a symbiotic relationship with Mesoamerican-domesticated common bean. The host allows replication of the virus which in turn does not cause apparent disease, but whether this interaction is of mutualistic nature is not known. The benefit or benefits, if any, which the virus provides to the host, are yet to be elucidated. Nevertheless, in the case of most common bean genotypes of Andean origin, the lack of these endornaviruses does not seem to have an adverse effect. It is possible that the putative beneficial effect that these endornaviruses may have on common bean was not needed for production during domestication in the Andean region.

It is not known why these endornaviruses are not present in the tested wild *P. vulgaris* of Andean origin. One possibility is that wild *P. vulgaris* which originated in Mesoamerica (Bitocchi et al., 2012) was first infected with these endornaviruses and only virus-free plants were disseminated to the Andean region. Alternatively, virus-infected plants were disseminated but not able to adapt or were not selected for domestication. A third possibility is that the

infection of wild *P. vulgaris* with these endornaviruses could have happened after the continental dissemination of this plant species.

Although not nearly-isogenic, preliminary studies with two lines of the cultivar Black Turtle Soup, one double-infected with PvEV1 and PvEV2 and the other one endornavirus-free, did not reveal obvious phenotype differences (Okada et al., 2013). Nevertheless, when planted in the field, these lines showed differences in traits of agronomic importance such as days to maturity. Moreover, inoculations of these two lines with *Tobacco ringspot virus* and seed germination tests yielded differential responses (Khankhum and Valverde, 2013).

PvEV1 or PvEV2 was not detected in 18 other *Phaseolus* species which included four other domesticated species: *P. acutifolius, P. coccineus, P. dumosus,* and *P. lunatus.* This suggests a relatively recent introduction of these two endornaviruses into *P. vulgaris* or that these two endornaviruses have a high degree of host specificity. In contrast, *Bell pepper endornavirus,* a close relative of PvEV2, has been shown to occur in many genotypes of four domesticated *Capsicum* species (Okada et al., 2011; Okada et al., 2013).

Limited nucleotide sequence information on selected genotypes suggests that the endornaviruses PvEV1 and PvEV2 infecting domesticated common bean are similar to those infecting wild *P. vulgaris*. This is particularly true for PvEV2 because nucleotide sequence identities among the various genotypes ranged from 98-99%. Although in the case of PvEV1, sequence identities of this endornavirus from some landraces and wild *P. vulgaris* genotypes were more divergent (95-96%) with respect to PvEV1 from Black Turtle Soup. This result was supported by phylogenetic analyses of the RNA-dependent RNA polymerase (RdRp) of PvEV1 and PvEV2 in which wild *P. vulgaris* genotypes infected with PvEV1 clustered in a specific clade, apart from PvEV1 infected landraces, cultivars, and breeding lines. The analysis of the

RdRp of PvEV2 suggests that this virus could be grouped into two major clades, regardless of infecting wild, landraces, cultivars, and breeding lines or their geographical origin.

The results obtained in this investigation can be added to the body of evidence that support the existence of two gene-divergent gene pools of common bean. Whereas the tested common bean cultivars and breeding lines from the Mesoamerican gene pool were almost 100% infected by endornaviruses, genotypes from the Andean gene pool were, with few exceptions, virtually endornavirus-free. It is interesting to mention that when common bean cultivars currently grown in countries of these two regions were tested, the two endornaviruses were detected in cultivars from both regions. Most likely, this is due to germplasm exchange among common bean breeders.

Currently, it is not known the effect that these viruses have in the common bean plant. It is possible that they may interact with acute viruses, other plant pathogens, or other biotic or abiotic agents. The development of isogenic lines and/or the development of an inoculation method for these viruses will be essential to address some of these questions.

CHAPTER 4.
ASSOCIATION OF *PHASEOLUS VULGARIS ENDORNAVIRUS 1* AND *PHASEOLUS VULGARIS ENDORNAVIRUS 2* WITH SEED GERMINATION, PLANT GROWTH, PIGMENT CONTENT, AND GRAIN YIELD OF BLACK TURTLE SOUP COMMON BEAN

4.1 Introduction

Pathological changes in plants due to abiotic and biotic stresses include changes in height, coloration, photosynthesis, reproductive behavior, low chlorophyll content, reduction in growth, and reduction in dry matter (Hull, 2014; Naylor and Giles, 1982). Systemic symptoms in plants caused by viral infection consist of foliar mosaic, yellows, chlorosis, necrosis, ring spots, wilting, leaf rolling, growth reduction, and tissue deformation. Chlorosis occurs when infected cells lose chlorophyll and other pigments (Hull, 2014). Reduced chlorophyll content in virus-infected plant affects photosynthetic capacity and chloroplast structure during leaf development (Funayama-Noguchi and Terashima, 2006; Guo et al., 2005). Not much information is available on the effect of plant viruses in common bean chlorophyll content. The reduction in photosynthesis of virus-infected plants is correlated with the reduction of rubisco and proteins associated with the photosynthetic pathway (Naidu et al, 1986; van Kooten et al., 1990). Reduction of morphological and growth characters of mustard (*Brassica juncea* var. *tsatsai*) infected with *Turnip mosaic virus* and banana (*Musa* spp.) infected with *Banana bunchy top virus* (BBTV) are other examples of the effects of virus infections (Guo et al., 2005; Hooks et al., 2008). Other effects of BBTV infection include reduced petiole length and distance between petioles, pseudostem diameter, plant height and canopy, leaf area and also significant decreases in chlorophyll a and b and total chlorophyll content (Guo et al., 2005; Hooks et al., 2008).

In common bean breeding programs, genotypes of broad genetic background have been used to improve the quantity and quality of common bean. Variation in growth type has been

used to prevent harvest losses (Kelly, 2010). In the case of green bean, pod size is important because it has marketable value (Morales, 2006). For dry bean, pod number, number of seeds per pod, and seed weight are recognized as important characters by breeders, as well as growers (Hampton, 1975; Rainey and Griffths, 2005). These phenotypic characters can be changed by virus infections. The potyviruses *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are the most prevalent viruses of common bean (Morales, 2006). Both BCMV and BCMNV occur worldwide and in some areas can limit common bean production (Hampton, 1975; Morales, 2006; Schwartz et al., 2005). BCMV-infected common bean produced curved pods, mottled pods and reduced size (Morales, 2006; Schwartz et al., 2005). *Bean yellow mosaic virus* (BYMV) is another potyvirus causing problems in common growing areas around the world (Morales, 2006; Schwartz et al., 2005). Symptoms of BYMV-infected common bean consist of plant malformation and plant death depending on the viral strain and the common bean cultivars. Other economically important common bean viruses include *Bean golden mosaic virus* (BGMV) and *Bean golden yellow mosaic virus* causing flower abortion, plant malformation, and pod distortion (Blair et al., 1995, Morales, 2006; Morales and Anderson, 2001; Schwartz et al., 2005). Pod malformation affects the number of pods per plant and number of seeds per pod (Román et al., 2004). *Cucumber mosaic virus* (CMV) is a virus with one of the broadest spectrums of host plants and includes common bean. Some strains of CMV have been shown to be seed-borne in common bean (Hampton and Francki, 1992).

With the exception of *Vicia faba endornavirus* (VfEV), which is associated with male sterility of faba bean (*Vicia faba*), most endormaviruses do not appear to affect the phenotype of the host (Pfeiffer, 1998). Until now, there are no other reports of effects of persistent plant viruses on plant phenotype. The common bean cv Black Turtle Soup (BTS) and other common

bean cultivars of various market classes were reported to be infected by two distantly related endornavirus species; *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) (Okada et al., 2013).

4.2 Objective

The objective was to investigate the effect PvEV1 and PvEV2 on seed germination, plant growth, pigment content, and grain yield of Black Turtle Soup common bean.

In this study, selections of two BTS common bean lines, one infected with PvEV1 and PvEV2 and another endornavirus-free were used in comparative experiments to evaluate seed germination, pigment content, growth, and phenotypic characters of endornavirus-infected and endornavirus-free lines.

4.3 Materials and Methods

4.3.1 Plant materials and growth conditions

Two common bean lines of the cv BTS, one double-infected with PvEV1 and PvEV2 and designated as BTS+ and the other one virus-free and designated BTS- obtained from previous investigations (Okada et al., 2013) and increased at least five generations by self-pollination, were used in all the comparative studies. Moreover, six selections from those lines designated BTS+8, BTS+13, BTS+18, BTS-9, BTS-16, and BTS-21were used as three replicates of each plant type. Plants were grown in a greenhouse and a microplot (Figure 4.1 A) located on the Baton Rouge campus of Louisiana State University. The greenhouse day/night temperatures averaged 25/18°C. The presence or absence of PvEV1 and PvEV2 were confirmed by dsRNA extraction (Valverde et al., 1990a) and RT-PCR using virus-specific primers (Okada et al., 2013).

Before planting, seeds of the two lines and respective selections were surface sterilized in 10% sodium hypochlorite for 10 min, washed in sterilized distilled water 3 times, and pre-germinated in the laboratory. Seeds were planted in 8-inch clay pots containing steam sterilized soil mixed as two parts soil, one part sand, and three parts sphagnum moss. The Potting Mix Miracle-Gro® soil (Miracle-Gro® Lawn Products, Inc., Marysville, OH) was used as sphagnum moss. Only a single plant was grown per pot. All tested plants were fertilized using Osmocote® Smart-Release® Plant Food Outdoor &Indoor (Miracle-Gro® The Scotts Company LLC, Marysville, OH) once, 2 weeks after planting. Plants were sprayed weekly for thrips and whiteflies using Imidacloprid (Bayer Environmental Science, Research Triangle PK, NC) or Avid (Syngenta Crop Protection, Inc., Greensboro, NC).

4.3.2 Seed germination

Experiments to evaluate seed germination were conducted following protocols for germination suggested by Dr. Marc A. Cohn (Department of Plant Pathology and Crop Physiology, Louisiana State University). Seeds from each line were surface sterilized and washed as described above and placed on sterilized plastic petri dishes containing a circle of sterilized filter paper (Whatman No. 2). Seventeen seeds were placed on the paper and another sterilized filter paper was placed on top. Sterile distilled water (8 ml) was added to each petri dish. The lid of the plate was then closed, and the plate was placed on the laboratory bench where the average room temperature was about 25^{o}C. Seeds were examined every 24 h until all seeds completed germination. Percentage of seed germination was calculated by obtaining the germination average of three replications. Radical root length was measured 3 days after germination. These experiments were done three times with three replications for each selection in each experiment. The experimental layout was a completely randomized design (CRD).

Statistical analyses were performed using SAS Proc Mixed (SAS v. 9.2, SAS Inc., Cary, NC). Fisher's LSD test at the 0.05 probability level was used to test for significant differences among means.

4.3.3 Foliar pigment content

Ten mature unfolded leaves from 1-month-old plants were collected from plants grown in the greenhouse. A pool of 10 leaves were ground with liquid nitrogen using mortar and pestle, and kept separately in a 2 ml microcentrifuge tubes. For anthocyanin analysis 0.4 g were used and 0.1 g for chlorophyll and carotenoid analyses. All tubes were covered with aluminum foil to protect samples from the light. Total anthocyanin content was determined following the method described by Neff and Chory (1998). Chlorophyll a and b content were determined following the method described by Arnon (1949). Carotenoid content was determined following the method described by Kirk and Allen (1965). The absorbance was measured with a BioMate^{TM} 3 spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) at 530 and 657 nm for anthocyanin (Neff and Chory, 1998) and at 663 and 645 nm for the two chlorophylls (Arnon, 1949). Carotenoid was measured at the same spectra as chlorophyll with additional measurement at 480 nm (Kirk and Allen, 1965). Pigment contents in leaves were calculated using equations described in the publications listed above. Common bean plants inoculated with *Sunn-hemp mosaic virus* (SHMV) were used as a control to measure decreased chlorophyll content due to virus infection. The two phenotypic characters, including plant wet weight and plant height were also measured before collecting trifoliate leaves to measure pigment content. Each line was tested three separate experiments with three biological replications. Means were obtained from the three separate tests. The experimental layout was a completely randomized design (CRD). Statistical analyses were performed using SAS Proc Mixed (SAS v. 9.2, SAS Inc., Cary, NC).

Fisher's LSD test at the 0.05 probability level was used to test for significant differences among means.

4.3.4 Agronomic characters

Common bean lines were planted in 6 L plastic pots and grown in a microplot (Figure 4.1 A) from May to July 2015. This experiment was conducted twice with a 2-weeks interval between plantings. Pots were placed in the ground using randomized complete block design. Single common bean plants were grown in pots containing 5 L of soil mix. There were three blocks per planting with five plots/block in which each plot contained both BTS+ and BTS- lines and one BTS+ and one BTS- plant inoculated with SHMV. Plants were watered with 4 L of water every 2 days, and watering stopped when the pods began to dry. After planting, the number of days before flowering was recorded (when the plant had five opened flowers, Figure 4.1 B) and before pod maturation (when the plant had five pods more than 5 cm long, Figure 4.1 C). When mature pods were ready to harvest, pods of every single plant were harvested. The number of pods per plant was recorded. Five pods were randomly selected to be measured (length and width). Length was measured from the peduncle connection point to pod apex, excluding the pod beak, and width and thickness were measured on the middle portion of the pod (Silva and Antunes, 2003) using digital caliper (Figure 4.1 D). Two seeds per pod were randomly collected and used to measure seed size. The dry weight of seed per plant was determined by collecting all the seeds from each plant. The dry weight of 100 seeds pooled from five plants (pooled from one plot) was determined. Common bean plants inoculated with SHMV were analyzed as described above and used as a control for evaluating the effects due to virus infection. Data were statistically analyzed with the combined data of the two planting set using

SAS Proc Mixed (SAS v. 9.2, SAS Inc., Cary, NC). Fisher's LSD test at the 0.05 probability level was used to test for statistical significance.

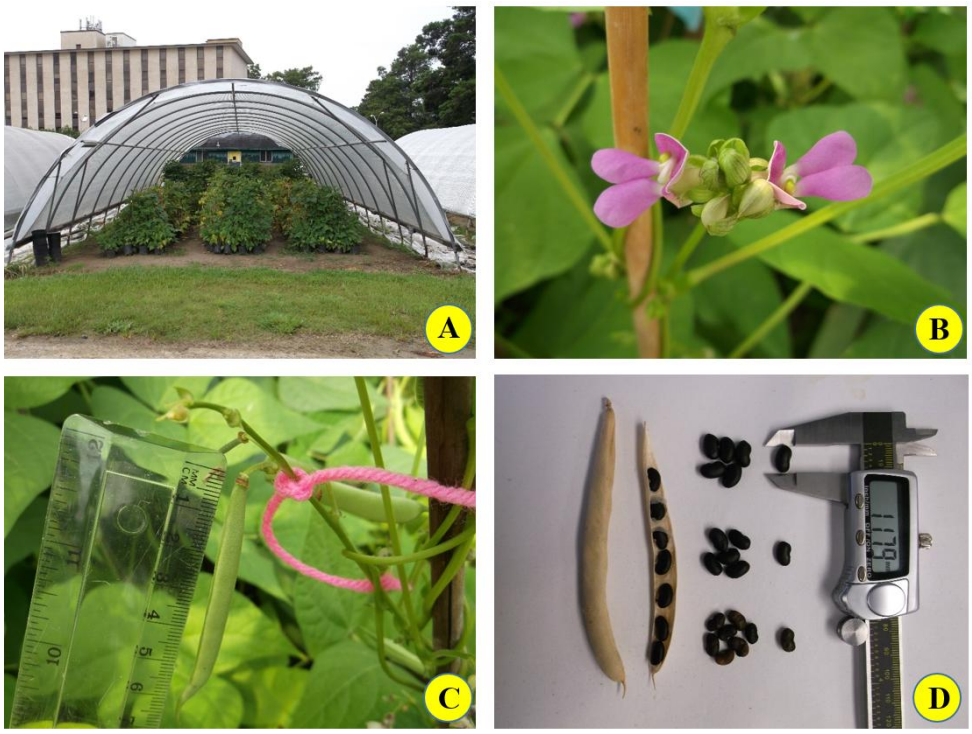

Figure 4.1. (A) common bean Black Turtle Soup plants growing in a microplot; (B) Black Turtle Soup flowers; (C) illustration of the recording of pod size; and (D) determination of the number of seeds per pod, and seed size.

4.4 Results

4.4.1 Seed germination

The BTS+ line and selections germinated faster than the BTS- line and selections. Two selections of BTS- (BTS-16 and BTS-21) had significantly lower percent seed germination than the BTS+ selections at day 2 but not at day 1. All tested seeds reached 100 percent germination at day 3 (Table 4.1).

Radical root lengths of the BTS+ and BTS- lines and selections measured 3 days after germination resulted in radical length ranging from 37.2 to 44.9 cm for BTS+ and selections and

26.5 to 34.8 cm for the BTS- lines and selections. However, there were two selections of BTS- (BTS-16 and BTS-21) that were significantly shorter in length of radical root than the other two BTS- as well as the four BTS+ and selections. The results are shown in Table 4.2.

Table 4.1. Germination of seed collected from two Black Turtle Soup lines and six selections. One line and three selections infected with PvEV1 and PvEV2 (BTS+) and one line and three selections endornavirus-free (BTS-).

Line/ selection	Number of germinated seeds			Percentage of seed germination		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
BTS+	10.9±2.1 b*	15.9±0.9 a	17±0 a	64.23±12.36 b	94.00±5.48 a	100±0 a
BTS+8	7.6±4.5 cd	15.9±0.7 a	17±0 a	45.06±26.65 cd	93.64±4.10 a	100±0 a
BTS+13	5.8±1.7 d	15.5±1.2 a	17±0 a	34.59±10.45 d	91.64±7.42 a	100±0 a
BTS+18	8.4±3.2 c	16.6±0.7 a	17±0 a	49.76±19.02 c	98.00±4.47 a	100±0 a
BTS-	3.0±2.7 e	15.7±1.4 a	17±0 a	18.00±16.43 e	92.47±8.51 a	100±0 a
BTS-9	2.3±2.2 e	15.8±1.2 a	17±0 a	14.00±13.42 e	93.29±7.44 a	100±0 a
BTS-16	1.2±1.4 e	11.4±1.5 b	17±0 a	7.18±8.32 e	67.41±9.31 b	100±0 a
BTS-21	1.9±1.9 e	11.1±2.2 b	17±0 a	10.29±11.96 e	55.76±16.17 bc	100±0 a

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).

Table 4.2. Radical root lengths of two Black Turtle Soup lines and six selections. One line and three selections infected with PvEV1 and PvEV2 (BTS+) and one line and three selections endornavirus-free (BTS-), measured three days after germination.

Line/Selection	Radical length (cm)
BTS+	37.0±1.2 bc*
BTS+8	41.7±1.0 ab
BTS+13	37.2±2.8 bc
BTS+18	44.9±3.8 a
BTS-	32.8±2.4 c
BTS-9	34.8±3.0 c
BTS-16	27.1±4.0 d
BTS-21	26.5±2.6 d

*Mean values followed by the same letter of a column do not differ significantly according to the LSD test ($p<0.05$).

4.4.2 Foliar pigment content

Carotenoid, chlorophyll a and b, and anthocyanin were recorded as milligrams per gram of trifoliate leaf, and results are shown in Table 4.3. The carotenoid content extracted from tested BTS+ line and selections was significantly greater than that of BTS- line and selections (Table 4.3). The amounts of carotenoid ranged from 0.081 to 0.084 mg per g for BTS+ line and selections, including BTS+ inoculated with SHMV. The carotenoid amounts in the BTS- line and selections ranged from 0.071 to 0.073 mg per g including the BTS- line inoculated with SHMV. The contents of chlorophyll a and b of the BTS+ line and selections were significantly lower than the BTS- lines. Total chlorophyll content was significantly lower in the BTS+ line and selections than in the BTS- lines (Table 4.3). The amount of total chlorophyll ranged from 2.76 to 2.75 mg per g of leaf in the BTS+ line and selections, while in the BTS- line and selections, it ranged from 3.39 to 3.43 mg per g wet weight. Both BTS+ and BTS- lines inoculated with SHMV yielded lower total chlorophyll content than non-inoculated BTS+ (2.64 mg per g in BTS+ and 3.31 mg per g in BTS-). However, in the case of chlorophyll b, BTS+ plants inoculated with SHMV yielded lower amounts than the BTS-, including the non-inoculated plants. Amount of anthocyanin varied among tested BTS lines and selections. They ranged from 0.071 to 0.095 mg per g wet weight of leaf (Table 4.3).

4.4.3 Wet weight and plant height

Only minor variation was detected in total plant weight with one BTS- selection exhibiting lower weight than three BTS+ selections and lower weight for the SHMV infected BTS- selection than most of the other selections of both lines (Table 4.3). The BTS- line inoculated with SHMV showed the lowest wet weight (23.9 g). Like total plant weight, plant height measured from the stem base to the apex resulted in no significant differences among

tested plants (Table 4.3). The only exception was BTS+8 which showed a higher height than all other plants.

4.4.4 Agronomic characters

During the two plantings, the average temperature of microplots was 34±2^{o}C. The lowest and highest temperature for both plantings were 32^{o}C and 38^{o}C, respectively. The time to flowering of each line was recorded (Tables 4.4 and Figure 4.1 B). In general, the results showed that the BTS+ line and selections did not differ in time to flowering from the BTS- line and selections (Table 4.4). BTS+ infected with SHMV had significantly longer time to flowering than BTS- inoculated with SHMV. BTS+ set flowers significantly faster than BTS+/SHMV.

The BTS+ line and selections generally did not differ in days to pod formation from the BTS- line and selections, including BTS- infected with SHMV (Table 4.4 and Figure 4.1 C). However, BTS+ infected with SHMV had significantly longer days to pod formation than BTS-, BTS+, and BTS-/SHMV. BTS- set pods significantly faster than BTS-/SHMV (Table 4.4).

The number of pods per plant did not show significant differences between the BTS+ and BTS- lines and selections including those infected with SHMV (Tables 4.4). The number of seeds per pod showed no differences between the BTS+ and BTS- lines and selections (Tables 4.4). However, BTS+ and BTS- infected with SHMV yielded significant lower number of seeds per pod (4.4-5.4 seeds) than non-SHMV infected lines which yielded 6.4 to 7.0 seeds per pod. BTS+ infected with SHMV yielded significantly lower number of seeds per pod than BTS- infected with SHMV (Table 4.4).

The BTS+ lines generally yielded longer pods (111.9-115.9 cm) than the BTS- lines (105.6-106.4 cm) (Tables 4.4). There were no significant differences between BTS+ infected with SHMV and BTS- infected with SHMV; however, the SHMV infected BTS+ plants yielded

shorter pod lengths than BTS+ lines and selections. In general, there were no differences in pod width and thickness among BTS+ and BTS- lines and selections (Table 4.4).

Seed size was not different among BTS+ and BTS- lines and selections (Table 4.5). There were generally no differences in seed weight per plant among BTS+ and BTS- lines and selections, including BTS+ and BTS- infected with SHMV (Tables 4.5). The weight of 100 seeds showed some variability among the BTS+ line and most selections, but they generally yielded significantly greater weight of 100 seeds than the BTS- line and selections (Tables 4.5). BTS+ infected with SHMV and BTS- infected with SHMV produced similar weight of 100 seeds but lower yield than the non-infected lines and selections of both types.

Plant height generally did not differ among the BTS lines and selections in both plantings (Tables 4.5). BTS+ infected with SHMV had significantly lower plant height compared to the most BTS selections of both types.

4.5 Discussion

Damage to the plant due to acute virus infections can consist of yield reduction, low product quality, and plant death (Gildow et al., 2008). However, these negative effects vary depending upon plant cultivars and time of infection (Spence and Walkey, 1995). In contrast, persistent viruses which do not cause apparent symptoms in plants, appear to be commensals or mutualists (Roossinck, 2011a, 2011b; Villarreal et al., 2000). Persistent viruses may provide some benefits to their plant hosts as well as additional functional proteins (Roossinck, 2010; Villarreal, 2009a, 2009b). An example of a three-way symbiosis involving a mutualistic interaction between an obligate mycovirus, *Curvularia* thermal tolerance virus, an endophytic fungus, and a plant has been reported (Márquez et al., 2007). This three-way interaction conferred the plant, tolerance to extreme high soil temperatures in Yellowstone National Park

Table 4.3. Leaf pigment contents, plant wet weight, and plant height of two Black Turtle Soup lines and six selections. One line and three selections infected with PvEV1 and PvEV2 (BTS+) and one line and three selections endornavirus-free (BTS-), measured for 1-month-old plants.

Sample	Carotenoid (mg)	Chlorophyll (mg/g)			Anthocyanin (mg)	Wet weight (g)	Height (cm)
		a	b	Total			
BTS+	0.083±0.001 ab*	1.015±0.007 b	1.721±0.04 c	2.735±0.03 cd	0.077±0.02 ab	33.8±4.2 a	62.2±4.6 bc
BTS+8	0.083±0.001 ab	1.012±0.005 b	1.747±0.04 c	2.759±0.04 c	0.08±0.01 ab	32.4±4.1 a	77.2±8.8 a
BTS+13	0.082±0.001 ab	1.011±0.008 b	1.713±0.05 c	2.722±0.04 cd	0.095±0.02 a	32.3±4.7 a	60.8±9.9 bc
BTS+18	0.084±0.001 a	1.01±0.005 b	1.722±0.03 c	2.733±0.03 cd	0.07±0.01 b	31.1±1.9 ab	60.9±10.0 bc
BTS+/SHMV	0.081±0.001 b	1.019±0.005 b	1.627±0.06 d	2.646±0.05 d	0.077±0.01 b	30.0±2.2 abc	62.5±3.3 b
BTS-	0.073±0.001 c	1.258±0.005 a	2.139±0.05 ab	3.396±0.05 ab	0.074±0.01 b	29.5±4.2 abc	66.1±6.7 b
BTS-9	0.072±0.001 cd	1.262±0.014 a	2.138±0.05 ab	3.434±0.11 a	0.077±0.01 ab	31.1±6.4 ab	66.2±6.3 b
BTS-16	0.072±0.001 cd	1.253±0.005 a	2.172±0.04 a	3.424±0.04 a	0.073±0.02 b	30.5±5.2 ab	66.7±6.2 b
BTS-21	0.072±0.001 cd	1.253±0.009 a	2.181±0.03 a	3.434±0.03 a	0.064±0.01 b	25.2±4.6 bc	56.8±4.4 bc
BTS-/SHMV	0.071±0.002 d	1.254±0.012 a	2.064±0.10 b	3.318±0.09 b	0.071±0.01 b	23.9±2.4 c	52.0±1.4 c

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$). *Sunn-hemp mosaic virus* (SHMV)-infected plants were used as control

Table 4.4. Comparison of the time to flowering, days to pod formation, number of pods per plant, number of seeds per pod, and pod size of two Black Turtle Soup lines and six selections. One line and three selections infected with PvEV1 and PvEV2 (BTS+) and one line and three selections endornavirus-free (BTS-).

Line/ selection	Time to flowering (days)	Days to pod formation	No. pods per plant	No. seeds per pod	Pod size (mm)		
					Length	Width	Thickness
BTS+	35.6±1.3 b*	43.5±1.9 bc	18.0±6.2 abc	6.5±0.7 a	113.8±5.1 a	6.3±0.5 ab	9.0±0.6 ab
BTS+8	33.1±1.9 d	40.3±2.3 e	21.3±10.3 ab	6.7±0.9 a	114.2±8.0 a	6.5±0.5 a	8.9±0.5 ab
BTS+13	34.0±1.7 cd	42.0±2.9 bcde	25.7±12.8 a	7.0±0.6 a	115.9±5.5 a	6.3±0.5 ab	9.1±0.6 a
BTS+18	35.4±1.8 bc	42.9±2.3 bcd	22.8±10.7 ab	6.6±0.9 a	111.9±4.3 a	6.3±0.4 ab	8.9±0.6 ab
BTS-	34.0±1.5 cd	41.1±2.3 de	22.3±10.7 ab	6.5±0.6 a	106.4±3.6 b	6.5±0.5 a	9.0±0.5 ab
BTS-9	34.2±1.7 bcd	41.6±2.9 bcde	21.5±10.6 ab	6.5±0.8 a	107.2±4.0 b	6.0±0.5 bc	8.9±0.5 ab
BTS-16	34.4±2.0 bcd	41.6±2.9 cde	23.7±10.5 ab	6.5±0.6 a	106.7±3.2 b	6.0±0.5 bc	8.9±0.6 ab
BTS-21	34.0±1.8 cd	40.7±2.1 e	22.4±8.8 ab	6.4±0.5 a	105.6±3.4 b	5.8±0.6 c	9.1±0.4 ab
BTS+/SHMV	38.0±2.0 a	46.8±2.6 a	12.8±5.9 c	4.4±1.2 c	106.4±6.9 b	6.2±0.6 abc	8.7±0.5 ab
BTS-/SHMV	35.5±2.7 bc	43.7±2.6 b	17.9±9.6 bc	5.4±1.0 b	105.3±4.9 b	6.5±0.4 a	8.7±0.4 b

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).
SHMV=*Sunn-hemp mosaic virus*.

Table 4.5. Comparison of seed size, seed weight, and plant height of two Black Turtle Soup lines and six selections. One line and three selections infected with PvEV1 and PvEV2 (BTS+) and one line and three selections endornavirus-free (BTS-).

Line/ selection	Seed size (mm)			Seed weight (g)		Plant height (cm)
	Length	Width	Thickness	Per plant	100 seeds	
BTS+	10.6±0.4 ab*	4.3±0.3 bc	5.8±0.2 bc	15.4±5.0 cd	20.8±0.2 b	181.2±24.2 abc
BTS+8	10.8±0.6 a	4.5±0.4 ab	6.1±0.3 a	23.8±12.5 ab	22.8±0.5 a	200.3±31.9 a
BTS+13	10.5±0.3 abc	4.4±0.3 bc	6.0±0.2 ab	27.3±12.8 a	21.1±1.1 b	190.6±33.5 a
BTS+18	10.5±0.4 abc	4.7±0.3 a	6.0±0.2 ab	23.2±11.4 abc	19.8±0.7 c	189.0±27.6 a
BTS-	10.5±0.4 abc	4.1±0.3 c	6.1±0.3 a	21.2±10.4 abc	17.9±0.7 d	181.2±33.0 abc
BTS-9	10.2±0.5 cd	4.2±0.4 c	6.0±0.3 ab	20.9±11.1 abc	17.9±1.3 d	195.4±24.8 a
BTS-16	10.2±0.3 bcd	4.2±0.3 c	6.0±0.2 a	22.7±11.5 abc	19.5±1.3 c	186.4±29.9 ab
BTS-21	10.1±0.5 d	4.3±0.3 bc	6.0±0.2 ab	22.8±9.7 abc	19.7±1.0 c	189.0±35.0 a
BTS+/SHMV	10.4±0.5 bcd	4.3±0.3 c	5.7±0.2 c	9.1±4.4 d	16.3±0.7 e	158.4±30.4 c
BTS-/SHMV	10.2±0.4 cd	4.3±0.3 bc	5.9±0.2 ab	16.2±8.5 bcd	16.3±0.5 e	162.3±35.6 bc

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).
SHMV=*Sunn-hemp mosaic virus*.

(Márquez et al., 2007; Redman et al., 2002). Another example of a possible mutualistic interaction between a plant and a persistent virus is *White clover cryptic virus* and white clover, its host plant. In this case, the virus encodes a gene that can affect nodulation. (Nakatsukasa-Akune et al., 2005). With the exception of VfEV, which is associated with male sterility, most endormaviruses do not appear to affect the plant phenotype (Pfeiffer, 1998).

In this comparative study, parameters evaluated included: seed germination, length of radical root, pigment content, growth characters, and grain yield components. The BTS+ line and selections showed faster seed germination and radical root growth than the BTS- line and selections. These two physiological characters appear to be affected by the presence of the two endornaviruses in the BTS+ line and selections. However, it is not known if one or both viruses are associated with seed germination and radical root growth of BTS.

Reduction of photosynthetic pigments, mainly chlorophyll can be caused by infection of plant viruses. Disease symptoms on leaves of virus-infected plants include mosaic, yellowing, and chlorosis, among others. In this study, the amounts of chlorophyll a and b and total chlorophyll of endornavirus-infected common bean lines and selections were significantly lower than the endornavirus-free lines and selections. Nevertheless, the leaves of the two BTS+ and BTS- lines did not show visually detectable phenotypic differences. There is no available information of the reduction of chlorophyll content in endornavirus-infected plants. However, in a study of the cellular localization of PvEV2 and PvEV1, it was found that PvEV2 is associated with the chloroplast fraction, while PvEV1 was associated with cytoplasmic vesicles (Okada et al., 2013). It is possible that the lower amount of chlorophyll is related to PvEV2 infection. However, the metabolism related to chloroplast development, chlorophyll production and maintenance, and photosynthesis was not studied in this investigation. It is well known that

viruses affect plant chloroplast and chlorophyll content. Reduction of the chlorophyll content and distortion of chloroplast have been observed in cassava leaves infected with *Cassava mosaic virus* (Ayanru and Sharma, 1982). The reduction of chlorophyll content in tomato plants infected with *Tomato yellow mosaic virus* (Leal and Lastra, 1984) and the wild plant *Eupatorium makinoi* infected with *Eupatorium* yellow vein virus (EpYVV) (Funayama et al., 1997b) have also been reported. Disorganization of the thylakoid system in the chloroplast of abutilon plants was observed when the plant was infected with *Abutilon mosaic virus* (Schuchalter-Eicke and Jeske, 1983). Associations between the decrease of maximum quantum yield of photosynthesis and the amount of light-harvesting proteins in *E. makinoi* plants infected with EpYVV has been reported (Funayama and Terashima, 1999; Funayama-Noguchi and Terashima, 2006). Those studies support the results of this investigation which showed a reduction of chlorophyll due to endornavirus and SHMV infections.

The carotenoid content of the BTS+ lines and selections was significantly greater than that of the BTS- lines and selections. The carotenoid content of BTS+ infected with SHMV was also greater than that of BTS- infected with SHMV. For the anthocyanin content, there were no differences between the BTS+ and BTS- lines and selections. There have been reports on the evaluation of amounts of carotenoid and anthocyanin in common bean and faba bean leaves (EL-Qudah, 2014). The analysis of carotenoids in faba bean cv Foul and common bean cv Fasolia Jaffeh showed that both of them contained low concentration of carotenoids, such as lutein, neoxanthin, violaxanthin, zeaxanthin, and β-Carotene (EL-Qudah, 2014). However, faba bean had higher lutein and β-Carotene contents than common bean. This test was done without knowing that faba bean was infected with the endornavirus VfEV (Grill and Garger, 1981; Pfeiffer, 1998). Anthocyanin is a polyphenolic compound present in black bean (Aparicio-

Fernandez et al., 2005). It exhibits strong antioxidant, antimutagenic and antigenotoxic activities, as well as preventing genetic damage due to chemical mutagens in animals (Azevedo et al., 2003; Wong et al., 2003). In this investigation, the content of anthocyanin showed little variation among all tested plants. High anthocyanin content has been reported in the black bean genotype T-39, which is a progenitor of Black Turtle Soup (Akond et al., 2011). This genotype (T-39) was positive for PvEV1 and PvEV2 (Khankhum et al., 2015). In another report, the anthocyanin content of the common bean cv Jaguar was higher than in cvs Vista and Othello (Akond et al., 2011). In this investigation (Chapter 3), the common bean cvs Jaguar and Vista tested positive for PvEV1 and PvEV2 while cv Othello was endornavirus-free.

A study on effects of TMV on tobacco plants and *Papaya ringspot virus* on papaya plants showed that infected plants had reduced total chlorophyll and phenolic antioxidant compounds (Dina and Sabah, 2008; Singh and Shukla, 2009). This data supports the results obtained in this investigation in which BTS+ infected with endornaviruses had higher carotenoid content. The reduction of chlorophyll content in endornavirus-infected common bean could affect growth and morphological characters, such as plant height, time to flower and pod formation, pod and seed size, and dry grain weight. In this study, plant morphological and physiological characters associated with infection by endornaviruses were evaluated using common bean BTS lines grown in microplots. Common bean is a heat sensitive plant, and the average temperature in the microplots (34^{o}C) was higher than the optimal temperature ($25\text{-}30^{o}$C) for common bean growing which could result in low yields (Rainey and Griffiths, 2005). Nevertheless, the conditions were similar to reported studies dealing with biomass, growth rate, and yield of common bean (Scully and Wallace 1990). The time to flowering and pod formation was not different between the BTS+ and the BTS- lines. The BTS+ inoculated with SHMV had significantly longer times to

flowering and pod formation than the BTS+ and BTS- lines that were not inoculated with SHMV. These results suggest that time to flower and pod formation of common bean was not affected by endornaviruses but was affected by SHMV infection. The results on delays of time to flower and fruit formation caused by acute virus infections were similar to results obtained with common bean plants infected with BYMV (Hampton, 1975; Schwartz et al., 2005).

BBTV infection in banana caused low productivity due to the low photosynthetic rate of the chlorotic leaves (Chia and He, 1999; Hooks et al., 2008). In common bean, some research has been conducted on the effects of growth and morphological characteristics due to acute virus infections (Azizi and Shams-bakhsh, 2014; Blair et al., 1995; Morales, 2006; Morales and Anderson, 2000). Common bean cultivars susceptible to CMV showed a reduction in plant fresh and dry weights (Azizi and Shams-bakhsh, 2014). BCMV and BCMNV caused yield losses depending on common bean varieties, environment and time of infection (Morales, 2006; Schwartz et al., 2005; Srivastava et al., 2012). Plants grown from BCMV-infected seed were stunted, had delay maturity, fewer and smaller pods, produced deformed pods, and fewer seeds per pod than healthy plants (Morales, 2006; Srivastava et al., 2012). *Bean pod mottle virus* significantly affected yield because it induced malformation of pod and seed abortion. Effects of BGMV infection included reduction of the number of pods, number of seeds per pod, and seed weight (Blair et al., 1995; Morales, 2006; Morales and Anderson, 2001; Schwartz et al., 2005).

In this study, the BTS+ and BTS- lines infected with SHMV yielded fewer seeds per pod than non-SHMV infected lines. Triple virus infected BTS+ (PvEV1, PvEV2, and SHMV) yielded significant lower numbers of seeds per pod than BTS- infected with SHMV. These results suggest that reduction of seeds per pod is associated with the presence of endornaviruses and they may increase the negative impact of infection by the acute virus SHMV. In contrast, the

weight of 100 seeds was higher for BTS+ lines compared to BTS- lines. BTS+ line and selections also yielded longer pods than BTS- line and selections. This suggests that endornaviruses may affect pod length, as well as seed weight. However, an effect on seed size was not detected.

In summary, the study results showed that endornavirus-infected BTS lines and selections had increased seed germination rate and radical root length. The chlorophyll content of endornavirus-infected BTS plants was lower than the chlorophyll content of virus-free plants. However, the carotenoid content of BTS+ lines was greater than that of BTS- lines and selections, while the anthocyanin content did not differ among tested plants. The BTS+ lines yielded longer pods and weight of 100 seeds compared to the BTS- lines. This comparative study needs to be validated using near-isogenic lines or ideally, making endornavirus inoculations after developing a successful virus-inoculation method.

In conclusion, BTS infected with the two endornaviruses yielded higher the weight of 100 seeds than endornavirus-free BTS plants. This may explain why all seed sources and selections (Chapter 3, Table 3.1) of BTS are infected with PvEV1 and PvEV2 and why virus-free plants were not selected in the breeding process. The increase in seed germination may be another important agronomic character selected by common bean breeders.

CHAPTER 5.
INTERACTION OF *PHASEOLUS VULGARIS ENDORNAVIRUS 1* AND *PHASEOLUS VULGARIS ENDORNAVIRUS 2* WITH *TOBACCO RINGSPOT VIRUS, TOBACCO MOSAIC VIRUS,* AND *SUNN-HEMP MOSAIC VIRUS*

5.1 Introduction

Based on host reaction, plant viruses can be divided into acute and persistent types (Roossinck, 2010). Acute viruses are the most studied and cause a variety effects on the phenotype and physiology of the host. Systemic symptoms due to plant virus infection can be mosaic, yellows, chlorosis, necrosis, ring spot, wilting, leaf rolling, growth reduction, deformation, and nodule reduction. Chlorotic symptom occurs when infected cells lose chlorophyll and other pigments (Hull, 2014). In general, single and double infections of acute viruses in plants cause different symptoms which depend on the viruses and the host. For example, leaves of *Impatiens walleriana* infected with *Tobacco ringspot virus* (TRSV) exhibited chlorotic ring patterns, while on *Nicotiana benthamiana* the virus caused local concentric chlorotic lesions, later necrotic rings, systemic leaf deformation, and dwarfing (Kundu et al., 2015). Mixed infections of plant viruses are common in field crops (Fuentes and Hamilton, 1991; Kundu et al., 2015). Double infection between the cowpea strain of *Southern bean mosaic virus* (SBMV-C) and S*unn-hemp mosaic virus* (SHMV) in pinto bean resulted in pinpoint necrotic local lesions on the inoculated primary leaves (Fuentes and Hamilton, 1991).

One common result of mixed infections of plants by two acute viruses is synergism. The synergism results from one virus being able to block the host immune system for the other virus. As a result, the host expresses more severe symptoms than when infected by one virus alone (Pruss et al., 1997). Interactions between viruses in mixed infected plants can be evaluated in terms of the accumulation of viruses by quantification techniques (Elena et al., 2014). The virus-

virus-host interactions can be observed as symptoms on the host. The virus titers, which indicate the efficiency of virus replication in an inoculated plant, can be obtained by immunological [the enzyme-linked immunosorbent assay (ELISA)] and PCR-based techniques. The ELISA technique is based on the measurement of antigen-antibody reaction. This reaction can be detected using an enzyme labelled antibody. The presence of the enzyme is detected with a substrate yielding a colored product that can be easily visualized or read in a microplate reader. Real-time or quantitative PCR (qPCR) is a technique that enhances detection, amplification, and quantification of a specific nucleic acid sequence (Fraga et al., 2008). The amplified product can be quantified during the cycles due to the detection of a fluorescence signal from a fluorogenic probe during amplification. There are two common methods used for the detection of the PCR products. The first is using a non-specific fluorescent dye, such as SYBR green. The second is a sequence-specific probe, such as the TaqMan fluorogenic probe. This probe is a specific oligonucleotide to the gene target that is labelled with a fluorescent reporter to the probe. The probe is hydrolyzed by the 5′ nuclease activity of Taq DNA polymerase when the primer is extended resulting in a fluorescence signal. TaqMan probes have been used extensively to investigate RNA titers in virus-infected plants.

Because persistent viruses are common, but in most cases not detected, it would not be surprising that they could interact with acute viruses and result in more severe diseases than those caused by the acute viruses alone. It is also possible that the activation of the plant immune system by persistent viruses could result in less severe diseases, such as in the case of cross protection. Single and/or double infections of plant endornaviruses have not been shown to cause detectable symptoms. Common bean cultivars Majesty, single infected with *Phaseolus vulgaris endornavirus 1* (PvEV1), Closeau, single infected with *Phaseolus vulgaris endornavirus 2*

(PvEV2), and Black Turtle Soup, double infected with PvEV1 and PvEV2 are examples of single and double endornavirus infections (Khankhum et al., 2015; Okada et al., 2013). The interactions of these persistent viruses with acute viruses infecting bean have not been determined.

5.2 Objectives

1. To evaluate the symptoms caused by single and mixed infections of the acute viruses *Tobacco mosaic virus* (TMV), TRSV, and SHMV in common bean lines and cultivars with single and double infections of PvEV1 and PvEV2.
2. To evaluate the virus and RNA titers of PvEV1 and PvEV2 when mixed infected with TRSV and SHMV.

In this chapter, common bean lines and cultivars infected with one or two endornaviruses were inoculated individually with each acute virus. The symptoms caused by the acute virus infections were evaluated. The accumulation in inoculated plants was evaluated for each acute virus using ELISA, and the relative virus titers of SHMV, PvEV1, and PvEV2 were determined by reverse transcription quantitative polymerase chain reaction qPCR.

5.3 Materials and Methods

5.3.1 Plant materials and growth conditions

Two lines of the black market class common bean cv Black Turtle Soup, one double-infected with PvEV1 and PvEV2 and designated BTS+ and the other one virus-free and designated BTS- obtained in previous investigations (Okada et al., 2013) and increased at least five generations by self-pollination were used in all the comparative studies. Two cultivars, Majesty and Red Hawk of the dark red kidney market class and two, Closeau and Celrk of the light red kidney market class also were included in the comparative studies. Majesty is infected by PvEV1 alone, and Closeau is infected by PvEV2 alone. Celrk and Red Hawk are both

endornavirus-free. Before using them, the presence or absence of PvEV1 and/or PvEv2 was confirmed by dsRNA extraction, electrophoretic analyses and RT-PCR using virus-specific primers (Khankhum et al., 2015; Okada et al., 2013). Plants were grown in a greenhouse located on the Baton Rouge campus of Louisiana State University. The greenhouse day/night temperatures averaged 25/18°C. Before planting, seeds of the two lines were surface-sterilized in 10% sodium hypochlorite for 10 min, washed in sterilized distilled water three times, and pre-germinated in the laboratory. Seeds were planted in 8-inch clay pots containing a steam-sterilized soil mix (two parts soil, one part sand, and three parts sphagnum moss). The Potting Mix Miracle-Gro® soil (Miracle-Gro® Lawn Products, Inc., Marysville, OH) was used as the sphagnum moss. There was a single experimental plant per pot. All plants were fertilized using Osmocote® Smart-Release® Plant Food Outdoor &Indoor (Miracle-Gro® The Scotts Company LLC, Marysville, OH) once, 2 weeks after planting. Common bean plants were sprayed weekly for thrips and whiteflies using Imidacloprid (Bayer Environmental Science, Research Triangle PK, NC) and Avid (Syngenta Crop Protection, Inc., Greensboro, NC).

5.3.2 Virus inoculations

Louisiana isolates of three mechanically transmitted viruses (TMV, TRSV, and SHMV) were used to evaluate virus-virus and virus-host interactions. These viruses were used because there were consistently transmitted by mechanical inoculations. These interactions were evaluated/recorded by symptom expression, serology, and in the case of SHMV, quantitative polymerase chain reaction (qPCR). The virus inoculum consisted of foliar tissue collected 2 weeks post inoculation, ground in liquid nitrogen, and stored at -70^{o}C to ensure inoculum consistency. The inoculum consisted of a 1:10 ratio one-part tissue and 10 parts buffer (0.02M Phosphate buffer, pH 7.2) containing 1.0 mg of carborundum per milliliter of inoculum.

Common bean plants were inoculated on the primary leaves 5 days after planting. After inoculation, leaves were washed with distilled water. Mock inoculations were also conducted on control plants. Three plants of each cultivar were inoculated with each virus separately. After inoculation, plants were covered with polypropylene garden fabric (Gardener's Supply Company, Burlington, VT) to avoid potential insect feeding. The virus inoculations were repeated three separate experiments. Foliar symptoms (primary and trifoliate) were evaluated visually 7 and 14 days after inoculations (DAI). In the case of TMV, the numbers of necrotic local lesions were counted from inoculated leaves.

5.3.3 Double antibody sandwich (DAS) ELISA

The DAS-ELISA was used to estimate virus titers. Polyclonal antisera to TRSV and SHMV were purchased from AC Diagnostics Inc. Fayetteville, AR and diluted according to the company instructions. Inoculated leaves and the first trifoliate leaves were collected and tested separately from each inoculated plant 7 and 14 days, respectively, after inoculation. Samples consisted of 0.1g of tissue. Tissue was ground in 1 ml of extraction buffer, pH 7.3 (1 L contains - 2 g powdered egg albumin, 10 g polyvinylpyrrolidone MW 24-40,000, 1.3 g sodium sulfite, 0.2 g sodium azide, and 10 g Tween-20). Grounded samples were placed in microcentrifuge tubes and centrifuged for 3 min at 3,000 rpm. The procedure for DAS-ELISA provided by AC diagnostics was followed. Absorbance (405 nm) representing virus titers was measured using a microplate auto reader (Model EL311 SX, Bio-Tek^{TM} Instrument Inc.) linked to Star NX-1001 Multifont printer. For all samples, three biological and two technical replications were conducted.

5.3.4 qPCR

To investigate possible interactions at the level of viral RNA titers between PvEV1, PvEV2 alone and in mixed infections with SHMV the qPCR technique was used.

- Total RNA extraction

One hundred milligrams of leaf tissue per sample was collected at 7 and 14 days after inoculation, placed in a 1.5 ml nuclease-free microcentrifuge tube, and immediately submerged in liquid nitrogen to avoid RNA degradation. Samples were kept at -70^{o}C until ready for RNA extraction. Total RNA was extracted following the extraction procedure of Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). Collected leaf tissues were ground in liquid nitrogen using a micro-pestle. To eliminate residual DNA contamination, RNA was DNase treated using the On-Spin Column DNase I Kit (MO BIO Laboratory, Inc, Carlsbad, CA) following the manufacturers' directions. Total RNA was eluted out from the column using nuclease-free water (Ambion®, Life Technologies^{TM}, Carlsbad, CA), the concentration was measured using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and the samples were kept at -70^{o}C until ready to use.

- Primer and probe development

Primer sets included forward and reverse and a fluorogenic universal probe (TaqMan® FAM/ MGB probe) for the three viruses PvEV1, PvEV2, and SHMV. Primers and probe for reference gene Actin-11 were designed according to Borges et al. (2012) who suggested the usefulness of this gene in the normalization of gene expression by RT-PCR analysis in common bean due to biotic stress. Primer sets and fluorogenic universal probes were designed based on nucleotide sequences available on GenBank (Table 5.1). These genes were subjected to the ProbeFinder version 2.50 software (Roche Diagnostics, https://lifescience.roche.com). All of

designed primer/probe sets (Table 5.1) were evaluated for hairpin and self-complementation properties and also compared to available sequences in the GenBank using the BLAST sequence alignment search tool, available online from the National Center for Biotechnology Information (NCBI). In addition, these primer/probe sets were tested for amplification efficiency against total RNA extracted from common bean cv Black Turtle Soup infected with PvEV1, PvEV2, and SHMV. The specific targets were identified through preliminary real-time PCR assays.

Table 5.1. Primers and probes used in qPCR reactions to quantify relative amounts of *Phaseolus vulgaris endornavirus 1* (PvEV1), *Phaseolus vulgaris endornavirus 2* (PvEV2), S*unn-hemp mosaic virus* (SHMV), and actin-11 reference gene.

Target gene	Accession#	Primer/universal probe	Fragment size (bp)
RdRp of PvEV1	AB719397	Forward 'agggaattggtggaatttga' Reverse 'cacatcttcaaaagttgatacacga' Probe 'gcaaccag' (#164, cat. no. 04694511001)	73
RdRp of PvEV2	AB719398	Forward 'ggcagcaataactgatgaagg' Reverse 'tcgaatctgcgtcttaatcg' Probe 'ggaccaga' (#93, cat. no. 04692101001)	69
Replicase of SHMV	U47034	Forward 'ctatcattatcgccgcctgt' Reverse 'tcaccacagaacccagcttt' Probe 'ggagaagg' (#133, cat. no. 04694171001)	73
Actin-11	62703083	Forward 'ttggcatgggtcaaaaagat' Reverse 'caaaataccctcttagactgtgc' Probe 'tggtgatg' (#9, cat. no. 04685075001)	62

- qPCR reactions

Relative RNA quantifications of PvEV1, PvEV2, and SHMV were conducted by one-step real-time PCR. Reaction mixtures were performed using iTaqTM Universal Probe One-Step Kit (Bio-Rad Laboratories, Inc., Hercules, CA) following the manufacturer's directions. Twenty

microliter of reactions consisted of 100 ng of RNA template (2 µl of 50 ng/µl), 1 ul of 10 nM of each primer, 0.4 µl of 10 nM of the probe, 10 µl of 2x iTaq universal probe reaction mix, 0.5 µl of iScript reverse transcriptase, and nuclease free water. The following qPCR thermal cycler conditions were used: 50°C for 10 min (cDNA synthesis), 95°C for 1 min (hot-start Taq DNA polymerase activation), followed by 40 cycles of denaturation at 95°C for 2 sec and annealing/extension at 60°C for 30 sec. Reaction mixtures of endogenous control and non-template control were performed as described above. qPCR reactions were performed on an CFX96 Touch™ Real-Time PCR Sequence Detection System using Hard-Shell Low-Profile 96-Well Semi-Skirted PCR plates that were sealed with Microseal 'B' Adhesive Seals (Bio-Rad Laboratories, Inc., Hercules, CA). To minimize the effects of any errors due to pipetting differences, triplicates of each sample were run on each plate, and their quantification cycle (Cq) values were averaged. Non-template water controls (NTC) was included on every plate. The ΔΔCq quantification method (CFX96 Touch™ Real-Time PCR Sequence Detection System Instruction Manual), which eliminates the need for standard curves on every plate, was implemented for the normalization of samples. Three biological replications were conducted for all tested common beans lines and cultivars as well as viruses. These experiments were repeated three times.

5.3.5 Statistical analysis

The experimental layout was a complete randomized design. The parameters related to viral infection were the means of three replicates per treatment. Statistical analyses were performed using SAS Proc Mixed (SAS v. 9.2, SAS Inc., Cary, NC). Fisher's LSD test at the 0.05 probability level was used to test for statistical significance.

5.4 Results

5.4.1 Foliar symptom evaluation

Common bean lines and cultivars showed different reactions depending on the acute virus used. Primary leaves inoculated with TMV showed different numbers of necrotic local lesions in different common bean lines and cultivars (Table 5.2). The number of lesions observed at 7 DAI did not change at 14 DAI, so only the 14 DAI results are reported. BTS+ and BTS- had significantly greater numbers of necrotic local lesions on primary leaves than light red kidney and dark red kidney bean cultivars with and without single endornavirus infections (Table 5.2 and Figure 5.1 A). There were no difference in the number of lesions among the four light red kidney and dark red kidney cultivars with and without single endornavirus infections (Table 5.2). The BTS+ line infected with PvEV1 and PvEV2 had significantly greater number of necrotic local lesions than the endornavirus-free BTS-. The average number of lesions was 19.5 and 11.2 respectively. No other symptoms observed on inoculated leaves and the virus did not cause systemic infections. Mock inoculated leaves did not show symptoms.

Symptoms induced by SHMV varied depending on the common bean cultivar inoculated (Table 5.2). Seven days after inoculation, vein necrosis was observed on the primary leaves (vnp) as well as on trifoliate leaves (vnt) of BTS +, BTS-, and the dark red kidney cultivars, but not observed on the light red kidney cultivars. However, 14 days after inoculation, vein necrosis with higher degree of severity on both primary and trifoliate leaves was observed with all common bean lines and cultivars. Seven days after inoculation, mosaic was observed on the primary (mp) and trifoliate (mt) leaves of BTS+, Majesty, Closeau and Celrk (Figure 5.1 D). This mosaic symptom was not observed on BTS-. At 14 DAI, all cultivars showed severe vein

necrosis. A third symptom induced by SHMV infection was leaf deformation which was observed only on the trifoliate leaves of BTS+ and Celrk.

Table 5.2. Symptoms on common bean lines and cultivars after inoculation with *Tobacco mosaic virus* (TMV), *Sunn-hemp mosaic virus* (SHMV), or *Tobacco ringspot virus* (TRSV) at 7 and 14 days after inoculation (DAI).

Market class	Line/ cultivar	Endornavirus	Symptoms				
			TMV	SHMV		TRSV	
			14 DAI	7 DAI	14 DAI	7 DAI	14 DAI
Black	BTS+	PvEV1 and PvEV2	19.5±5.2 a*	vnp, mt	vnp, ft	rs, c, np	rs, c, nt
	BTS-	Virus free	12.4±2.6 b	vnp	vnp	nd	nd
Dark red kidney	Majesty	PvEV1	2.5±1.3 c	vnp, mp, mt, vnt	vnp	rs, mt, nt	rs, mt, nt
	Red Hawk	Virus free	0.4±0.7 c	vnp	vnp	mt, nt	rs, mt, nt
Light red kidney	Closeau	PvEV2	2.3±2.0 c	mp, mt	vnp, vnt	rs, mt, nt	rs, mt, nt
	Celrk	Virus free	0.8±0.7 c	mp, mt	vnp, vnt, ft	rs, mt, nt	rs, mt, nt

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).
Abbreviations for symptoms: vein necrosis on primary leaves (vnp); vein necrosis on trifoliate leaves (vnt); mosaic on primary leaves (mp); mosaic on trifoliate leaves (mt); deformation of trifoliate leaves (ft); ring spot (rs); chlorosis (c); necrosis on primary leaves (np); necrosis on trifoliate leaves (nt); not detected (nd).

Except for BTS- plants that were symptomless, symptoms exhibited by plants infected with TRSV were variable and depended on the common bean cultivar inoculated (Table 5.2). Initial symptoms caused by TRSV consisted of ring spots (rs) observed on both primary and trifoliate leaves of BTS+, Majesty, Celrk, and Closeau at 7 DAI, excluding BTS- and Red Hawk (Figure 5.1 B). At 14 DAI, all common bean cultivars exhibited a higher number of ring spots. A second symptom observed only on BTS+ was leaf chlorosis in both primary and trifoliate leaves.

Necrosis of primary leaves (np) was observed only in BTS+ at 7 DAI (Figure 5.1 B). Except for the BTS-, all cultivars exhibited necrotic symptom on the trifoliate leaves (nt) at 7 DAI, and severity increased at 14 DAI (Figure 5.1 C). Mosaic symptoms were observed on trifoliate leaves (mt) of Majesty (infected withPvEV1), Closeau (infected with PvEV2), and endornavirus-free cultivars Celrk and Red Hawk at 7 DAI. The severity of the mosaic increased at 14 DAI.

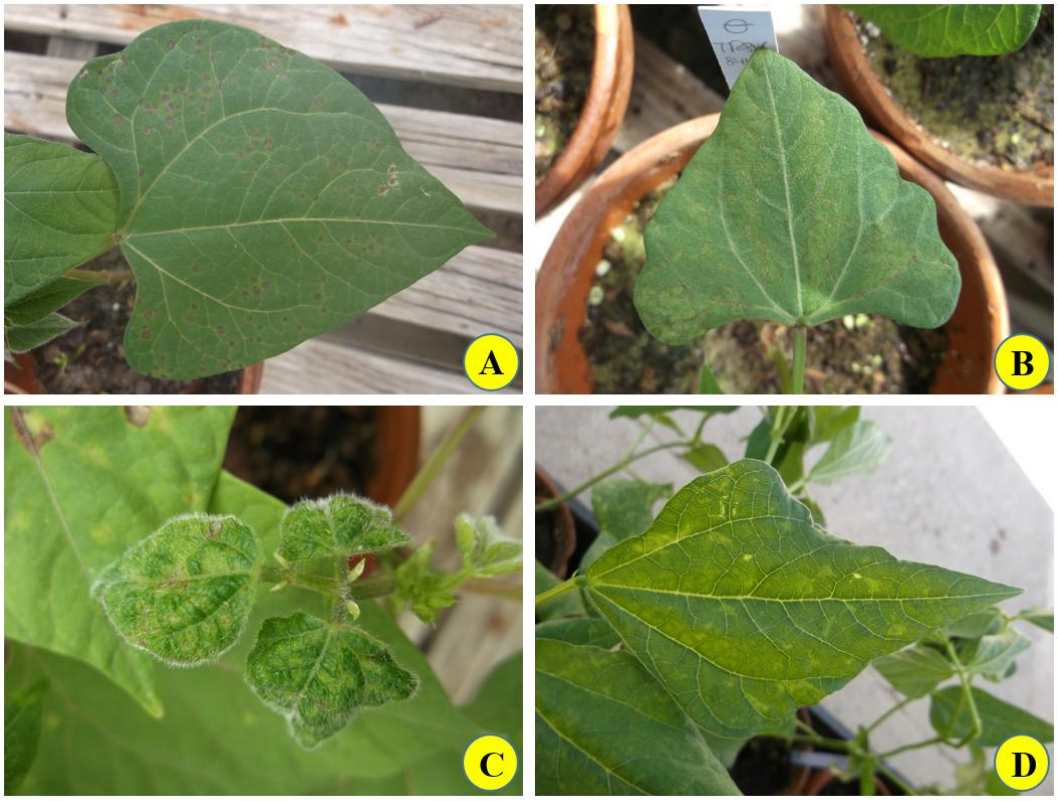

Figure 5.1. Symptoms on different common bean caused by acute virus infections. (A) necrotic local lesion caused by *Tobacco mosaic virus* a primary leaf of Black Turtle Soup infected with PvEV1 and PvEV2 (BTS+); (B) necrosis and ring spots caused by *Tobacco ringspot virus* (TRSV) on a primary leaf of BTS+ line; (C) necrosis and mosaic on a trifoliate leaf of cv Majesty caused by TRSV; (D) mosaic on a trifoliate leaf of cv Closeau caused by *Sunn-hemp mosaic virus.*

5.4.2 Virus titer measured by ELISA

Titers of SHMV and TRSV were measured by ELISA in inoculated common bean lines and cultivars at 7 and 14 DAI (Table 5.3). There were no significant differences in virus titers

among common bean lines and cultivars inoculated with SHMV regardless of endornavirus infections. Similarly, the red kidney common bean lines and cultivars inoculated with TRSV did not show significant differences in titers in both primary and trifoliate leaves. However, BTS- trifoliate leaves had lower virus titers than BTS+ trifoliate leaves at 7 and 14 DAI (Table 5.3). These titers also were lower than those detected in primary leaves of BTS- and BTS+ plants.

Table 5.3. Double antibody sandwich ELISA readings (405 nm) of *Sunn-hemp mosaic virus* (SHMV) and *Tobacco ringspot virus* (TRSV) infected primary and trifoliate leaves of common bean lines and cultivars of different market class with and without infection by two endornaviruses.

Market class	Line/ cultivar	Endornavirus	Type of leaf	Days after inoculation	ELISA	
					SHMV	TRSV
Black	Black Turtle Soup	PvEV1 and PvEV2	Primary	7	0.87±0.23ab*	2.07±0.02 a
				14	1.01±0.11 ab	1.70±0.62 a
			Trifoliate	7	0.98±0.14 ab	1.72±0.30 a
				14	1.04±0.29 ab	1.53±0 ab
	Black Turtle Soup	Endornavirus-free	Primary	7	0.76±0.12 b	2.17±0.62 a
				14	0.98±0.12 ab	1.69±0.45 a
			Trifoliate	7	0.90±0.10 ab	0.54±0.49 cd
				14	1.09±0.34 a	0.64±1.09 cd
Dark Red	Majesty	PvEV1	Primary	7	0.83±0.19 ab	1.68±0.44 a
				14	0.98±0.13 ab	1.56±0.74 ab
			Trifoliate	7	0.99±0.19 ab	1.58±0.56 ab
				14	1.07±0.29 ab	1.65±0.36 a
	Red Hawk	Endornavirus-free	Primary	7	0.84±0.22 ab	1.69±0.42 a
				14	0.99±0.12 ab	1.53±0.81 ab
			Trifoliate	7	0.95±0.12 ab	1.30±0.47 abc
				14	1.04±0.29 ab	1.39±0.75 abc
Light Red	Closeau	PvEV2	Primary	7	0.82±0.17 ab	1.65±0.46 a
				14	0.94±0.11 ab	1.62±0.87 a
			Trifoliate	7	0.98±0.16 ab	1.61±0.71 ab
				14	1.02±0.23 ab	1.42±0.60 abc
	Celrk	Endornavirus-free	Primary	7	0.83±0.15 ab	1.65±0.46 a
				14	0.95±0.10 ab	1.57±0.76 ab
			Trifoliate	7	0.95±0.22 ab	1.25±0.52 abc
				14	1.05±0.32 ab	1.58±0.45 ab

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).

5.4.3 Relative virus titers measured by qPCR

Virus titers in common bean lines and cultivars were determined at 7 and 14 DAI by qPCR and expressed as relative virus RNA titers of PvEV1, PvEV2, and SHMV in triple and double viral infections (Table 5.4). At 7 DAI with SHMV, the titer of PvEV1 in double virus infected Majesty was significantly greater than titer that measured from triple virus infected BTS+ (Table 5.4). This result was the same at 14 DAI. The titer of PvEV1 in Majesty at 7 DAI was significantly greater than titer at 14 DAI, while in BTS+, the titers were similar. At 7 DAI, titer of PvEV2 in triple virus infected BTS+ was not significantly different from the titer in double virus infected Closeau (infected with PvEV2). However, at 14 DAI, the titer of PvEV2 in Closeau was significantly greater than the titer in BTS+.

Titers of the acute virus SHMV at 7 DAI measured from triple virus infected BTS+ was not statistically different from double virus infected Majesty and Closeau. Double virus infection of Majesty and Closeau resulted in no differences in titers of SHMV at 7 DAI. At 14 DAI, titer of SHMV measured from triple infected BTS+ was not statistically different from double virus infected Majesty and Closeau. Titer of SHMV in double virus infection of Majesty was not different from double virus infection of Closeau at 14 DAI. Titers of SHMV measured between assessment dates of the same cultivar were not differences.

5.5 Discussion

In this study, the symptoms of single, double, and triple infections of TMV, TRSV, and SHMV and two endornaviruses in common bean lines and cultivars were evaluated. Common bean lines and cultivars showed different reactions depending on the acute virus inoculated. Necrotic local lesions were induced in primary leaves of TMV-inoculated common bean lines and cultivars, while various systemic symptoms were induced by SHMV and TRSV.

Table 5.4. Relative virus titers of *Phaseolus vulgaris endornavirus 1* (PvEV1), *Phaseolus vulgaris endornavirus 2* (PvEV2), and *Sunn-hemp mosaic virus* (SHMV) determined by qPCR in double or triple infection of different common bean lines and cultivars inoculated with SHMV at 7 and 14 days after inoculation.

Market class	Line/cultivar	Endornavirus	RT-qPCR	Days after inoculation	Relative virus titer	
Black	Black Turtle Soup	PvEV1 and PvEV2	PvEV1	7	1.128±0.31	d*
				14	0.99±0.29	d
			PvEV2	7	0.981±0.34	d
				14	1.323±0.59	d
			SHMV	7	1.023±0.40	d
				14	0.965±0.30	d
Dark Red	Majesty	PvEV1	PvEV1	7	21.314±5.49	a
				14	9.549±2.08	b
			SHMV	7	2.736±1.56	cd
				14	1.953±0.52	cd
Light Red	Closeau	PvEV2	PvEV2	7	1.776±0.83	d
				14	4.812±1.35	c
			SHMV	7	2.357±0.73	cd
				14	1.192±0.48	cd

*Mean values followed by the same letter of the same column do not differ significantly according to the LSD test ($p<0.05$).

TMV, the type member of the genus *Tobamovirus*, has been reported as a serious pathogenic virus of many field crops such as brassicas, cucurbits, solanaceous crops, various ornamental plants, and greenhouse grown crops (Alishiri et al., 2013; Cherian and Muniyapppa, 1998; Chitra et al., 2002; Hull, 2014; Kumar et al., 2011). Typical symptoms of TMV-infected plants include malformations, yellow spotting on leaves, vein yellowing, mosaic patterns of light and dark green on the leaves and fruits, interveinal and systemic chlorosis, leaf roll, necrosis, and stunting (Alishiri et al., 2013; Hull, 2014; Kumar et al., 2011). In this study, the bean cultivars used did not react with systemic symptoms after inoculation with TMV.

The necrotic local lesion reaction, or hypersensitive response, in virus-infected plants is a host resistance mechanism which limits the spread of virus and restricts it to cells around the

point of entry (Loebenstein, 2009). It is one of the most notable resistance responses and has been used by breeders to obtain virus-resistant cultivars of many crops. This mechanism affects virus multiplication and movement which results in incompatibility of viral and host factors (Hull, 2014; Loebenstein, 2009; Loebenstein and Akad, 2006). The number of necrotic local lesions corresponds to relative infectivity of the virus, as well as the degree of resistance of the plant (Loebenstein and Akad, 2006). In this study, the results from TMV inoculation to different common bean lines and cultivars suggest that the necrotic local lesion reaction may be associated with the presence of endornaviruses.

Various systemic symptoms induced by TRSV and SHMV were observed in primary and trifoliate leaves of inoculated common bean lines and cultivars. TRSV is a ssRNA virus transmitted by nematodes of the genus *Xiphinema*. The severe bean strain of TRSV (TRSV-SB) caused bud blight in inoculated white beans and the recovered plants from the initial infection produced deformed leaves with mosaic (Tu, 1981). Pinto bean responded to TRSV infection by producing local necrotic lesions (Sehgal, 1992). In this study, symptoms caused by TRSV consisted of ring spots, chlorosis, necrosis, and mosaic. These symptoms were obtained when the BTS+ was inoculated with TRSV but not when the BTS- was inoculated. BTS- did not react with apparent symptoms. The titer of TRSV measured by ELISA was correlated with the host reaction. The TRSV titer was high in systemically infected leaves of BTS+ and low in BTS- leaves. This result suggested that a synergistic effect was obtained in triple infections of PvEV1, PvEV2, and TRSV in BTS+ which made common bean more susceptible to TRSV. Mosaic symptoms were obtained after inoculation of four common bean cultivars regardless of the presence of single endornaviruses. This suggests that in the case of TRSV, double and not single endornaviruses can cause the synergistic reaction.

Limited studies have been conducted with SHMV-infected common bean plants. Pinto bean reacted with pinpoint necrotic local lesions in the inoculated primary leaves in mixed infections between the cowpea strain of SBMV-C and SHMV (Fuentes and Hamilton, 1991). The symptoms caused by SHMV infection included vein necrosis, mosaic, and leaf malformation. Vein necrosis caused by SHMV in Majesty and BTS+ was obtained 7 DAI. However, at 14 DAI, these two cultivars and Closeau showed vein necrosis with higher degree of severity on both primary and trifoliate leaves. This result suggests that the synergistic effect between PvEV1 and SHMV in Majesty resulted in vein necrosis and mosaic symptoms. The results of ELISA were not practical to evaluate this interaction because all tested common bean genotypes exhibited high virus titers. However, in the case of Majesty, the relative PvEV1 titers determined by qPCR showed an increase titer when coinfected with SHMV while in the case of other viruses, the titer did not change significantly. The increase of PvEV1 titer in Majesty was possible due to its synergistic reaction with SHMV. This interaction might be caused by SHMV suppressing the common bean immune system resulting in an increase of PvEV1 replication. Mixed infection of SHMV and endornaviruses in other tested common bean cultivars resulted in similar SHMV accumulation when measured by ELISA or qPCR.

The result of synergism between PvEV1 and SHMV in Majesty with higher relative PvEV1 RNA titers has not been reported. In these studies, the host showed more symptom severity and higher relative virus titer in a mixed infection of two viruses when compared with a single infection.

The symptoms obtained from single, double, and triple infections of endornaviruses and three acute viruses in common bean cultivars were variable. A synergism was observed in triple infections of PvEV1, PvEV2, and TRSV in the BTS+ line. The BTS+ plants showed ring spots,

chlorosis, and necrosis on the leaves. In this study, most of the symptoms were evident by 7 DAI which was the first time that samples were collected for ELISA and qPCR tests. It is possible that it was already too late to measure the gradient of virus titers. Therefore, in future experiments, testing should be done using samples collected 3, 6, and 9 days after inoculation. Furthermore, to obtain data on virus accumulation, studies of the ultrastructure of infected cells are also important. Previous studies on the ultrastructure of plants infected with helper viruses showed that they can help a second virus to move systemically in infected plants (Fuentes and Hamilton, 1991). A similar study possibly can be done to evaluate the transmission of endornaviruses when co-inoculated with different acute viruses. However, to confirm the synergistic effects that result from the interactions between common bean endornavirus and acute viruses in mixed infections, endornavirus-infected and endornavirus-free near-isogenic lines should be used.

CHAPTER 6.
CONCLUSIONS

Common bean (*Phaseolus vulgaris*), a legume in the family *Fabaceae*, is the main grain legume for direct human consumption. It represents a rich source of protein, vitamins, minerals, and fiber, and serves as a nutritious food for the poor populations (Broughton *et al*., 2003). Common bean originated and was domesticated in the new world and is now grown worldwide. The domestication of common bean took place in two geographical locations, Mesoamerica and the Andes (Singh *et al*., 1991). Based on DNA analysis, there appears to have been limited domestication events in the Andean gene pool resulting in less genetic diversity. In contrast, multiple domestication events are recorded in the Mesoamerican gene pool. These multiple domestication events resulted in a greater genetic diversity in this pool and suggest that Mesoamerica is likely the origin of common bean (Bitocchi et al., 2012; Kwak and Gepts, 2009).

Generally, endornaviruses have been identified by electrophoretic analyses of large dsRNAs. Most dsRNA extraction methods are based on phenol extraction combined with the dsRNA-binding to fibrous cellulose. The requirement of large amounts of tissue and reagents are limitations of most published methods. In this investigation, a modification of the "non-phenol batch procedure" published by Morris et al. (1983) was developed and validated. The modified dsRNA extraction method was efficient, fast, economic, versatile, and required small amounts of tissue. The method was successfully used to extract dsRNAs from plants infected with acute and persistent viruses and from biotrophic fungi infecting plants. The modified method included several improvements from previously described methods. These included short processing time, small amount of tissue, relatively high dsRNA yields, and most important low cost and low amounts of toxic waste. Furthermore, this method allowed a large number of samples to be processed in a short period of time using low amounts of reagents. The level of detection was

improved by staining dsRNAs with GelRed™, a safe alternative to ethidium bromide. Replicative forms of viral dsRNAs obtained with the modified method were used successfully as templates in RT-PCR reactions. In addition, this method was used to extract dsRNAs from virus-infected desiccated tissues. The use of desiccated tissues provides a practical alternative to many laboratories that do not have access to liquid nitrogen. The modified method is similar to other previously published dsRNA extraction methods; however, it contains several improvements that increase the overall extraction efficiency and the practicality of using dsRNA as reagent for plant and fungal virus diagnosis, identification, and sequencing. Furthermore, this method could be very helpful to researchers interested in virome analyses of phytobiomes.

In the common bean cultivar Black Turtle Soup (BTS), two endornaviruses have been identified; *Phaseolus vulgaris endornavirus 1* (PvEV1) and *Phaseolus vulgaris endornavirus 2* (PvEV2) (Okada et al., 2013). The modified dsRNA extraction method developed in this investigation was used in a study to determine the occurrence of PvEV1 and PvEV2 in common bean germplasm from the two centers of common bean domestication. The results of this investigation showed that Mesoamerican-domesticated common bean genotypes are often double-infected with PvEV1 and PvEV2. Moreover, these endornaviruses were detected in some wild *P. vulgaris* from this region. In contrast, these viruses were not detected in wild *P. vulgaris* from the Andean region and were present in a low percentage in Andean-domesticated common bean genotypes. It is likely that the infection of domesticated *P. vulgaris* genotypes from the Andean region with endornaviruses is the product of introgression among gene pools (Mesoamerica and Andean) by modern plant breeding. This is not surprising, because endornaviruses are transmitted at relatively high percentages through both gametes (Fukuhara and Moriyama, 2008). It is possible that in Mesoamerica, endornavirus-infected wild *P. vulgaris*

were selected during crop domestication and introduced to some cultivars during the breeding process. Lower (15%) occurrence of PvEV1 and PvEV2 of wild species, medium (39%) of land races, and high (93%) of cultivars and breeding-lines supports this idea.

Common bean endornaviruses do not seem to have an adverse effect on common bean domesticated in Mesoamerica. All tested endornavirus-infected common bean cultivars and breeding-lines of Mesoamerican origin were infected simultaneously with both PvEV1 and PvEV2. These two endornaviruses seem to coexist in the plant host as double-infections. It appears that PvEV1 and PvEV2 are in a symbiotic relationship with Mesoamerican-domesticated common bean. Nevertheless, in the case of most common bean genotypes of Andean origin, the lack of these endornaviruses does not seem to have an adverse effect. It is possible that the putative beneficial effect that these endornaviruses may have on common bean was not needed in the Andean region.

The endornaviruses PvEV1 or PvEV2 were not detected in 18 other *Phaseolus* species which included four other domesticated species: *P. acutifolius, P. coccineus, P. dumosus,* and *P. lunatus*. Endornaviruses PvEV1 and PvEV2 infecting domesticated common bean are similar to those infecting wild *P. vulgaris* genotypes. This is particularly true for PvEV2 because nucleotide sequence identities among the various genotypes ranged from 98-99%. Although in the case of PvEV1, sequence identities of this endornavirus from some landraces and wild *P. vulgaris* genotypes were more divergent (95-96%) with respect to PvEV1 from Black Turtle Soup. This result was supported by phylogenetic analyses using partial sequences of the RNA-dependent RNA polymerase (RdRp) of PvEV1 and PvEV2 in which wild *P. vulgaris* genotypes infected with PvEV1 clustered in a specific clade, apart from PvEV1 infected landraces, cultivars, and breeding lines. The analysis of the RdRp of PvEV2 suggests that this virus could

be grouped into two major clades, regardless of infecting wild, landraces, cultivars, and breeding lines or their geographical origin.

A comparative study of morphological and physiological characters was conducted with two lines of BTS, one double-infected with PvEV1 and PvEV2 (BTS+) and the other one virus-free (BTS-). Three selections from each line were used as replicates. The BTS+ and BTS- lines and selections were inoculated with *Sunn-hemp mosaic virus* (SHMV). The BTS+ line and selections showed faster seed germination and longer length of the radical root than the BTS- line and selections. These results suggest that endornaviruses may promote seed germination and root elongation.

The chlorophyll content of the BTS+ line and selections was significantly lower than the content in the BTS- line and selections. Nevertheless, the leaves of these two common bean lines did not show detectable phenotypic differences. Although, there is no available information on the reduction of chlorophyll content in endornavirus-infected plants, a study of the cellular localization of PvEV2 and PvEV1, found that PvEV2 was associated with the chloroplast fraction, while PvEV1 was associated with cytoplasmic vesicles (Okada et al., 2013). It is possible that the lower amount of the chlorophyll in BTS+ is related to the localization of PvEV2 in that organelle. A chlorophyll reduction was also obtained when the acute virus SHMV was inoculated to the BTS+ line.

The carotenoid content of the BTS+ line and selections was significantly greater than that of the BTS- line and selections. The carotenoid content of BTS+ inoculated with SHMV was also greater than that of the BTS- inoculated with SHMV. These results suggest that the higher carotenoid content in the BTS+ line and selections might be associated with the presence of the endornaviruses. For the anthocyanin content, there were no differences between the BTS+ and

the BTS- lines. A similar anthocyanin content in the common bean cultivars T-39, Jaguar, and Vista (endornavirus-infected) and Othello (endornavirus-free) reported by Akond et al. (2011) supports the lack of association of endornaviruses and the amount of anthocyanin.

There were no differences in the time required to reach flowering between the BTS+ and the BTS- lines and selections. However, triple virus infection of BTS+ infected with SHMV increased significantly the time to flowering when compared to BTS- inoculated with SHMV. In addition, both BTS+ and BTS- lines inoculated with SHMV had a significantly longer time to flowering than BTS+ and BTS- lines that were not inoculated with SHMV. The results of the effect on pod formation showed a similar trend. These investigations suggest that the time to flower and pod formation of common bean do not appear to be affected by the presence or absence of endornaviruses, although, they were affected by SHMV infection. In this study, BTS+ and BTS- lines infected with SHMV yielded significant lower number of seeds per pod than the non-SHMV infected lines. Triple virus infected BTS+ yielded significant lower number of seeds per pod than BTS- infected with SHMV. These results suggest that reduction of seeds per pod is not associated with the presence of endornaviruses but it was associated to SHMV infection.

Like the number of seeds per pods and time to flowering, plant height was not affected by the presence of endornaviruses. The only exception was BTS+ inoculated with SHMV which showed significantly less plant height than the other tested lines and selections. The BTS+ line and selections yielded longer pods than the BTS- line and selections. This result suggests that pod length was associated with the presence of endornaviruses in common bean. The BTS+ line infected with SHMV showed significant less seed weight per plant than the most lines and selections, however, it did not differ from BTS- infected with SHMV. It is clear that the BTS+

and BTS- lines inoculated with SHMV differed significantly in weight of 100 seeds from the non-virus inoculated lines, but between them, there were no differences.

The BTS+ and BTS- lines, and cvs Majesty, Closeau, Celrk, and Red Hawk exhibited different reactions to the various acute virus inoculations. Inoculations of TMV to the different lines and cultivars resulted in necrotic local lesions which are associated with resistance in common bean cultivars. The BTS+ line reacted with more necrotic local lesion when compared to the BTS- line. The different numbers of necrotic local lesions between the BTS+ and BTS- lines suggest that endornaviruses may be associated with the local lesion reaction.

Synergistic symptoms caused by TRSV infection in BTS+ included ring spots, leaf chlorosis, necrosis, and mosaic. This synergistic effect was not observed when endornaviruses were not present such as the case of the BTS- line. The ELISA data supported these findings. The, mosaic symptom did not appear to be related to synergism because it was observed in all common bean cultivars.

Symptoms caused by SHMV were variable and depended upon the common bean cultivar inoculated and included vein necrosis, mosaic, and leaf malformation. Vein necrosis caused by SHMV in Closeau (infected with PvEV2) and BTS+ was observed at 7 DAI, however this was not the case with Majesty (infected with PvEV1). Nevertheless, at 14 DAI, these two cultivars and the BTS+ line exhibited vein necrosis with higher degree of severity on both primary and trifoliate leaves. The results of ELISA were not useful to evaluate interactions because all tested common bean genotypes exhibited high acute virus titers. However, in the case of Majesty the relative PvEV1 titer, determined by qPCR, increased when co-infected with SHMV while in the case of Closeau, PvEV2 titer did not change. These results suggest a synergistic interaction between PvEV1 and SHMV in Majesty. In the case of the BTS+ line infected by both PvEV1

and PvEV2, virus titers did not change, although the symptoms consisted of foliar vein necrosis. Mosaic or leaf malformation symptoms were not associated with acute virus/endornavirus interactions. Most likely, they were associated with the genetics of the common bean cultivars used. In summary, a practical dsRNA extraction method was developed and used to detect endornviruses from common bean. The method was used to determine the occurrence of common bean endornaviruses in the two main centers of common bean domestication. A study on the interactions between endornaviruses and the host revealed that in the case of common bean, endornaviruses may promote seed germination and pod length. However, endornaviruses were associated with lower chlorophyll content of common bean plants. When these interactions were conducted with two acute viruses (TRSV and SHMV), a synergistic effect between these viruses and common bean endornaviruses was obtained. More investigations should be conducted to determine the type of symbiotic interaction that exists between common bean and endornaviruses.

REFERENCES

Adams, M. J, E. J. Lefkowitz, A. M. Q. King, and E. B. Carstens. 2014. "Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses." *Archives of Virology* 159:2831-2841.

Akond, A. S. M. G. M., L. Khandaker, J. Berthold, L. Gates, K. Peters, H. Delong, and K. Hossain. 2011. "Anthocyanin, total polyphenols and antioxidant activity of common bean." *American Journal of Food Technology* 6:385-394.

Al Rwahnih, M., S. Daubert, J. R. Urbez-Torres, F. Cordero, and A. Rowhani. 2011. "Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses." *Archives of Virology* 156:397-403.

Alishiri, A., F. Rakhshandehroo, H.-R. Zamanizadeh, and P. Palukaitis. 2013. "Prevalence of Tobacco mosaic virus in Iran and evolutionary analyses of the coat protein gene." *The Plant Pathology Journal* 29:260-273.

Aparicio-Fernandez, X., L. Manzo-Bonilla, and G. Loarca-Pina. 2005. "Comparison of antimutagenic activity of phenolic compounds in newly harvested and stored common beans *Phaseolus vulgaris* against aflatoxin B1." *Journal Food Science* 70:73-78.

Arnon, D. I. 1994. "Copper enzymes in isolated chloroplasts, polyphenol oxidase in *Beta vulgaris*." *Plant Physiology* 2:1-15.

Ayanru, D. K. G., and V. C. Sharma. 1982. "Effects of cassava mosaic disease on certain leaf parameters of field-grown cassava clones." *Phytopathology* 72:1057-1059.

Azevedo, L., J. C. Gomes, P. C. Stringheta, A. M. M. C. Gontijo, C. R. Padovani, L. R. Ribeiro, and D. M. F. Salvadori. 2003. "Black bean (*Phaseolus vulgaris* L.) as a protective against DNA damage in mice." *Food Chemical Toxicology* 41:1671-1676.

Azizi, A., and M. Shams-bakhsh. 2014. "Impact of cucumber mosaic virus infection on the varietal traits of common bean cultivars in Iran." *Virus Disease* 25:447-454.

Balijja, A., A. Kvarnheden, and T. Turchetti. 2008. "A non-phenol-chloroform extraction of double-stranded RNA from plant and fungal tissues." *Journal of Virological Methods* 152:32-37.

Bar-Joseph, M., A. Rosner, M. Moskovitz, and R. Hull. 1983. "A simple procedure for the extraction of double-stranded RNA from viral infected plants." *Journal of Virological Methods* 6:1-8.

Becerra-Velásquez, V. L., and P. Gepts. 1994. "RFLP diversity in common bean (*Phaseolus vulgaris* L.)." *Genome* 37:256-263.

Bitocchi, E., E. Bellucci, A. Giardini, D. Rau, M. Rodriguez, E. Biagetti, R. Santilocchi, P. S. Zeuli, T. Gioia, G. Logozzo, G. Attene, L. Nanni, and R. Papa. 2013. "Molecular analysis of the parallel domestication of the common bean (*Phaseolus vulgaris*) in Mesoamerica and the Andes." *New Phytologist* 197:300-313.

Bitocchi, E., L. Nanni, E. Bellucci, M. Rossi, A. Giardini, P. S. Zeuli, G. Logozzo, J. Stougaard, P. McClean, G. Attene, and R. Papa. 2012. "Mesoamerican origin of the common bean (*Phaseolus vulgaris* L.) is revealed by sequence data." *Proceedings of the National Academy of Sciences of the United States of America* 109:E788-E796.

Blair, M. W., M. J. Bassett, A. M. Abouzid, E. Hiebert, J. E. Polston, R. T. Jr. McMillan, and W. Graves, and M. Lamberts. 1995. "Occurrence of bean golden mosaic virus in Florida." *Plant Disease* 79:529-533.

Bliss, F. A. 1980. *Common Bean in Hybridization of Crop Plant*. American Society of Agronomy-Crop Science Society of America, Madison, WI, p. 273-284.

Broughton, W. J., G. Hernández, M. Blair, S. Beebe, P. Gepts, and J. Vanderleyden. 2003. "Beans (*Phaseolus* spp.)-model food legumes." *Plant and Soil* 252:55-128.

Buck, K. W. 1999. "Replication of tobacco mosaic virus RNA." *Philosophical Transactions of The Royal Society B Biological Sciences* 354:613-627.

Candresse, T., A. Marais, C. Faure, and P. Gentit. 2013. "Association of Little cherry virus 1 (LChV1) with the Shirofugen stunt disease and characterization of the genome of a divergent LChV1 isolate." *Phytopathology* 103:293-308.

Candresse, T., A. Marais, R. Sorrentino, C. Faure, S. Theil, V. Cadot, M. Rolland, J. Villemot, and F. Rabenstein. 2016. "Complete genomic sequence of barley (*Hordeum vulgare*) endornavirus (HvEV) determined by next-generation sequencing." *Archives of Virology* 161:741-743.

Castillo, A., L. Cottet, M., Castro, and F. Selpulveda. 2011. "Rapid isolation of mycoviral double-stranded RNA from *Botrytis cinerea* and *Saccharomyces cerevisiae.*" *Virology Journal* 8:38.

Cherian, S., and V. Muniyapppa. 1998. "ELISA based survey and host range of tomato mosaic Tobamovirus." *Indian Journal of Virology* 14:65-69.

Chia, T. F., and J. He. 1999. "Photosynthetic capacity in *Oncidium* (Orchidaceae) plants after virus eradication." *Environmental and Experimental Botany* 42:11-16.

Chitra, T. R., H. S. Prakash, S. E. Albrechtsen, H. S. Shetty, and S. B. Mathur. 2002. "Indexing of leaf and seed samples of tomato and bell pepper for tobamoviruses." *Indian Phytopathology* 55:84-86.

Coetzee, B., M. J. Freeborough, H. J. Maree, J. M. Celton, D. J. G. Rees, and J. T. Burger. 2010. "Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard." *Virology* 400:157-163.

Colinet, D., M. Nguyen, J. Kummert, and P. Lepoivre. 1998. "Differentiation among potyviruses infecting sweet potato based on genus- and virus-specific reverse transcription polymerase chain reaction." *Plant Disease* 82:223-229.

Davis, R. F., and R. O. Hampton. 1986. "Cucumber mosaic virus isolates seedborne in Phaseolus vulgaris: serology, host-pathogen relationships, and seed transmission." *Phytopathology* 76:999-1004.

Deker, C. J., and R. Parker. 2014. "Analysis of double-stranded RNA from microbial communities identifies double-stranded RNA virus-like elements." *Cell Reports* 7:898-906.

DePaulo, J. J., and C. A. Powell. 1995. "Extraction of double-stranded RNA from plant tissues without the use of organic solvents." *Plant Disease* 79:246-248.

Dina, M. B., and A. A. E-M. Sabah. 2008. "Quantitative and qualitative changes of phytochemical composition in viral infested *N. tabacum*." *Journal of Applied Sciences Research* 4:1083-1091.

Drijfhout, E., M. J. Silbernagel, and D. W. Burke. 1978. "Differentiation of strains of bean common mosaic virus." *Netherlands Journal of Plant Pathology* 84:15-26.

Elena, S. F., G. P. Bernet, and J. L. Carrasco. 2014. "The games plant viruses play." *Current Opinion in Virology* 8:62-67.

EL-Qudah, J. M. 2014. "Estimation of Carotenoid contents of celected Mediterranean legumes by HPLC." *World Journal of Medical Sciences* 10:89-93.

Enebak, S. A., B. I. Hillman, and W. L. Macdonald. 1994. "A hypovirulent isolate of *Cryphonectria parasitica* with multiple, genetically unique dsRNA segments." *Molecular Plant-Microbe Interactions* 7:590-595.

Espach, Y., H. J. Maree, and J. T. Burger, 2012. "Complete genome of a novel endornavirus assembled from next-generation sequence data." *Journal of Virology* 86:131-142.

Fraga, D., T. Meulia, and S. Fenster. 2008. *Real-Time PCR*. In Current Protocols Essential Labolatory Techniques. John Wiley & Sons, Inc. p 10.3.1-10.3.33. DOI: 10.1002/9780470089941.et1003s00.

Franklin, R. M. 1966. "Purification and properties of replicative intermediate of the RNA bacteriophage R17." *Proceedings of the National Academy of Sciences of the United States of America* 55:1504-1511.

Fuentes, A. L., and R. I. Hamilton. 1991. "Sunn-hemp mosaic virus facilitates cell-to-cell spread of southern bean mosaic virus in a nonpermissive host." *Phytopathology* 81:1302-1305.

Fukuhara, T. 1999. "Double-stranded RNA in rice." *Journal of Plant Research* 112:131-136.

Fukuhara, T., and M. J. Gibbs, 2012. *Family Endornaviridae*. In: King, A.M.Q., Adams, M.J., Carstens, E. B., Lefkowitz, E. J. (Eds.), Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, UK, pp. 519-521.

Fukuhara, T., and H. Moriyama. 2008. *Endornaviruses*: In Encyclopedia of Virology, 3rd ed. Elsevier, Oxford, pp 109-116.

Funayama, S., and I. Terashima. 1999. "Effects of geminivirus infection and growth irradiance on the vegetative growth and photosynthetic production of *Eupatorium makinoi*." *New Phytologist* 142:483-494.

Funayama-Noguchi, S., and I. Terashima. 2006. "Effects of *Eupatorium* yellow vein virus infection on photosynthetic rate, chlorophyll content and chloroplast structure in leaves of *Eupatorium makinoi* during leaf development." *Functional Plant Biology* 33:165-175.

Gaut, B.S. 2014. "The complex domestication history of the common bean." *Nature Genetics* 46:663-664.

Gepts, P., T. C. Osborn, K. Rashka, and F. A. Bliss. 1986. "Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): Evidence for multiple centers of domestication." *Economic Botany* 40:451-468.

Ghabrial, S. A., and N. Suzuki. 2009. *Fungal Viruses*. In Encyclopedia of Virology; Granoff, A., Webster, R., Eds.; Elsevier: Amsterdam, The Netherlands, Volume 1, pp. 284-291.

Gildow, F. E., D. A. Shah, W. M. Sackett, T. Butzler, B. A. Nault, and S. J. Fleischer. 2008. "Transmission efficiency of cucumber mosaic virus by aphids associated with virus epidemics in snap bean." *Phytopathology* 98:1233-1241.

Grill, L. K., and S. J. Garger. 1981. "Identification and characterization of double-stranded RNA associated with cytoplasmic male sterility in *Vicia faba*." *Proceedings of the National Academy of Sciences of the United States of America* 78:7043-7046.

Guo D. P., Y. P. Guo, J. P. Zhao, H. Liu, Y. Peng, Q. M. Wang, J. S. Chen, and G. Z. Rao. 2005. "Photosynthetic rate and chlorophyll fluorescence in leaves of stem mustard (*Brassica juncea* var. *tsatsai*) after turnip mosaic virus infection." *Plant Science* 168:57-63.

Hacker, C. V., C. M. Brasier, and K. W. Buck. 2005. "A double-stranded RNA from a *Phytophthora* species is related to the plant endornaviruses and contains a putative UDP glycosyltransferase gene." *Journal of General Virology* 86:1561-1570.

Hampton, R.O. 1975. "The nature of bean yield reduction by bean yellow and bean common mosaic virus." *Phytopathology* 65:1342-1346.

Hampton, R. O., and R. I. B. Francki. 1992. "RNA-1 dependent seed transmissibility of cucumber mosaic virus in *Phaseolus vulgaris*." *Phytopathology* 82:127-130.

Herrero, N., S. S. Márquez, and I. Zabalgogeazcoa. 2009. "Mycoviruses are common among different species of endophytic fungi of grasses." *Archives of Virology* 154:327-330.

Hillman, B. I., R. Foglia, and W. Yuan. 2000. "Satellite and defective RNAs of *Cryphonectria hypovirus* 3-Grand Haven 2, a virus species in the family *Hypoviridae* with a single open reading frame." *Virology* 276:181-189.

Hooks, C. R. R., M. G. Wright, D. S. Kabasawa, R. Manandhar, and R. P. P. Almeida. 2008. "Effect of banana bunchy top virus infection on morphology and growth characteristics of banana." *Annals of Applied Biology* 153:1-9.

Huang, Q., L. Baum, and W. L. Fu. 2010. "Simple and practical staining of DNA with GelRed in agarose gel electrophoresis." *Clinical Laboratory* 56:149-52.

Hull, R. 2014. *Plant Virology*. 5th ed. Academic Press, London, UK, 1104 pp.

Jelkmann, W., R. R. Martin, and E. Maiss. 1989. "Cloning of four viruses from small quantities of double-stranded RNA." *Phytopathology*. 79:1250-1253.

Jo, Y., H. Choi, and W. K. Cho. 2015. "De novo assembly of a bell pepper endornavirus genome sequence using RNA sequencing data." *Genome Announcements* 3(2):e00061-15. doi: 10.1128/genomeA.00061-15.

Jo, Y., H. Choi, J. Y. Yoon, S. K. Choi, and W. K. Cho. 2016. "*In silico* identification of Bell pepper endornavirus from pepper transcriptomes and their phylogenetic and recombination analyses." *Gene* 575:712-717.

Kelly, J. D. 2010. *The story of bean breeding*. Michigan State University publication prepared for BeanCAP and Works, PBGJune 2010. http://www.css.msu.edu/Bean/_pdf/Story_of_Bean_Breeding_in_the_US.pdf.

Khalifa, M. E., and M. N. Pearson. 2014. "Molecular characterization of an endornavirus infecting the phytopathogen *Sclerotinia sclerotiorum.*" *Virus Research* 189:303-309.

Khankhum, S., and R. A. Valverde. 2013. "Association of endornaviruses of Black Turtle Soup common bean with seed germination, grain yield, and host reaction to *Tobacco ringspot virus*." *Phytopathology* 103:S2.73.

Khankhum, S., R. A. Valverde, M. A. Pastor-Corrales, J. M. Osorno, and S. Sabanadzovic. 2015. "Two endornaviruses show differential infection patterns between gene pools of *Phaseolus vulgaris*." *Archives of Virology* 160:1131-1137.

Kirk, J. T. O., and R. L. Allen. 1965. "Dependence of chloroplast pigment synthesis on protein synthetic effects on actilione." *Biochemical and Biophysics Research Journal of Canada*. 27:523-530.

Koenig, R., and P. Gepts. 1989. "Allozyme diversity in wild *Phaseolus vulgaris*: Further evidence for two major centers of genetic diversity." *Theoretical and Applied Genetics* 78:809-817.

Kokkinos, C. D., and C. A. Clark. 2006. "Real-time PCR assays for detection and quantification of sweetpotato viruses." *Plant Disease* 90:783-788.

Kokkinos, C. D., C. A. Clark, C. E. McGregor, and D. R. LaBonte. 2006. "The effect of sweet potato virus disease and its viral components on expression levels in sweetpotato." *Journal of the American Society for Horticultural Science* 131:657-666.

Kundu J. K., S. Gadiou, G. Schlesingerová, M. Dziaková, and V. Čermák. 2015. "Emergence of quarantine *Tobacco ringspot virus* in *Impatiens walleriana* in the Czech Republic." *Plant Protection Science* 51: 115-122.

Kwak, M., and P. Gepts. 2009. "Structure of genetic diversity in the two major gene pools of common bean (*Phaseolus vulgaris* L., Fabaceae)." *Theoretical and Applied Genetics* 118:979-992.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. "ClustalW and ClustalX version 2." *Bioinformatics* 23:2947-2948.

Larson, G., D. R. Piperno, R. G. Allaby, M. D. Purugganan, L. Andersson, M. Arroyo-Kalin, L. Barton, C. C. Vigueira, T. Denham, K. Dobney, A. N. Doust, P. Gepts, M. T. P. Gilbert, K. J. Gremillion, L. Lucas, L. Lukens F. B. Marshall, K. M. Olsen, J. C. Pires, P. J. Richerson, R. R. de Casas, O. I. Sanjur, M. G. Thomas, and D. Q. Fuller. 2014. "Current perspectives and the future of domestication studies." *Proceedings of the National Academy of Sciences of the United States of America* 111:6139-6146.

Leal, N., and R. Lastra. 1984. "Altered metabolism of tomato plants infected with tomato yellow mosaic virus." *Physiological Plant Pathology* 24:1-7.

Liu, H., Y. Fu, J. Xie, J. Cheng, S. A. Ghabrial, G. Li, X. Yi, and D. Jiang. 2012. "Discovery of novel dsRNA viral sequences by *in silico* cloning and implications for viral diversity host range and evolution." *PLoS ONE*. 7: e42147. doi:10.1371/journal.pone.0042147.

Liu, W., X. Zhao, P. Zhang, T. T. Mar, Y. Liu, Z. Zhang, C. Han, and X. Wang. 2013. "A one step real-time RT-PCR assay for the quantitation of *Wheat yellow mosaic virus* (WYMV)." *Virology Journal* 10:1-9.

Loebenstein, G. 2009. "Local lesions and induced resistance." *Advances in Virus Research* 75:73-117.

Loebenstein, G., and F. Akad. 2006. *The local lesion response*. *In* "Natural Resistance Mechanisms of Plants to Viruses" (G. Loebenstein and J. P. Carr, eds.) pp. 99-124. Springer, Dordrecht, The Netherlands.

Márquez, L. M., R. S. Redman, R. J. Rodriguez, and M. J. Roossinck. 2007. "A virus in a fungus in a plant-three way symbiosis required for thermal tolerance." *Science* 315:513-515.

Mensack M. M., V. K. Fitzgerald, E. P. Ryan, M. R. Lewis, H. J. Thompson, and M. A. Brick. 2010. "Evaluation of diversity among common beans (*Phaseolus vulgaris* L.) from two centers of domestication using 'omics' technologies." *BMC Genomics* 11: 686. doi: 10.1186/1471-2164-11-686.

Morales, F. J. 2006. Common beans. In G. Loebenstein and J. P. Carr (eds.), *Natural resistance mechanism of plants to viruses*. p367-382. The Netherlands, Springer.

Morales, F. J., and P. K. Anderson. 2001. "The emergence and dissemination of whitefly-transmitted germiniviruses in Latin America." *Archives of Virology* 146:415-441.

Morales, F. J., and L. Bos. 1988. *Bean common mosaic*. C.M.I./A.A.B. Descriptions of Plant Viruses No. 337. Kew, Surrey, England, 4 p.

Moriyama, H., H. Horiuchi, R. Koga, and T. Fukuhara. 1999. "Molecular characterization of two endogenous double-stranded RNAs in rice and their inheritance by interspecific hybrids." *Journal of Biological Chemistry* 274:6882-6888.

Moriyama, H., K. Kanaya, J. Z. Wang, T. Nitta, and T. Fukuhara. 1996. "Stringently and developmentally regulated levels of a cytoplasmic double-stranded RNA and its high efficiency transmission via egg and pollen in rice." *Plant Molecular Biology* 31:713-719.

Moriyama, H., T. Nitta, and T. Fukuhara. 1995. "Double-stranded RNA in rice: a novel RNA replicon in plants." *Molecular and General Genetics* 248:364-369.

Morris, T. J., and J. A. Dodds. 1979. "Isolation and analysis of double-stranded RNA from virus infected plant and fungal tissue." *Phytopathology* 69:854-858.

Morris, T. J., J. A. Dodds, B. Hillman, R. Jordan, S. A. Lommel, and S. Tamaki. 1983. "Viral specific dsRNA: diagnostic value for plant disease identification." *Plant Molecular Biology Reporter* 1:27-30.

Naidu, R. A., M. Krishnan, M. V. Nayadu, and A. Gnanam. 1986. "Studies on peanut green mosaic virus infected peanut (*Arachis hypogaea* L.) leaves: III Changes in the polypeptides of Photosystem II particles." *Physiological and Molecular Plant Pathology* 29:53-58.

Nakatsukasa-Akune, M., K. Yamashita, Y. Shimoda, T. Uchiumi, M. Abe, T. Aoki, A. Kamizawa, S.-i. Ayabe, S. Higashi, and A. Suzuki. 2005. "Suppression of root nodule formation by artificial expression of the TrEnodDR1 (coat protein of *White clover cryptic virus 2*) gene in Lotus japonicas." *Molecular Plant-Microbe Interactions* 18:1069-1080.

Naylor, W. A., and L. J. Giles. 1982. "Growth, pigment synthesis, and ultrastructural responses of *Phaseolus vulgaris* L. cv. Blue Lake to intermittent and flashing light 1,2." *Plant Physiology* 70:257-263

Neff, M. M., and J. Chory. 1998. "Genetic interactions between phytochrome A, phytochrome B and cryptochrome 1 during *Arabidopsis* development." *Plant Physiology* 118:27-35.

Nerva, L., M. Ciuffo, M. Vallino, P. Margaria, G. C. Varese, G. Gnavi, and M. Turina. 2015. "Multiple approaches for the detection and characterization of viral and plasmid symbionts from a collection of marine fungi." *Virus Research* (In press). doi:10.1016/j.virusres.2015.10.028.

Nuss, D. L., and Y. Koltin. 1990. "Significance of dsRNA genetic elements in plant pathogenic Fungi." *Annual Review of Phytopathology* 28:37-58.

Okada, R., E. Kiyota, H. Moriyama, T. Fukuhara, and T. Natsuaki. 2015. "A simple and rapid method to purify viral dsRNA from plant and fungal tissue." *Journal of General Plant Pathology* 8:103-107.

Okada, R., E. Kiyota, S. Sabanadzovic, H. Moriyama, T. Fukuhara, P. Saha, M. J. Roossinck, A. Severin, and R. A. Valverde. 2011. "Bell pepper endornavirus: molecular and biological properties and occurrence in the genus *Capsicum*." *Journal of General Virology* 92:2664-2673.

Okada R., C. K. Young, R. A. Valverde, S. Sabanadzovic, N. Aoki, S. Hotate, E. Kiyota, H. Moriyama, and T. Fukuhara. 2013. "Molecular characterization of two evolutionarily distinct endornaviruses co-infecting common bean (*Phaseolus vulgaris*)." *Journal of General Virology* 94:220-229.

Osaki H., H. Nakamura, A. Sasaki, N. Matsumoto, and K. Yoshida. 2006. "An endornavirus from a hypovirulent strain of the violet root rot fungus, *Helicobasidium mompa*." *Virus Research* 118:143-149.

Papa, R., and P. Gepts. 2003. "Asymmetry of gene flow and differential geographical structure of molecular diversity in wild and domesticated common bean (*Phaseolus vulgaris* L.) from Mesoamerica." *Theoretical and Applied Genetics* 106:239-250.

Pfeiffer, P. 1998. "Nucleotide sequence, genetic organization and expression strategy of the double-stranded RNA associated with the '447' cytoplasmic male sterility trait in *Vicia faba*." *Journal of General Virology* 79:2349-2358.

Pruss, G., X. Ge, X. M. Shi, J. C. Carrington, and V. B. Vancea. 1997. "Plant Viral Synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses." *The Plant Cell* 9:859-868.

Quito-Avila, D. F., W. Jelkmann, I. Tzanetakis, K. Keller, and R. R. Martin. 2011. "Complete sequence and genetic characterization of *Raspberry latent virus*, a novel member of the family *Reoviridae*." *Virus Research* 155:397-405.

Quito-Avila, D. F., and R. R. Martin. 2012. "Real-time RT-PCR for detection of Raspberry bushy dwarf virus, Raspberry leaf mottle virus and characterizing synergistic interactions in mixed infections." *Journal of Virological Methods* 179:38-44.

Rainey, K. M., and P. D. Griffiths. 2005. "Differential response of common bean genotypes to high temperature." *Journal of the American Society for Horticultural Science* 130:18-23.

Rao, G. P., R. A. Valverde, and C. I. Dovas. 2008. *Techniques in Diagnosis of Plant Viruses*. Studium Press, Texas, USA, 302 pp.

Redman, R. S., K. B. Sheehan, R. G. Stout, R. J. Rodriguez, and J. M. Henson. 2002. "Thermotholerance generated by plant/fungal symbiosis." *Science* 298:1581.

Román, M. A., A. M. Castañeda, J. C. A. Sánchez, C. G. Muñoz, and J. S. Beaver. 2004. "Inheritance of normal pod development in bean golden mosaic resistant common bean." *Journal of the American Society for Horticultural Science* 129:549-552.

Roossinck, M. J. 2010. "Lifestyles of plant viruses**."** *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 365:1899-1905.

Roossinck, M. J. 2011a. "Changes in population dynamics in mutualistic *versus* pathogenic viruses." *Viruses* 3:12-19.

Roossinck, M. J. 2011b. "The good viruses: viral mutualistic symbioses." *Nature Reviews Microbiology* 9:99-108.

Roossinck, M. J. 2013. "Plant Virus Ecology." *PLoS Pathogen* 9: e1003304. doi:10.1371/journal.ppat.1003304

Roossinck, M. J., P. Saha, G. B. Wiley, J. Quan, J. D. White, H. Lai, F. Chavarria, G. Shen, and B. A. Roe. 2010. "Ecogenomics: Using massively parallel pyrosequencing to understand virus ecology." *Molecular Ecology* 19:81-88.

Rott, M. E., and W. Jelkmann. 2001. "Characterization and detection of several filamentous viruses of cherry: Adaptation of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol." *European Journal* of *Plant Pathology* 107:411-420.

Sabanadzovic, S., D. M. Ingram, and A. M. Lawrence. 2010. "First report of *Tobacco ringspot virus* in Joe-pye weed (*Eupatorium purpureum*) in Mississippi". *Plant Disease* 94:126.

Sabanadzovic, S., and R. A. Valverde. 2011. "Properties of two cryptoviruses from pepper (*Capsicum annuum*)." *Virus Genes* 43:307-312.

Sabanadzovic, S., R. A. Valverde, J. K. Brown, R. R. Martin, and I. E. Tzanetakis. 2009. "Southern tomato virus: the link between the families *Totiviridae* and *Partitiviridae*." *Virus Research* 140:130-137.

Sabanadzovic, S., W. M. Wintermantel, R. A. Valverde, J. D. McCreight, and N. Aboughanem-Sabanadzovic. 2016. "Cucumis melo endornavirus: genome organization, host range and co-divergence with the host." *Virus Research* 214:49-58.

SAS Institute Inc. 2010. *The SAS System for Windows*. Release 9.2. Statistical Analysis Systems Institute, Cary, NC, USA.

Schmutz, J., P. E. McClean, S. Mamidi, G. A. Wu, S. B. Cannon, J. Grimwood, J. Jenkins, S. Shu, Q. Song, C. Chavarro, M. Torres-Torres, V. Geffroy, S.M. Moghaddam, D. Gao, B. Abernathy, K. Barry, M. Blair, M. A. Brick, M. Chovatia, P. Gepts, D. M. Goodstein, M. Gonzales, U. Hellsten, D. L. Hyten, G. Jia, J. D. Kelly, D. Kudrna, R. Lee, M.M. S. Richard, P. N. Miklas, J.M. Osorno, J. Rodrigues, V. Thareau, C.A Urrea, M.Wang, Y. Yu, M. Zhang, R.A. Wing, P. B. Cregan, D. S. Rokhsar, and S. A. Jackson. 2014. "A reference genome for common bean and genome-wide analysis of dual domestications." *Nature Genetics* 46:707-713.

Schuchalter-Eicke, G., and H. Jeske. 1983. "Seasonal changes in the chloroplast ltrastructure in *Abutilon Mosaic Virus* (AbMV) infected *Abutilon* spec. (Malvaceae)." *Journal of Phytopathology* 108:172-184.

Schwartz, H. F., J. R. Steadman, R. Hall, and R. L. Forster. 2005. *Compendium of bean diseases*. 2nd ed. The American Phytopathological Society Press. St. Paul, MN, 109 pp.

Scully, B. T., and D. H. Wallace. 1990. "Variation in and relationship of biomass, growth rate, harvest index, and phenology to yield of common bean**."** *Journal of the American Society for Horticultural Science* 115:218-225.

Segundo, E., M. P. Carmona, E. Sáez, L. Velasco, G. Martín, L. Ruiz, D. Janssen, and I. M. Cuadrado. 2008. "Occurrence and incidence of viruses infecting green beans in south-eastern Spain." *European Journal of Plant Pathology* 122:579–591.

Sehgal, O. P. 1992. “Pathogenesis-related proteins in lima bean leaves infected with tobacco ringspot virus and their distribution within and around local lesions.” *Plant Cell Reports* 12:55-59.

Sela, N., N. Luria, and A. Dombrovsky. 2012. “Genome assembly of bell pepper endornavirus from small RNA.” *Journal of Virology* 86:7721.

Silva, H. T.-D., and I. F. Antunes. 2003. “Pod class definition based on length and width in common bean (*Phaseolus vulgaris* L.) .” In Reports of Bean Improvement Cooperative and National Dry Bean Council Research Conference. Annual report p.131-132.

Singh, S. P. 1992. “Common bean improvement in the tropics.” *Plant Breeding Reviews* 10:199-269.

Singh, S. P., P. L. Gepts, and D. G. Debouck. 1991. “Races of common bean (*Phaseolus vulgaris*, Fabaceae).” *Economic Botany* 45:379-396.

Singh, S. P., and H. F. Schwartz. 2010. “Breeding common bean for resistance to diseases: A review.” *Crop Science* 50:2199-2223.

Singh, V., and K. Shukla. 2009. “Effect of PRSV infection on pigment content and assimilation of carbohydrate in *Carica papaya* L.” *Annals of Plant Protection Sciences* 17:1.

Song, D., W. K. Cho, S. H. Park, Y. Jo, and K. H. Kim. 2013. “Evolution and horizontal gene transfer in the Endornavirus genus.” *PLoS One* 8: e64270. doi:10.1371/journal.pone.0064270

Spence, N. J., and D. G. A. Walkey. 1995. “Variation for pathogenicity among isolates of bean common mosaic virus in Africa and a reinterpretation of the genetic relationship between cultivars of *Phaseolus vulgaris* and pathotypes of BCMV.” *Plant Pathology* 44:527–46.

Srivastava, M., U. P. Gupta, and A. Sinha. 2012. “Influence of bean common mosaic virus infection on chlorophyll content and primary productivity in hyacinth bean (*Dolichos lablab* L.).” *Crop Research* 44:174-181.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. “MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.” *Molecular Biology and Evolution* 30:2725-2729.

Tu, J. O. 1981. “Identification of a severe strain of tobacco ringspot virus from white beans (*Phaseolus vulgaris* L.).” *Phytopathology* 101:153-162.

Tzanetakis, I. E., G. B. Halgren, K. E. Keller, S. C. Hokanson, J. L. Maas, P. L. McCarthy, and R. R. Martin. 2004. “Identification and detection of a virus associated with strawberry pallidosis disease.” *Plant Disease* 88:383-390.

Tzanetakis, I. E., and R. R. Martin. 2008. “A new method for extraction of double-stranded RNA from plants.” *Journal of Virological Methods* 149:167-70.

Valverde, R. A., and J. A. Dodds. 1986. “Evidence for a satellite RNA associated naturally with the U5 strain and experimentally with the U1 strain of tobacco mosaic virus.” *Journal of General Virology* 67:1875-1884.

Valverde, R. A., J. A. Dodds, and J. A. Heick. 1986. “Double stranded RNAs from plants infected with viruses having elongated particles and undivided genomes.” *Phytopathology* 76:459-465.

Valverde, R. A., and D. L. Gutierrez. 2007. “Transmission of a dsRNA in bell pepper and evidence that it consists of the genome of an endornavirus.” *Virus Genes* 35:399-403.

Valverde, R. A., S. Nameth, O. Abdallha, O. Al-Musa, P. Desjardins, and J. A. Dodds. 1990b. “Indigenous double-stranded RNA from pepper (*Capsicum annuum*).” *Plant Science* 67:195-201.

Valverde, R. A., S. T. Nameth, and R. L. Jordan. 1990a. “Analysis of double-stranded RNA for plant virus diagnosis.” *Plant Disease* 74:255-258.

Valverde, R. A., and S. Sabanadzovic. 2009. “A new plant virus with unique properties infecting Japanese holly fern.” *Journal of General Virology* 90:2542-2549.

Valverde, R. A., S. Sabanadzovic, and M. C. Rush. 2011. “Identification of Oryza sativa endornavirus in rice genotypes from breeding programmes in the United States.” *Plant Breeding* 130:271-274.

van Kooten, O., C. Meurs, and L.C. van Loon. 1990. “Photosynthetic electron transport in tobacco leaves infected with tobacco mosaic virus.” *Physiologia Plantarum* 80: 446-542.

Velez, J. J., M. J. Basset, J. S. Beaver, and A. Molina. 1998. “Inheritance of resistance to bean golden mosaic virus in common bean.” *Journal of the American Society for Horticultural Science* 123:628-631.

Villanueva, F., S. Sabanadzovic, R. A. Valverde, and J. Navas-Castillo. 2012. “Complete genome sequence of a double-stranded RNA virus from avocado.” *Journal of Virology* 86:1282-1283.

Villarreal, L. P. 2009a. “Persistence pays: how viruses promote host group survival.” *Current Opinion in Microbiology* 12:467-472.

Villarreal, L. P. 2009b. “The source of self: genetic parasites and the origin of adaptive immunity.” *Annals of the New York Academy of Sciences* 1178:194-232.

Villarreal, L. P., V. R. Defilippis, and K. A. Gottlieb. 2000. "Acute and persistent viral life strategies and their relationship to emerging diseases." *Virology* 272:1-6.

Wakarchuk, D. A., and R. I. Hamilton. 1990. "Partial nucleotide sequence from enigmatic dsRNAs in *Phaseolus vulgaris*." *Plant Molecular Biology* 14:637-639.

Whitham, S. A., S. Quan, H-R. Chang, B. Cooper, B. Estes, T. Zhu, X. Wang, and Y-M. Hou. 2003. "Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants." *The Plant Journal* 33:271-283.

Wong, Y. S., Y. T. Chiang, and C. C. Chan. 2003. "Evaluation of antioxidant activity of *Vigna sinensis* seed extract and its protective effect on hydrogen peroxide-induced DNA damage." *Institute of Food Technology Annual Meeting,* Abstract 14E-21. http://ift.confex.com/ift/2003/techprogram/paper_18744.htm

Zabalgogeazcoa, I. A., and F. E. Gildow. 1992. "Double-stranded ribonucleic acid in 'Barsoy' Barley." *Plant Science* 83:187-194.

Zabalgogeazcoa, I., D. C. Cox-Foster, and F. E. Gildow. 1993. "Pedigree analysis of the transmission of a double-stranded RNA in Barley cultivars." *Plant Science* 91:45-53.

Zhang, Y. P., and A. Rowhani. 2000. "A strategy for rapid cDNA cloning from double-stranded RNA templates isolated from plants infected with RNA viruses using Taq DNA polymerase." *Journal of Virological Methods* 84:59-63.

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VITA

Surasak Khankhum was born in Ubonratchathani, Thailand. He graduated from Mahasarakham University, Mahasarakham, Thailand, in 2001 with a Bachelor of Science in Biology, with a concentration in microbiology. In 2007, he graduated from Kasetsart University, Khamphaengsean Campus, Nakhon Prathom, Thailand with a Master of Science in Agricultural Biotechnology, under the direction of Prof. Dr. Pissawan Chiemsombat who opened the world of plant viruses to him. He conducted research on *Capsicum chlorosis virus*, an important causal agent of peanut bud blight in Thailand. In 2006, during his masters study, he obtained a summer internship to learn laboratory techniques at Louisiana State University. He joined the plant virology laboratory of Dr. Rodrigo A. Valverde in the Department of Plant Pathology and Crop Physiology. After that, he went back to Kasetsart University to finish his master degree. Due to his outstanding master research, in 2007 he obtained a position to be instructor in the Department of Biology, Faculty of Science, Mahasarakham University to teach microbiology and to conduct research on plant viruses and biological control of plant diseases. He was awarded a scholarship by the Ministry of Science and Technology of Thailand to conduct studies and research toward a doctoral degree. In 2011, he joined the Department of Plant Pathology and Crop Physiology at LSU under the direction of Dr. Valverde. Throughout his time at LSU, he conducted research on *Phaseolus vulgaris* endornaviruses and acute viruses that infect common bean. Currently, he is a candidate for the degree of Doctor of Philosophy for the 2016 Spring Commencement.