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Sweetpotato Virus C and Its Contribution to the Potyvirus Complex in Sweetpotato (Ipomoea batatas)

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SWEETPOTATO VIRUS C AND ITS CONTRIBUTION TO THE POTYVIRUS COMPLEX IN SWEETPOTATO (*IPOMOEA BATATAS*)

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To mom and dad, I love you both.

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TABLE OF CONTENTS

ABSTRACT

In Louisiana, sweetpotato (*Ipomoea batatas*) is infected in Louisiana by the four ubiquitous potyviruses: *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus G* (SPVG), *Sweetpotato virus 2* (SPV2) and the strain of SPFMV previously known as the common strain, recently renamed as *Sweetpotato virus C* (SPVC). These four viruses belong to the *Potyviridae* family, with single stranded RNA of ~11kb. In this group of plant viruses, a single polyprotein is coded entirely but later cleaved into ten mature proteins: P1, HC-pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro Nib and Coat Protein (CP). In sweetpotato potyviruses, two additional open reading frames produced by polymerase slippage called PIPO and PISPO act as RNA silencing suppressors. Despite the minimal differences at the nucleotide level in these four viruses, their titers, vector transmissibility and presence in the field are different. The objectives of this research were: (i) redesign the qPCR assay of SPFMV and SPVC and determine the best organ and sampling time after sweetpotato transplanting to detect each of these four viruses; (ii) determine if SPVC is the missing element in reproducing the observed yield reduction of natural infections that occur in the field and; (iii) determine the complete sequences of nine isolates from sweetpotato production fields in Louisiana and analyze the genetic structure and variability compared to other isolates present in the world. Results suggested that leaf tissue at the 3rd week after transplanting is the best organ to sample to determine if the plant is infected with the four potyviruses. The inclusion of SPVC did not reproduce the storage root reduction observed under naturally infected plants and, the molecular variation was not high from other isolates previously sequenced but six isolates report recombination events in the CP and P1 region of their genome.

CHAPTER 1: LITERATURE REVIEW

1.1 The plant

Sweetpotato [*Ipomoea batatas* L. (Lam); Convolvulaceae] is the 7th most important agricultural commodity in the world. It ranks 1st by quantity and value in China, and it is 6th and 14th in the United States, respectively. (FAO, 2012). Sweetpotato is a versatile plant being able to be cultivated under high and low input agricultural systems. How it is used depends on the regions and the way that it is produced; varying from animal feed, industrial (ethanol production), to being one of the primary sources of carbohydrates, protein and nutrients (such as carotenoids, vitamin C, iron, among others) in some countries (Clark *et al.*, 2013a). In the United States, it has been traditionally consumed during the holidays and was an important source of food during the depression in the 1930's, but is becoming more popular because of its nutritional value and availability of value-added products (Clark *et al.*, 2012b). Sweetpotato is known by other names such as batatas, camote, Louisiana yams or kumara. These differences in nomenclature led growers to confuse it with other crops creating agricultural management problems as well as researchers when they describe sweetpotato morphology in comparison to other root and tuber crops (Villordon *et al.*, 2014). Sweetpotato is a dicotyledonous plant of the morning glory family. It is believed to have originated in central and south America, but evidence suggests that it might have had a prehistoric distribution in Oceania. Sweetpotato is a vegetatively propagated perennial crop that is grown as an annual. It can form storage roots from the adventitious roots produced from the leaf gaps in nodes (Firon *et al.*, 2009). The genes involved in storage root initiation have not been fully described yet due to the hexaploid genome of 90 chromosomes of the plant, compared to the 30 chromosomes that most diploid species in the *Ipomoea* genus have (used as ornamentals or common weeds) (Kays, 1985). However, it has been reported that external and internal stimuli determine if an adventitious root differentiates to become a storage root (Firon *et al.*, 2009; Villordon *et al.*, 2012).

1.2 The viruses

The reduction of the storage root quality and yield due to virus accumulation, pathogens, and mutations is known as cultivar decline (Villordon and Labonte, 1995). The most important stimuli that are associated with yield variations are pathogens, where plant viruses of the Potyviridae family have been described as the culprit behind cultivar decline in the U.S. (Clark and Hoy, 2006). Potyviruses belong to the family Potyviridae and the genus *Potyvirus*, where *Potato virus Y* is the type species of this group (Adams *et al*., 2011). Sweetpotato potyviruses have filamentous particles approximately 850 nm long, restricted host range (affecting primarily the Convolvulaceae family) and are vectored in a non-persistent manner by many aphid species, some of them more efficiently than others in sweetpotato (Wosula *et al.*, 2012). Eriophyid mites, the fungus *Polymyxa graminis,* and the whitefly *Bemisia tabaci* (Shukla *et al.*, 1994) transmit more distantly related viruses in the family. The genome of sweetpotato potyviruses ranges from 10,731 to 10,800 nt excluding the 3' poly (A) tail (Li *et al.*, 2012). The genome includes several genes such as P1 (proteinase; terminal step in polyprotein processing, host identification); HC-pro (aphid transmission; proteinase, polyprotein processing); P3 (unknown); 6K1 (unknown, possibly polyprotein genome replication); CI (polyprotein genome replication, RNA helicase, unwinding of dsRNA, membrane attachment); 6K2 (unknown, possibly polyprotein genome replication); NIa-VPg (polyprotein genome replication, primer); NIa-pro (proteinase, major

aspects of polyprotein processing); Nib (polyprotein genome replication, RNA-dependent RNA polymerase) and CP (RNA encapsidation, aphid transmission, cell-to-cell movement) (Shukla *et al.*, 1994; Salvador *et al.*, 2008). In sweetpotato potyviruses, another open reading frame, called Pretty Interesting Sweet Potato Potyvirus ORF (PISPO) is much conserved among this group of viruses (Chung *et al.*, 2008).

In the United States, four potyviruses: *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus 2* (SPV2); have been documented to date (Clark *et al*., 2012). This group of viruses has been transmitted by grafting, but not seed or contact between plants (Loebenstein *et al.*, 2009). Genetically, they are conserved in the C-terminal half of their coat protein gene (CP) (Li *et al.*, 2012). Titers in infected plants and vector transmissibility are most efficient when they are co-infected with other viruses (Kokkinos *et al*., 2006; Wosula *et al*., 2012). The best-known example of mixed-infections is called the Sweet potato virus disease (SPVD) when the potyviruses are co-infected with *Sweetpotato chlorotic stunt virus* (SPCSV), increasing potyvirus titers (Gutierrez *et al.*, 2003). Their detection has been based on biological (grafting), serological (ELISA) or nucleic acid (Polymerase Chain Reaction (PCR)/ quantitative polymerase chain reaction (qPCR)) assays. However, similar symptoms have been documented in indicator plants such as *I. setosa* (Untiveros *et al.*, 2008). Similarly, cross reactions with antibodies between SPVG-SPV2 and SPVC-SPFMV (Souto *et al.*, 2003), which may be due to high sequence similarity in the CP region (Li *et al.*, 2012), leave only nucleic acid methods available for accurate detection of individual viruses. A one-step multiplex RT-PCR for the four viruses was developed (Li *et al.*, 2012) that allows simultaneous detection of SPFMV, SPVC, SPCG, and SPV2. For quantification, individual qPCR tests for the relative quantification of SPVG, SPV2, SPCSV, and *Sweet potato leaf curl virus* (SPLCV) were developed (Kokkinos *et al.*, 2006). Another set of qPCR assays were developed for detection of SPFMV, SPVG, and SPV2 (Ling *et al*., 2010). Unfortunately, SPVC and SPFMV were reclassified as different species (Untiveros *et al.*, 2010) after the design of the Kokkinos primers and probes, which are not specific enough to differentiate these species. Conserved regions such as CP (Li *et al.*, 2012) and P1 (Untiveros *et al.*, 2010) have been used to differentiate the four viruses. It is worth mentioning that the recently described Pretty Interesting Sweet Potato virus open reading frame (PISPO; 207-239 AA residues), produced by polymerase slippage (Univeros *et al*., 2016) is not conserved among the four viruses (Li *et al.*, 2012).

SPVG was first described in China, where it is also widespread (Colinet *et al.*, 1998). It has been reported in other parts of the world such as Peru and the United States (Untiveros *et al.*, 2007; Souto *et al*., 2003). This virus is vectored by the aphids *A. gossypii* and *M. persicae* (Wosula *et al.*, 2012), it is also mechanically transmissible to various *Ipomoea* spp. such as *I. codatotriloba, I. hederacea, I. nil, I. setosa* and *I. tricolor* (Brunt *et al.*, 1996; Souto *et al.*, 2003). Genetically, SPVG is very similar to SPV2 in amino acid length (618aa), but differs from SPVC and SPFMV (664aa-724aa). All are significantly larger than other potyviruses (Li *et al.*, 2012). Together they share identities of 63.5-64.6% with SPFMV and 62.6-64.1% with SPVC, which makes them closely related but different potyvirus species, according to the criteria to describe species (73 and 86% homology for complete genome and polyprotein respectively) in potyviruses (Adams *et al.*, 2005).

SPVC was proposed to be separated as a different species from SPFMV due to differences in the P1 region (Untiveros *et al.*, 2010). It was previously known as SPFMV-C (common strain) a distinct strain of SPFMV [East African (EA); ordinary (O) and russet crack (RC)] classified based on the CP sequences (Kreuze *et al.*, 2000). Isolates of strains RC, O and EA are closely related to each other, but are phylogenetically distant from strain C (Tairo *et al.*, 2005). Strains RC, O and C are distributed worldwide, whereas isolates of the EA strain have been largely restricted to countries in East Africa (Kreuze *et al.*, 2000; Mukasa *et al.*, 2003). Besides serology, there is no pertinent information about vector efficiency or symptoms that differentiate SPVC from the other potyviruses (Kennedy and Moyer, 1982).

SPFMV was first described and characterized in 1978 (Moyer and Kennedy, 1978). It remained as the only characterized virus known in sweetpotato until 1998, but advances in molecular biology lead to a characterization of several species that diverge in their sequence. SPFMV is non-persistently transmitted by aphids *M. persicae* (Sulzer), and *A. gossypii* Glover (Souto *et al.*, 2003; Wosula *et al.*, 2012). It can be mechanically transmitted to various *Ipomoea* spp. such as *I. batatas, I. setosa, I. nil, I. incarnata* and *I. purpurea*, and some strains of *Nicotiana benthamiana, N. clevelandii, Chenopodium amaranticolor* and *C. quinoa* (Brunt *et al.*, 1996).

SPV2 was first described in Taiwan (Loebenstein *et al.*, 2009). It was also known as Sweet potato virus II, Sweet potato virus Y and Ipomoea vein mosaic virus. It is found in several places in the world including the United States (Souto *et al*., 2003). It induces chlorotic bands along sections of veins and discrete mosaic along the entire length of the veins in *I. setosa*, and vein mosaic in *I. nil* and *I. tricolor* (Ateka *et al.*, 2007; Souto *et al.*, 2003). It is mainly found in mixed infections with SPVG and SPFMV and spreads slowly in the field (Clark *et al.*, 2012). The isolate found in Taiwan is non-persistently transmitted by *M. persicae* similarly to a California isolate (Ateka *et al.*, 2004; Clark *personal communication*), but the Louisiana isolate has not been successfully transmitted by *A. gossypii* or *M. persicae* (Souto *et al.*, 2003). It is mechanically transmitted to *I. nil, I. setosa, I. tricolor*, and several species of the genera *Chenopodium*, *Datura*, *Nicotiana*, and *Ipomoea* (Ateka *et al.*, 2007; Loebenstein *et al.*, 2009; Souto *et al.*, 2003).

1.3 The stimuli for storage root development and cultivar decline

Cultivar decline is defined as the reduction of the storage root quality and yield due to accumulation of viruses, other pathogens and mutations in the propagating material (Bryan *et al*., 2003; Villordon and Labonte, 1995; Clark *et al*., 2002). It is not entirely clear what biotic or abiotic internal/external stimuli can affect the storage root initiation and/or storage root bulking (Villordon and Clark, 2014). In sweetpotato, the most important physiological process is storage root initiation, which is defined as the appearance of cambia around the protoxylem and secondary xylem elements and determines sweetpotato yield (Wilson and Lowe, 1973; Firon *et al*., 2009).

Sweetpotato root architechture has been affected by several factors. For example, in storage root initiation, differential expression profiles between fibrous roots and initiating storage roots indicate down-regulation of classical root functions like transport and lignin biosynthesis and upregulation of carbohydrate metabolism and starch biosynthesis (Firon *et al*., 2013).

Lateral root development was associated with the competency of adventitious roots to undergo storage root initiation (Villordon *et al*., 2012). Together, root system architecture (lateral root initiation, morphogenesis, emergence and growth), promotes a better water-efficiency and nutrient uptake (Casimiro *et al*., 2003). These previous studies suggested that both internal and external cues could drive the sweetpotato root system in different development rates.

Internal cues for lateral root formation include auxins (De Smet *et al.*, 2012; Wang and Estelle, 2014), ethylene (Ivanchenko *et al.*, 2008), abscisic acid (Lopez-Buncio *et al.*, 2002), cytokinin/strigolactones (Koltai, 2011) and carbohydrate availability (Ruyter-Spira *et al.*, 2011). External cues include water availability in the growth substrate (Deak and Malamy, 2005) and nutrients such as ammonium (NH_4) (Lima *et al.*, 2010), nitrate (NO_3) (Zhang *et al.*, 1998), phosphorus (Johnson *et al.*, 1996), sulfate (Kutz *et al.*, 2002) and iron (Lopez-Bucio *et al.*, 2003). Water availability in the growth substrate (Villordon *et al.*, 2012) and nitrogen availability (Villordon *et al.*, 2013) altering root architecture have been recently validated to affect storage root production in 'Beauregard' sweetpotato leading the rest of the stimuli for further investigation.

In terms of sweetpotato plant viruses, potyviruses have been attributed as the main factor in yield decline in the U.S. due to their ubiquity in field surveys (Valderde *et al*., 2007) and their accumulation due to the vegetative propagation of the sweetpotato crop (Clark *et al*., 2012). Mixed infections of SPVG, SPFMV and SPV2 did not replicate the amount of yield lost observed in natural infections (Clark and Hoy, 2006), leaving the question of what is the missing component of sweetpotato cultivar decline in the U.S. Since SPVC had not been evaluated previously for its role, the hypothesis was considered the SPVC was the missing component. SPVC was reported to have higher number of clean read tags in sweetpotato roots compared to other parts of the plant and to the other three potyviruses in next-genetion sequencing data, further suggesting its potential importance (Guo *et al*., 2014).

1.4 Real-time PCR

Real-time polymerase chain reaction (PCR) or quantitative PCR (qPCR) was introduced in 1992 as a modification of regular PCR (Huguchi *et al.*, 1992). The reaction starts as a regular PCR where theoretically the amount of initial DNA is doubled after each cycle resulting in an exponential amplification, but the efficiency starts to decrease when the reagents in the reaction are depleted. Due to this factor, qPCR is divided into three phases: exponential (where the reaction proceeds with 100% of efficiency); linear or non-exponential (where the reagents start to decrease) and plateau (where the reagents are depleted and the reaction stops). The exponential line is visualized due to probes that emit fluorescence after every cycle amplification.

During the past decade, qPCR has been used for genotyping, quantifying viral load in patients, assessing gene copy number and gene expression levels. It offers several advantages over other methods for quantification. These advantages include small amounts of template, high reproducibility; the capability of analyzing more than one target in the same reaction, increased speed due to reduced cycle number, lack of post-PCR gel electrophoresis for the visualization of the products and higher sensitivity (Fraga *et al.*, 2008). Despite these advantages, it also requires a strategic planning by several steps.

The first step in the strategic planning requires obtaining high quality of template (DNA or RNA). RNA compared to DNA is very unstable and RNases -enzymes that degrade RNA, are ubiquitous in nature and highly stable compared to DNases –enzymes that degrade DNA. This problem can be solved with clean laboratory techniques and the addition of RNase inhibitors at the end of the extraction. The RNA template differs from DNA that it needs to be converted into protein-encoding genes (cDNA) by an RNA-dependent DNA polymerase enzyme called reverse transcriptase. This enzyme is derived from retroviruses such as an *Avian myeloblastosis virus* (AMV) and the Moloney strain of *Murine leukemia virus* (MMLV). The second step requires optimizing the technical aspects in the experiment. These include the design of primers (specific to the target of interest, amplify short amplicons -<300bp and that do not from dimers), probes (non-specific dyes -SYBR green or strand-specific fluorescent probes -Taqman), annealing temperatures and optimal concentration of the other reagents. Finally, the real-time analysis and quantification that include negative and positive controls and replication of the same sample to avoid pipetting errors.

To quantify the expression of the different genes of interests and make comparisons, the cycle threshold (CT) is used. The threshold is described as the fluorescence signal above the background to be considered a reliable signal. If the threshold is set too low, it could lead to unreliable data and, if it is too high, a detection of the product when it has left the exponential phase. To determine the CT value, a baseline is needed, which is determined from a plot of fluorescence versus cycle number. The number of cycles usually are the first ones (3 to 15) and the CT value is set at three standard deviations above the baseline value.

To be able to compare between two samples, it is important that they have similar amplification efficiencies. Each efficiency is calculated by the formula $E= 10 (-1/slope)^{-1}$ obtained from the line plotted from PCR on a serial dilution series of the template. In theory a 100% efficiency would require 3.3 cycles to increase amplicon concentration by 10 fold. A slope of -3.6 and -3.1 corresponds to an efficiency of 90% and 110%. When the slope of the line is <0.1, amplification efficiencies are comparable, if it is >0.1 primer redesign or improvement of the amplification is required (Bustin and Nolan, 2004). To compare two samples for relative quantification titers, the equation: $2(CT_1-CT_2)$ = fold difference in the amount of starting target; where CT_1 (of sample 1) and CT_2 (of sample 2) is used for the calculations. To determine the limit of detection the formula $LoD= LoB + 1.645 \times \sigma_{lowconcentrationsample}$; where LOD= limit of detection, LoB= limit of blank ($LoB=mean_{blank} +1.645 \times \sigma_{blank}$) (Forootan *et al*., 2017) is employed.

In qPCR, there are two types of quantifications. The first one, absolute quantification expresses the amount of target expressed as copy number or concentration, which also requires identical amplification efficiencies for the control and the target sequence, which is more accurate but labor intensive, and usually requires knowing the amount of target. The second one, relative quantification measures the change in gene expression in response to different treatments or the state of tissue. It requires internal standards to control variability against different samples, which serves as normalization of the curve. They are calculated as a ratio between the CT value of the experimental primers against the average of the CT values of the different housekeeping genes used for normalization (Pfaffl *et al.*, 2001).

1.5 Reference genes for relative quantification in sweetpotato

The importance to have stable internal reference genes for the normalization of real-time PCR reactions is crucial for the data analysis. When plants face different stresses, the type of gene used for relative quantification can vary affecting quantification results and reliability of the data. Some traditional genes considered housekeeping such as actin (ACT), tubulin (TUB), glyceraldehyde-3-phosphate dehydrogenase (GAP), elongation factor-1 alpha (EF1α) and 18S rRNA are commonly used for normalization. Under two algorithms, geNorm and Normfinder, sweetpotato plants were evaluated under different abiotic conditions such as cold, drought, salt and oxidative stress (Park *et al.*, 2012).

GeNorm algorithm examines the stability of expression as well as the optimal number of reference genes needed for normalization. It first calculates an expression stability value (M) for each gene and then the pairwise variation (V) of this gene with the others. The lowest stability value represents the gene with the most stable expression within the gene set examined (Vandemsopele *et al.*, 2002). NormFinder algorithm determines the stability of expression as well as the optimal gene or combination of genes for normalization purposes. It ranks the set of candidate normalization genes according to the stability of their expression in a given sample set under a given experimental design (Andersen *et al.*, 2004).

In an experiment conducted to determine the best reference gene in sweetpotato, several genes such as β-actin (ACT), ribosomal protein L (RPL), glyceraldehyde-3-phosphate dehydrogenase (GAP), cyclophilin (CYC), α-tubulin (TUB), ADP-ribosylation factor (ARF), histone H2B (H2B) and ubiquitin extension protein(UBI), cytochrome c oxidase subunit Vc (COX) and phospholipase D1α (PLD) were used. After the results were analyzed and tabulated by GeNorm and Normfinder, it was concluded that the number of reference genes depends on the cultivar used and the stress imposed to the plants and that COX was one of the best candidates (Park *et al.*, 2012).

1.6 Next-generation sequencing of plant viruses using Hiseq2000

There are over 30 viruses infecting sweetpotato in the world and full genome sequencing has become a tool for their analysis. The use of the next-generation sequencing (NGS) of viral genomes provides a highly sensitive method for virus detection compared to Sanger and overlap consensus sequence assemblies since it does not require previous knowledge of the virus. Additionally, the technology allows detection of unknown sequences in the sample. The former, is more sensitive than the other two, however, it is cost prohibitive for some laboratories.

The Hiseq2000 sequencing system can produce 200 GB per run with high yield data. The technology enables sequencing millions of fragments by using a reversible terminator-based method that detects single bases as they are incorporated into the growing DNA strands. Each base is detected and, since all dNTP's are present in the sequencing process, natural competition lowers bias incorporation. The result is highly accurate since they exclude homopolymers or sequence-context errors.

The workflow of the Hiseq2000 consists of three basic steps. First, libraries are prepared from any nucleic acid sample, which are amplified to produce local clusters and sequenced using massively parallel synthesis (Illumina, 2010). Second, a sample of pure DNA/RNA is sent using

the kits provided by the company who is offering the services for sequencing. At this stage is important to take into consideration if the sample is multiplexed or not with others since the future analysis will compromise the quality of results. Finally, the data obtained from NGS comes as a FASTA file with all reads that the machine provides. Since usually these files are large, the use of High-processing computers (HPC) is required since they cannot be opened in a regular computer.

In most cases, the FASTA reads are not free from host DNA, so viral reads need to be assembled using overlapping sequences present in the file using references from a database. In virology, to assemble the contigs, free software such as Velvet, Galaxy, Bowtie, or paid software as DNAStar are preferred based on costs.

The final step is the assessment of the genome. To accomplish this, programs such as Mauve of ClustalW are used. NGS detection is possible when virus identities are at least 30-40% of the total viral genome (Kreuze *et al.*, 2009). When libraries are completed, it is necessary to confirm the samples by PCR and complete the ends by 5'RACE and/or 3' RACE. Finally, the sequences could be uploaded to NCBI and analyzed as the project requires.

1.7 5'/3' RACE

Rapid amplification of cDNA ends (RACE) is used to identify 5' and 3' ends of a cDNA transcript from partial cDNA (Frohman *et al.*, 1988). The technique has been modified by several laboratories and commercialized (Scotto-Lavino *et al.*, 2006; Clontech Laboratories, 2006). RACE utilizes RT-PCR to convert the mRNA into cDNA, and PCR to amplify the ends of transcripts.

To perform "classic" RACE, a partial or a complete sequence of the mRNA of interest has to be known, from where three gene specific primers are designed. The first primer will reverse-transcribe the mRNA into cDNA. Then, the reaction proceeds to dephosphorylate the cDNA with shrimp alkaline phosphatase (SAP) which leaves the full cDNA with the methylated "G" caps intact. The methylated "G" cap is removed with tobacco acid pyrophosphatase (TAP) which exposes the ends for ligation to the linker or homopolymer. The second primer is used to amplify a PCR product from the poly (A) tail to the known region (to obtain the 3' end); while the appended homopolymer tail obtains the 5' end. Finally, a nested PCR, using the third specific primer allows reducing unwanted products.

The moment of appending the homopolymer led to the discovery of three different methods of RACE (Yeku and Frohman, 2011). In the "classic" RACE, the homopolymer is appended after the mRNA is reverse transcribed. In the "new" RACE, the homopolymer is appended before the reverse transcription reaction that improves the recognition of the transcription start site. Finally, "circular" RACE allows the recognition of both 5' and 3' in the same reaction, but it requires substantial optimization before an accurate end is acquired. "Circular" RACE has been mostly utilized in eukaryotes like *Caenorhabditis elegans* (McGrath, 2011).

1.8 Molecular characterization in *Potyviridae*

Before the advent of sequence data, species and strains of potyviruses were differentiated using host range, symptomatology and serology (Adams *et al.*, 2005). However, as molecular biology techniques improved, molecular characterization of the whole genome and its different genes has been used to describe them.

A potyvirus consists of a positive-sense, single-stranded RNA genome, which encodes a large polyprotein processed into several genes by cleavage sites which are conserved (Adams *et al.*, 2005). The polyprotein starts with a nucleotide consensus of TGAAATGGC in plants (Lutcke *et al.*, 1987) and starts the coding of the polyprotein as a whole. The polyprotein then cleaves in some conserved amino acid regions, which allowed recognizing the following genes. P1 gene has been characterized in *Tobacco vein mottling virus* (TVMV) and *Turnip mosaic virus* (TuMV) with the functions of proteinase activity and single-stranded RNA binding activity, it has also been suggested to be an accessory factor for genome amplification (Verchot and Carrington, 1995). The HC-Pro is a helper component for virus transmission by aphids, has proteinase activity in its C-terminal and is involved in long distance movement (Shukla *et al.*, 1994). P3 has been reported with cylindrical inclusions with a possible event in replication (Restrepo and Carrington, 1994). The 6K2 protein is believed to be involved in virus replication. The CI is a cytoplasmic inclusion protein with a conserved RNA helicase sequence suspected to be involved in virus replication (Shukla *et al.*, 1994). The NIa is composed of VPg and a proteinase, both of which are thought to be involved in RNA replicase for virus multiplication (Murphy *et al.*, 1990). The NIb is also probably involved in virus replication by RNA replicase and finally, the CP is involved in assembly, transmission and spread of the virus (Dolja *et al.*, 1994). In sweetpotato potyviruses, two additional proteins produced by polymerase slippage called Potyvirus open reading frame (PIPO) and Pretty interesting open reading frame (PISPO) are probably involved in RNA silencing (Olspert *et al.*, 2015; Untiveros *et al*., 2016).

To describe variability among the different species at a molecular level, phylogenetic trees of the 5' untranslated region (UTR), 3' UTR, the whole polyprotein and the different proteins that they produce have been used both at the amino acid and nucleotide level. The encoded proteins can be inferred by the nucleotide sequence and analogy with other potyviruses. The amino acid cleavage site between P1 and HC-Pro are tyrosine (Y) and serine (S). Between HC-Pro and P3 between glycine (G) and glycine (G). In the middle of P3 and 6K1 is composed of a consensus of glutamine (Q) / alanine (A), serine (S) and glutamic acid (E) / arginine (R). Between 6K1 and C is glutamine (Q) / serine (S), threonine (T). Next, CI and 6K2 are glutamine (Q) / serine (S). Following, 6K2 and NIa-VPg are glutamine (Q) / glycine (G). Next are NIa-VPg and NIa-Pro with a glutamic acid (E) / alanine (A), glycine (G) and serine (S). NIa-Pro and NIb by glutamine (Q) / alanine (A), glycine (G) and serine (S). Finally, NIb and CP are separated by glutamine (Q) / alanine (A) or serine (S). The end of the polyprotein is followed by a polyadenylated tail (Shukla *et al*., 1994). The polyprotein and each gene has its own thresholds of nucleotide and amino acid similarity to be classified at the genera and species level as previously determined by Adams *et al*. in 2005 (Table 1.1).

Table 1.1. Nucleotide and amino acid identity between genera and species in *Potyviridae* (Modified from Adams *et al*., 2005)

Between genera				
	% nucleotide identity		% amino acid identity	
	Different genus	Same genus	Different genus	Same genus
P1	34.7-47.6	34.6+	00.-45.5	0.0+
HC-Pro	35.1-46.5	47.1+	19.3-31.7	36.0+
P3	33.4-44.7	36.9+	0.0-32.6	0.0+
CI	38.4-55.4	49.4+	21.6-51.8	42.3+
VPg	33.2-55.7	42.3+	15.1-47.6	28.4+
NIa-Pro	33.6-52.8	45.2+	9.1-47.6	28.4+
NIb	42.2-59.4	55.5+	29.2-58.1	51.9+
CP	35.6-59.8	41.2+	13.2-56.5	30.6+
Polyprotein	38.6-50.6	49.3+	24.4-41.1	42.2+
5'-untraslated	33.8-62.8	32.0+	-	-
3'-untranslated	31.6-51.8	30.9+	-	-
Between species				
	% nucleotide identity		% amino acid identity	
	Different species	Same species	Different species	Same species
P1	34.6-68.9	41.4+	0.0-71.6	27.8+
HC-Pro	35.1-75.7	76.3+	19.3-85.0	85.2+
P3	33.4-79.6	74.6+	0.0-86.7	76.6+
CI	38.4-78.2	78.3+	21.6-91.3	88.0+
VPg	33.2-79.1	76.2+	15.1-87.2	81.4+
NIa-Pro	33.6-77.5	76.9+	9.1-85.2	88.5+
NIb	42.2-77.8	76.6+	29.2-88.4	89.0+
CP	36.6-81-1	78.0+	13.2-88.6	79.6+
Polyprotein	38.6-74.7	77.1+	24.4-80.9	82.9+
5'-untraslated	32.0-74.2	39.7+	-	-
3'-untranslated	30.9-84.0	71.9+	-	-

1.9 Recombination analysis

Potyviruses have been described as prone to recombination events (Revers *et al.*, 1996). Most of these events have targeted the P1, CI, 6K2 and VPg in several viruses in this family like *Turnip mosaic virus* (TuMV) (Ohshima *et al.*, 2007), *Sweetpotato mild mottle virus* (SPMMV) (Valli *et al.*, 2007) and *Potato virus Y* (PVY) (Galvino-Costa *et al.*, 2012).

Several studies prove that different recombination events through the SPFMV family could lead to the phylogenetic lineages of East African (EA), Russet Crack (RC), Ordinary (O) and Common (C); now reclassified as SPVC (Untiveros *et al.*, 2008; Untiveros *et al.*, 2010). This evidence provides an indication that recombination analysis is necessary when new isolates are being described at a molecular level.

Several programs have been used to detect recombination events like Simplot, Dual Brothers, Jphmm, Scueal and RDP4. However, RDP4 has been preffered over the others because of the flexibility of the software to configure which sequence is the recombinant of interest and the parent (Martin *et al.*, 2010). These software have been used in previous research to detect recombination events in SPFMV (Untiveros *et al.*, 2008; Untiveros *et al.*, 2010).

1.10 Hypothesis and objectives

- Objective 1:

  Design primer-probe sets for RT-qPCR that differentiate *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC).

  Question to be answered:

  Where is the best part of the sweetpotato plant to test for SPVG, SPVC, SPFMV and SPV2 presence?

  Hypothesis:

  Hypothesis (H0): SPVG, SPVC, SPFMV and SPV2 have higher titers in roots and than in stems or leaves.

  Alternative hypothesis (H1): SPVG, SPVC, SPFMV and SPV2 do not have higher titers in roots.

- Objective 2:

  Determine the effects of SPVC on sweetpotato storage root number under greenhouse-controlled conditions.

  Question to be answered:

  Is SPVC the missing component for the differences in the storage root number of sweetpotato plants inoculated in combination of SPFMV, SPVG and SPV2 compared to naturally infected plants?

  Hypothesis:

  H0: Sweetpotato 'Beauregard' plants infected with SPVG, SPVC, SPFMV and SPV2 together (4-way interaction) will produce similar number of storage roots than plants naturally infected (B14-G7).

  H1: Sweetpotato 'Beauregard' plants infected with SPVG, SPVC, SPFMV and SPV2 together (4-way interaction) will not produce similar number of storage roots than plants naturally infected (B14-G7).

- Objective 3:

  Determine full genome sequences of the isolates present in Louisiana and describe and compare them at a molecular level with other isolates originated in other parts of the world.

Question to be answered:

Are SPFMV or SPVC isolates moleculary different from other isolates previously reported?

Hypothesis:

H0: Molecular variation of the United States potyvirus isolates describe them as new strains and molecular variation is high.

H1: Molecular variation of the United States potyvirus isolates will not describe them as new strains and molecular variation is low.

CHAPTER 2: VIRAL DISTRIBUTION AND TITERS OF SPVG, SPVC, SPFMV AND SPV2 (*POTYVIRIDAE*) IN 'BEAUREGARD' SWEETPOTATO (*IPOMOEA BATATAS*)

2.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is ranked 7th in world staple food production (expressed on a dry matter basis). The crop is particularly important in South-East Asia, Oceania and Latin America with China accounting for more than half of the total world production (Worldatlas, 2017). Sweetpotato is a vegetatively propagated perennial crop, which is generally grown as an annual. Slips (sprouts from storage roots) are used for propagation in the temperate zone, and the final consumed products are storage roots that are differentiated from adventitious roots that arise at or near nodes on the stems (Firon *et al*., 2009). Cultivar decline is defined as the reduction of the storage root quality and yield due to accumulation of viruses, other pathogens and mutations in the propagating material (Bryan *et al*., 2003; Villordon and Labonte, 1995; Clark *et al*., 2002). While several pathogens affect the crop, in Louisiana, plant viruses are thought to primarily account for the cultivar decline effect. The most prevalent sweetpotato viruses in the U.S. are members of the *Potyviridae* family (Clark and Hoy, 2006). In the United States, four potyviruses: *Sweet potato virus G* (SPVG), *Sweet potato virus C* (SPVC), *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato virus 2* (SPV2); are commonly found in field surveys (Valverde *et al*., 2007). Symptom severity, distribution in the field, titers in infected plants and vector transmissibility are greater when plants are co-infected with these potyviruses than when any one of the viruses is present alone (Kokkinos *et al*., 2006; Wosula *et al*., 2012). Therefore, it is important not only to know whether a plant is infected with one of the four potyviruses, but it is also important to know specifically which and how many of viruses are present.

Methods for sweetpotato virus detection have included biological (grafting to the indicator host *Ipomoea setosa*) (Moyer and Salazar, 1989), serological (ELISA) (Hammond *et al*., 1992) or nucleic acid (PCR/qPCR) assays (Li *et al*., 2012; Kokkinos *et al*., 2006). However, each of these methods have some limitations. For example, similar symptoms have been documented in indicator plants such as *I. setosa* when infected with the different potyviruses, making it difficult to distinguish which potyvirus is present (Untiveros *et al*., 2008). Cross-reactions with polyclonal antibodies between SPVG-SPV2 and SPVC-SPFMV (Souto *et al*., 2003) have been observed probably due to high amino acid sequence similarity in the coat protein region (Li *et al*., 2012). All of these limitations led to the polymerase chain reaction (PCR) becoming the preferred detection method and to serve as a primary tool in quarantine and certification programs.

Currently, for a sweetpotato plant to obtain virus-tested status, the procedure to test them for viral infections starts with total RNA extraction from the leaves of the sweetpotato plant (Li *et al*., 2008). The total RNA extraction is used as a template to test for potyviruses (Ha *et al*., 2008; Li *et al*., 2012; Zheng *et al*., 2010), *Sweetpotato chlorotic stunt virus* (SPCSV) (Wei and Nakhla, *personal communication*) and *Sweetpotato leaf curl virus* (SPLCV) (Li *et al*., 2004; Ling *et al*., 2010). There are also additional qPCR primers that allow the detection of SPFMV, SPVG, SPV2, *Sweet potato chlorotic stunt virus* (SPCSV), and SPLCV (Kokkinos *et al*., 2006). The common strain of SPFMV (now named SPVC) was reclassified as a different species due to differences in nucleotide sequences in the P1 region (Untiveros *et al*., 2010). Unfortunately, this occurred after the design of the first set of primers and probes (Kokkinos, 2006), which

amplified both SPFMV and SPVC and did not allow independent quantification of each virus. Additionally, Kokkinos used a predesigned housekeeping gene, 18S rRNA, for gene normalization (AppliedBiosystems, Foster City, CA). However, the reagents for that gene are not produced anymore and under our experimental conditions, amplification of the gene occurs in the first 10 cycles, which can produce errors in relative quantification experiments (Pfaffl, 2001). Recently, to obtain a housekeeping gene for relative quantification in sweetpotato, the plant was stressed using different abiotic conditions and 10 genes were tested to analyze which one remains more stable under geNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004) algorithms. The analysis suggested that *Cytochrome C oxidase subunit Vc* (COX) was one of the most stable (Park *et al*., 2012).

The potyvirus genome is composed of ten mature proteins, which are cleaved following translation on a single large polyprotein (Adams *et al*., 2010). SPFMV has two additional proteins, PIPO and PISPO, produced by polymerase slippage (Untiveros *et al*., 2016; Figure 2.1). Due to its high level of nucleotide sequence conservation compared to the other proteins produced, the coat protein (CP) gene has been chosen as an optimal target for primer design (Li *et al*., 2012). However, the P1 region appears to be the region of greatest diversity between SPFMV and SPVC compared to the other 10 mature proteins (Untiveros *et al*., 2010). Despite this low level of genetic diversity among the four potyviruses, there is evidence that their respective titers vary among different locations within an infected sweetpotato plant. For example, the number of reads for SPVC were four-fold higher than the other three potyviruses and the greatest number of reads were from fibrous roots for each virus, except SPV2, for which the expanding roots had a greater number of reads according to next generation sequence data (Gu *et al*., 2014). However, only one sample was taken at the end of the growing season in that study thus it did not take into consideration differences that might occur at different phenological stages of sweetpotato development. To understand how the potyvirus complex affects yield and to develop the most sensitive protocol for detection of these viruses in plants that often do not show symptoms, it is important to know within which organs in the plant the viruses replicate and accumulate.

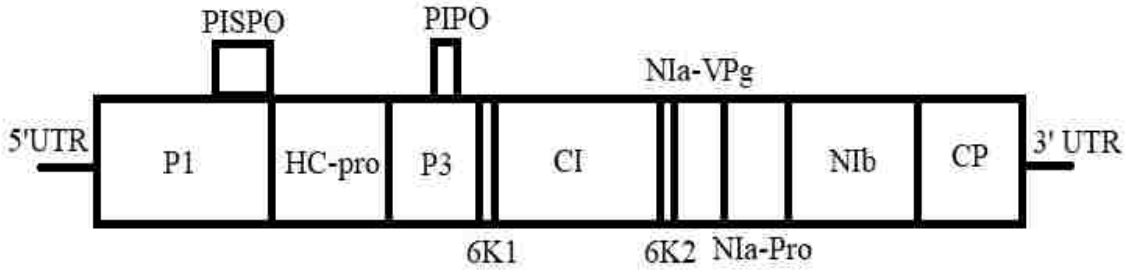

Figure 2.1. Genome organization of *Sweetpotato feathery mottle virus* (~10.8Kb). The polyprotein is coded from 5' to 3' and then cleaved into the 10 mature proteins: P1, HC-pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP. Two additional proteins named PISPO and PIPO are produced by polymerase slippage.

This study was undertaken to develop methods to independently quantify SPFMV and SPVC, and to use those methods along with previously developed methods for quantifying SPVG and SPV2. In addition, the study was aimed to compare the effects of different sweetpotato organs, phenological stages, and virus combinations on titers of each for the four

common sweetpotato potyviruses (Villordon *et al*., 2013). The objective of this experiment is to test if roots have higher virus accumulation compared to leaves and stems plus, at the same time, if the SR3 stage (presence of at least one storage root), accumulate greater virus titers compared to SR1 (presence of at least one adventitious root) and SR2 (observation of the onset of anomalous cambium (AC) in a minimum of one AR in at least 50% of transplants) stages.

2.2 Materials and methods

2.2.1 Potyvirus isolates

Potyviruses were collected in previous studies, either from sweetpotatoes from the U.S. showing potyvirus-like symptoms (Souto *et al*., 2003), or from *I. setosa* sentinel plants placed in sweetpotato fields in Louisiana (Wosula *et al*., 2013). Individual viruses were transferred by mechanical inoculations from graft-inoculated or sentinel *I. setosa* to *I. nil* 'Scarlet O'Hara' (SOH). Isolates of individual potyvirus species were obtained by single aphid transmission from infected to healthy SOH and by single lesion transfers from mechanically inoculated *Chenopodium quinoa* plants. The potyviruses present in each plant were confirmed using the multiplex PCR method of Li *et al*. (2012). Isolates were maintained in SOH by periodic mechanical inoculations using leaves triturated with a mortar and pestle in 0.02M phosphate buffer, pH 7.2, amended with 0.1M sodium diethyl dithiocarbamate (DIECA) and rubbing the inoculum on Carborundum-dusted leaves. Their separation was confirmed by a multiplex RT-PCR, which detects SPVG, SPVC, SPFMV and SPV2 in the same reaction (Li *et al*., 2012). Each isolate was kept in a rearing and observation cage of 12" cube white with vinyl window (model 1466AV) (Bioquip products, CA) in a greenhouse.

Table 2.1. Potyvirus isolates of sweetpotato and used in this study. Isolates were separated using differential host assay or single aphid probe transmissions. Isolates were mechanically transmitted into *Ipomoea nil* 'Scarlet O'Hara' and renewed every three weeks.

Isolate	Species	Location	Method used for isolation
LSU-1	SPVG	Louisiana, U.S.	*Aphis gossypii* single probe
95-6	SPVC	North Carolina, U.S.	*Nicotiana benthamiana* mechanical inoculation
Ark-1	SPFMV	Arkansas, U.S.	*Chenopodium quinoa* single local lesion
CA-6	SPV2	California, U.S.	*C. quinoa* single local lesion

2.2.2 Plant material for *Potyvirus* quantification

Ipomoea setosa seedlings were mechanically inoculated with SPVG (isolate LSU-1), SPVC (isolate 95-6), SPFMV (isolate Ark-1) and SPV2 (isolate CA-6) to create scions for graft-inoculation into virus-tested *I. batatas* 'Beauregard' that were clonally propagated under controlled greenhouse conditions (Souto *et al*., 2003). After two weeks, plants with viral symptoms were graft-inoculated into virus-tested 'Beauregard' sweetpotato plants. Two *I. setosa* plants, with virus symptoms, were graft-inoculated per sweetpotato plant for each isolate

to produce singly infected plants. To produce plants infected with all four potyviruses together, four grafts were made to each plant, one each with an *I. setosa* scion infected with either SPFMV, SPVC, SPVG, or SPV2. After three weeks, plants with scions that survived grafting were tested by the Li *et al*. (2012) potyvirus multiplex PCR, to confirm whether or not they were infected with SPVG, SPVC, SPFMV and SPV2. Plants with single infections, the four potyviruses together and a naturally infected plant propagated during seven generations (B14-G7) were used as source for growing in aeroponics detailed in the next step.

After grafting B14-G7 on *I. setosa*, it was tested using RT-PCR, qRT-PCR and NCM-ELISA, and found to be infected with SPVG, SPVC, SPFMV and SPV2. B14-G7 tested negative for *Sweet potato mild mottle virus*, *Sweet potato latent virus*, *Sweet potato chlorotic fleck virus*, *Sweet potato mild speckling virus*, *Sweet potato leaf curl virus*, *Sweet potato chlorotic stunt virus*, *Sweet potato collusive virus*, and *Cucumber mosaic virus*. However, the possibility that it was infected by viruses not yet recognized in sweetpotato cannot be eliminated.

2.2.3 Plant growth and sample collection

Vine cuttings with two nodes from the infected and virus-tested plants were used for transplanting for tests in aeroponics. The dark container (Sterilite® 20 Gallon Aquarium Latch Tote with Titanium Latches - 22-3/4" L x 18-1/2" W x 16-1/4" H, United States Plastic Corp.; Lima, OH) was covered with aluminum foil to exclude light to the root zone and filled with seven liters of Hoagland's solution which was renewed every week (Hoagland and Arnon, 1950). The Hoagland's solution provided the nutrients and water during the whole experiment to the slips via an intermittent mist (AgroMax Digital Cycle Timer; HTGSupply U.S.) irrigation system. The irrigation system was composed of a dual outlet air pump (Active Aqua Air Pump, 2 Outlets, 3W, 7.8 L/min; Hydrofarm, Inc., Petaluma CA), which connects to venturi-misters (19-8400-1, Hummert International; Topeka, KS) via hoses (Heavy-Duty 3/4" FLEXIBLE Black Tubing; HTGSupply U.S.). On the top of the containers, six circles were made to fit black foam clone collars (HTGSupply U.S.) into which the sweetpotato slips were placed. Quantum T5 Fluorescent Light Fixtures (Hydrofarm; U.S.) provided supplemental light for 16 hrs per day. Samples of stems, leaves and roots were collected during the first, third, and fifth week after planting based on the SR1, SR2, and SR3 phenology stages described by Villordon *et al*. (2013). Each of the SR's are calculated based on a growing degree day (GDD) formula. To calculate GDD the formula is: maximum daily temperature (T_{max}) – base temperature (B), where if T_{max}> ceiling temperature (C, 32.2^{o}C), then T_{max}=C, and where GDD=0 if T_{min}<B (15.5^{o}C). GDD of 56, 278 and 468 were used to demarcate the SR1, SR2, and SR3 stages. Based on those considerations, samples from different organs were collected after seven, twenty-one and thirty-five days.

A weekly insecticide program was applied to control aphids and whiteflies. The experiment was conducted three times with three replicated plants each time for each treatment. At each collection date, samples of whole stems, leaves and roots were placed immediately in liquid nitrogen, and kept at -80^{o}C until RNA extraction.

2.2.4 Total nucleic acid extraction

Samples of stems, leaves and roots from weeks one, three and five after transplanting were ground into powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the CTAB method of (Li *et al*., 2008). Leaf tissue (100 mg) previously ground in liquid

nitrogen using a mortar and pestle was transferred to FastPrep-24™ (MP Biomedicals; Eschwege, Germany) and mixed with 1ml of CTAB/beta-mercaptoethanol. Samples were placed in a freezer at -20 ºC for 15 min. Tubes were homogenized using a Fastprep FP120 (Qbiogene, Inc.; North America) at 4.5 speed for 30 sec, cooled on ice for five min and the homogenization step was repeated. Samples were incubated at 65 ºC for 15 min in a water bath and centrifuged at 5,220 g in an Eppendorf 5415 microcentrifuge (Eppendorf, U.S.) for five min. 650 µl of the supernatant were mixed with 650 µl of 24:1 chloroform:isoamyl alcohol. Samples were mixed using a vortexer and centrifuged at 16,300 g for 10 min. 500 µl of the aqueous phase were mixed with 350 µl of isopropanol (2-propanol) and centrifuged again at high-speed (16,300 g) for 10 min. The supernatant was discarded and the pellet was washed twice with 500 µl of 70% ethanol after two high-speed (16,300 g) centrifugations of two min. Finally, the pellet was dried, resuspended in 50 µl of 20 mM Tris-HCl pH 8.0 and dissolved on ice for 15 min. To eliminate RNAses, Invitrogen RNase Out (40 units/µl) (ThermoFisher) was added at 1 µl per 50 µl of extract. To standardize the initial concentration of RNA, samples were measured by spectrophotometry (Nanodrop; Thermo Scientific) and adjusted to a concentration of 250 ng/µl, and 260/230 and 280/230 ratios both above 2.0. Samples were kept at -20ºC until qPCR testing.

2.2.5 Primer and probe development

To develop primer sequences to differentiate SPFMV and SPVC, sequences from different strains of SPFMV and SPVC were analyzed previously by Li *et al*. (2012) who designed forward species-specific primers for SPFMV and SPVC. Briefly, GenBank accession numbers: NC001841 (SPFMV-RC strain), FJ155666 (SPFMV-EA strain), AB439206 and AB439208 (SPFMV-O strain) and SPVC (AB509453 and GU207957) were aligned using MUSCLE on MEGA7 software (Kumar *et al*., 2017). Due to the low amount of information on the P1 gene, its low percentage of nucleotide conservation (Adams *et al*., 2005) and the reports of P1 being prone to recombination (Ohshima *et al*., 2007; Salvador *et al*., 2008); the primers were designed from the CP region taking advantage of the small mismatches of the 3' side of the primer and the cDNA template (Crouse and Vincek, 1995). A reverse primer for SPFMV and SPVC were designed manually but their properties were analyzed using the OligoCal website (Kibbe WA, 2007) to avoid self-complementarity between primers and to adhere to correct primer design standards. Sequences are indicated in Table 2.2 and were tested against different isolates maintained at Louisiana State University (Fig. 2.2).

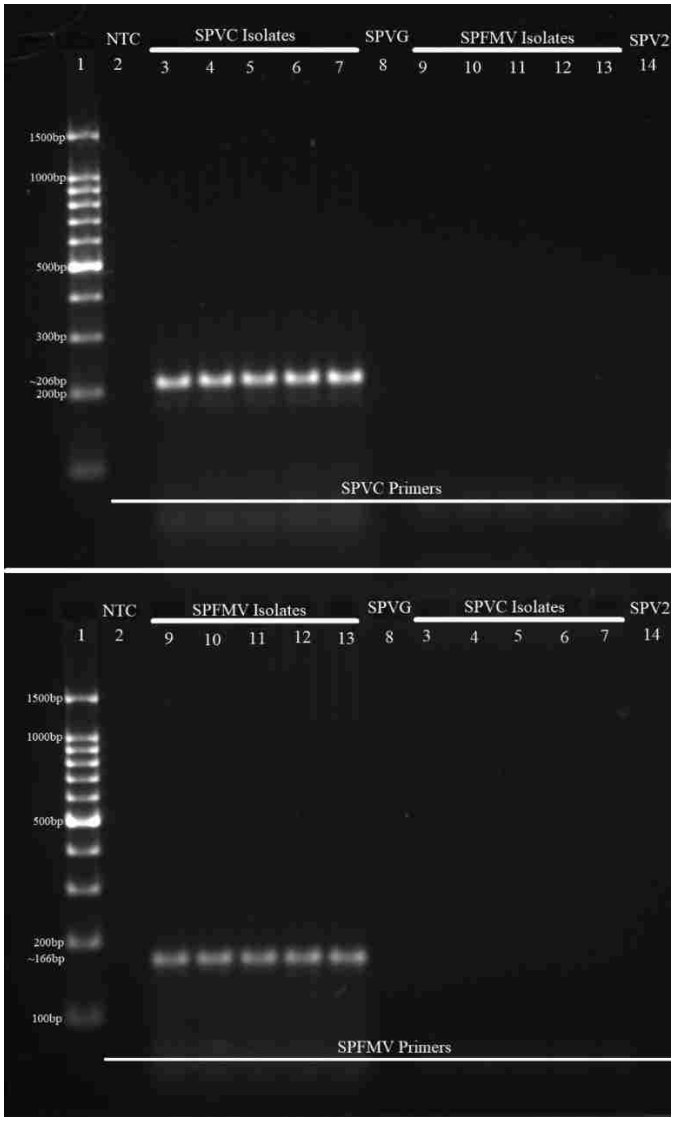

Figure 2.2. Electrophoresis of amplicons from different *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus C* (SPVC), *Sweetpotato virus G* (SPVG) and *Sweetpotato virus 2* (SPV2) isolates using primers for SPVC (top gel) and SPFMV (bottom gel). Lane 1: Bio-Rad 100bp Molecular Marker. Lane 2: No-template control (NTC). From 3 to 7: SPVC isolates: Moyer C, 95-6, SPVC PR3, 11-5, TFSW1-E. Lane 8: SPVG (isolate LSU-1). From 9 to 13: SPFMV isolates: 95-2 04R, 95-2T, 11-1, TFSW1-J, ARK-1. Lane 14: SPV2 (isolate LSU-2). Samples were run in a 2.5% agarose/TBA buffer pH 7.0 gel at 60 volts for 4 hrs. Numbers on the left correspond to the molecular marker nucleotide size provided by Bio-Rad Molecular Marker and the expected fragment size for SPFMV (~166bp) and SPVC (~206bp).

Table 2.2. Primer Sequences of SPFMV, SPVC and COX used for qPCR analysis

Primer Name	Primer Sequence	Expected fragment size
SPFMV-forward	GGATTAYGGTGTTGACGACACA	166 bp
SPFMV-reverse	TAGGCACTGCATGATCCAAC	
SPFMV-probe	**FAM**-AATGATGGACGGTGACGAGCAAGT-**MGB**	
SPVC-forward	GTGAGAAAYCTATGCGCTCTGTT	206 bp
SPVC-reverse	TTGAGCGTGTATTCCCAATG	
SPVC-probe	**FAM**-CATACTAGCAAAATGCGCCA-**MGB**	
COX-forward	ACTGGAACAGCCAGAGGAGA	156 bp
COX-reverse	ATGCAATCTTCCATGGGTTC	
COX-probe	**FAM**-ATCAGTGTTGTTGCCGATGA-**MGB**	
SPVG-forward	GAATCAAAGGTGAGGAGCAAGAC	160 bp
SPVG-reverse	GCTATGAGCAAATCGTCACCATT	
SPVG-probe	**FAM**-AGGTTTGCGTCTACTTC-**MGB**	
SPV2-forward	GAGACAGCACTGAAAGCTCTGTACA	170 bp
SPV2-reverse	CACGAACATACTCGGACAAATCTT	
SPV2-probe	**FAM**-TGTGTTGAACCATCAGC-**MGB**	

To analyze the data using relative quantification, the previously designed primers for the *Cytochrome C oxidase* gene (COX) housekeeping genes were used (Park *et al*., 2012; Table 4.2). Since Park *et al*., 2012 used SYBR green technology for the analysis, the probe was changed to make it consistent with the Taqman chemistry used for the rest of the probes as mentioned before (Table 2.2).

To design probes for SPFMV and SPVC, the Primer3 website (Rozen and Skaletzky, 1998) was used. The FAM reporter was used in the 5' end of the probe, and a Minor Groove binder (MGM) was used in the 3' end to increase the melting temperature (Tm) of the probe due to the low GC content of the chosen region, as indicated in Table 4.2. This chemistry was used to standardize the probes for SPFMV and SPVC to be consistent with the previously designed SPVG and SPV2 probes designed by Kokkinos *et al*. (2006). To set up the reactions, each sample consisted of 500ng sample template, 10 µl of 1X of iTaq Universal Probe master mix (Bio-Rad; U.S. CA), 0.5 µl of 40X reverse transcriptase iTaq Universal Probe (Bio-Rad; U.S. CA), 2 µl (2.5 uM) of forward and reverse primer, 0.4 µl (5 uM) of probe and 5.1 µl of water for a reaction of 20 µl per tube. Duplicates of each sample were run in 96-well PCR plate low-profile semi-skirted (BioRad; U.S. CA) in a CFX-96 Connect Real-Time System (BioRad; U.S. CA) at 48°C for 30 min (cDNA synthesis), 95°C for 10 min (AmpliTaq Gold® activation), followed by 40 cycles of denaturation at 95°C for 15 sec annealing/extension at 60°C for one min. The Ct value was determined from each sample using the ΔΔCq quantification method (CFX96 TouchTM Real-Time PCR Sequence Detection System Instruction Manual).

2.2.6 qPCR relative quantification and data collection

To determine the amplification efficiency and limit of detection of the primers used in this experiment, standard curves of at least five duplicated sample dilutions were generated for the two viral targets and the mRNA COX reference control (Figure 2.3, 2.4, 2.5). Since the

correlation between Ct values and log relative amounts was very high with R-squared values (R^2) exceeding 0.99 in all standard curves, the ΔΔCq quantification method (CFX96 TouchTM Real-Time PCR Sequence Detection System Instruction Manual) was used which eliminates the use of standard curves on every plate and sample normalization. From the standard curves generated, SPVC primer/probe set can detect $2.1x10^{-7}$ mg/ml, SPFMV primer/probe set can detect $1.39x10^{-7}$ mg/ml and COX can detect $2.58x10^{-7}$ mg/ml. Data collected represented relative quantification of the Ct (crossing point) values of 108 samples per organ and time run in duplicate. These values were analyzed in Analysis of Variance (ANOVA) in SAS 9.4 ($p<0.05$).

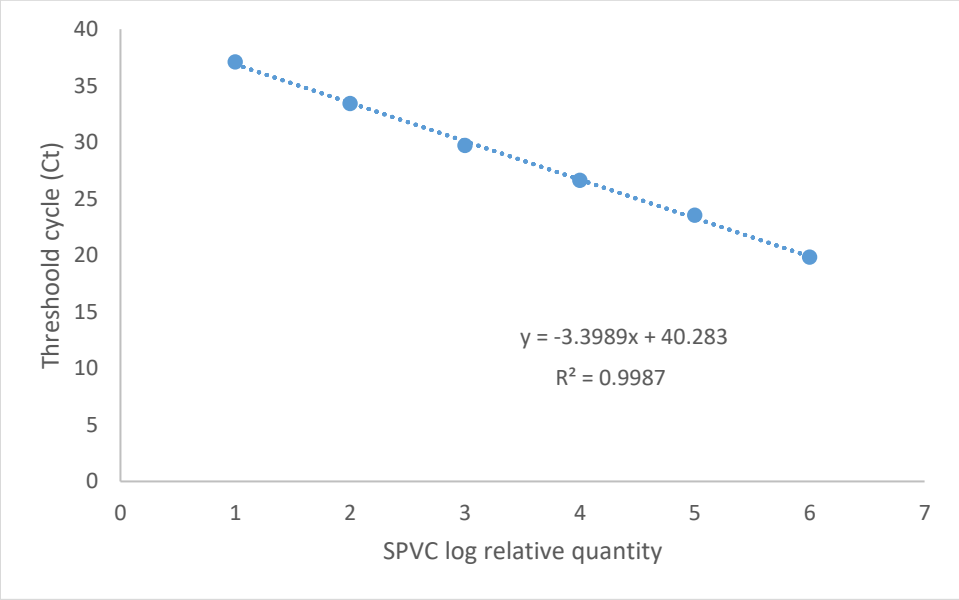

Figure 2.3. Standard curve generated by plotting the log relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweetpotato virus C* (SPVC).

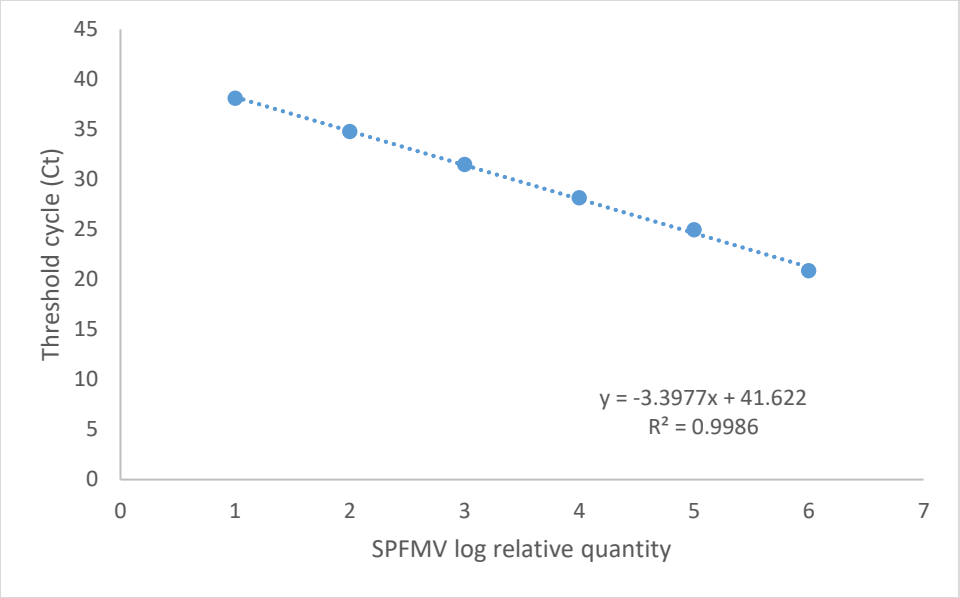

Figure 2.4. Standard curve generated by plotting the log relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweetpotato feathery mottle virus* (SPFMV).

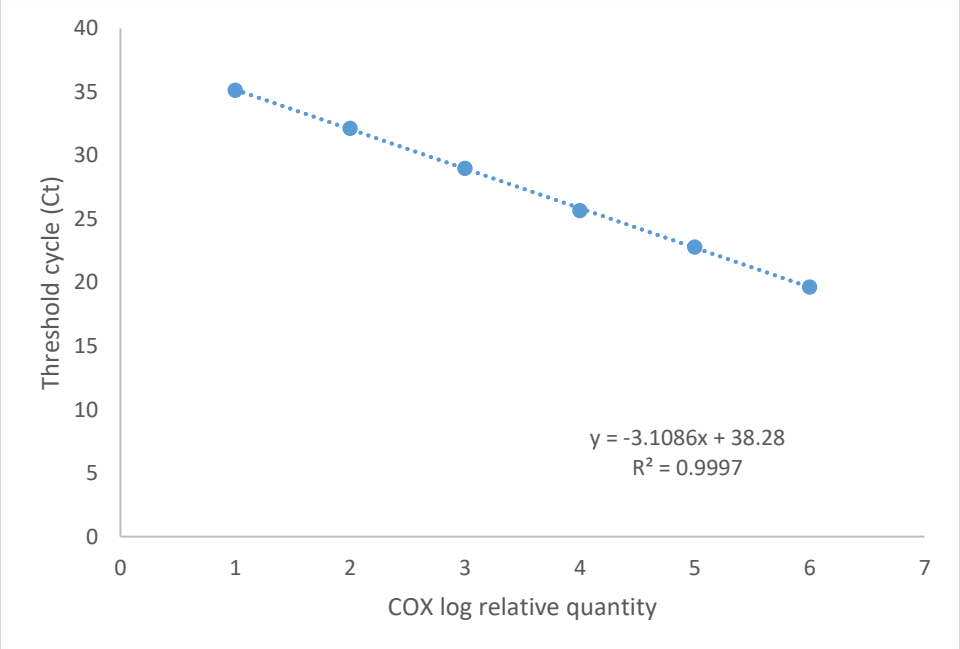

Figure 2.5. Standard curve generated by plotting the log relative quantity of a total RNA virus tested 'Beauregard' sweetpotato against critical threshold values from real-time PCR assays for *Cytochrome C oxidase* (COX).

2.3 Results

Naturally infected sweetpotato plants 'Beauregard' that had been exposed in the field for seven generations were used as a source previously determined to have relatively high titers of each of the four potyviruses. Titers for SPVG, SPVC, SPFMV and SPV2 were analyzed alone and in combination using the primers and probes designed for SPFMV and SPVC in this study and the primers for SPVG and SPV2 designed previously (Kokkinos *et al*., 2006). SPVG relative quantification titers were significantly greater in 'Beauregard' sweetpotato plants singly infected and in plants with the 4-way infection than in the naturally infected plants. For SPVC there were no statistical differences among treatments. In the case of SPFMV, titer was significantly greater in singly infected plants than in plants inoculated with the 4-way combination but the naturally infected plants were intermediate and not significantly different from either of the other treatments. Only for SPV2 did the naturally infected plants have greater titer than single infections but the 4-way multiple infection was intermediate and did not differ from the other treatments (Fig. 2.6).

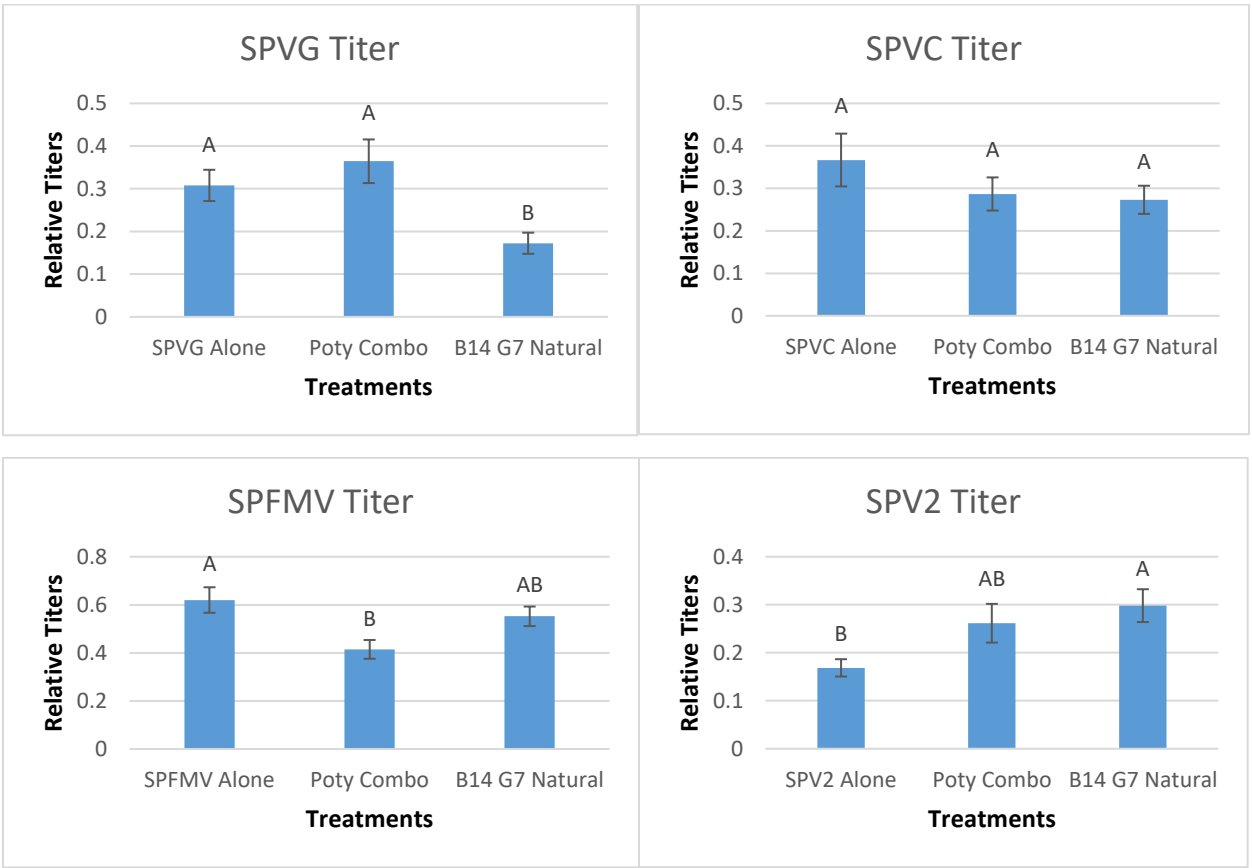

Figure 2.6. Relative quantification titers of treatments of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' in singly infected plants, plants artificially inoculated with all four potyviruses (Poty Combo) and plants naturally infected during seven generations of propagation in the field (B14 G7 Natural). Analyzed data is a combination of the different organs (leaf, root and stem) and times (week one, three and five). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

Relative titers were compared among three types of organs: roots, stems and leaves. For both SPVG and SPFMV, leaves had a greater relative quantification titer than roots, and stems were intermediate and not significantly different from leaves or roots. There were no significant differences among organ types for SPVC. Finally, in SPV2, there was no statistical difference among the sampled organs (Fig. 2.7).

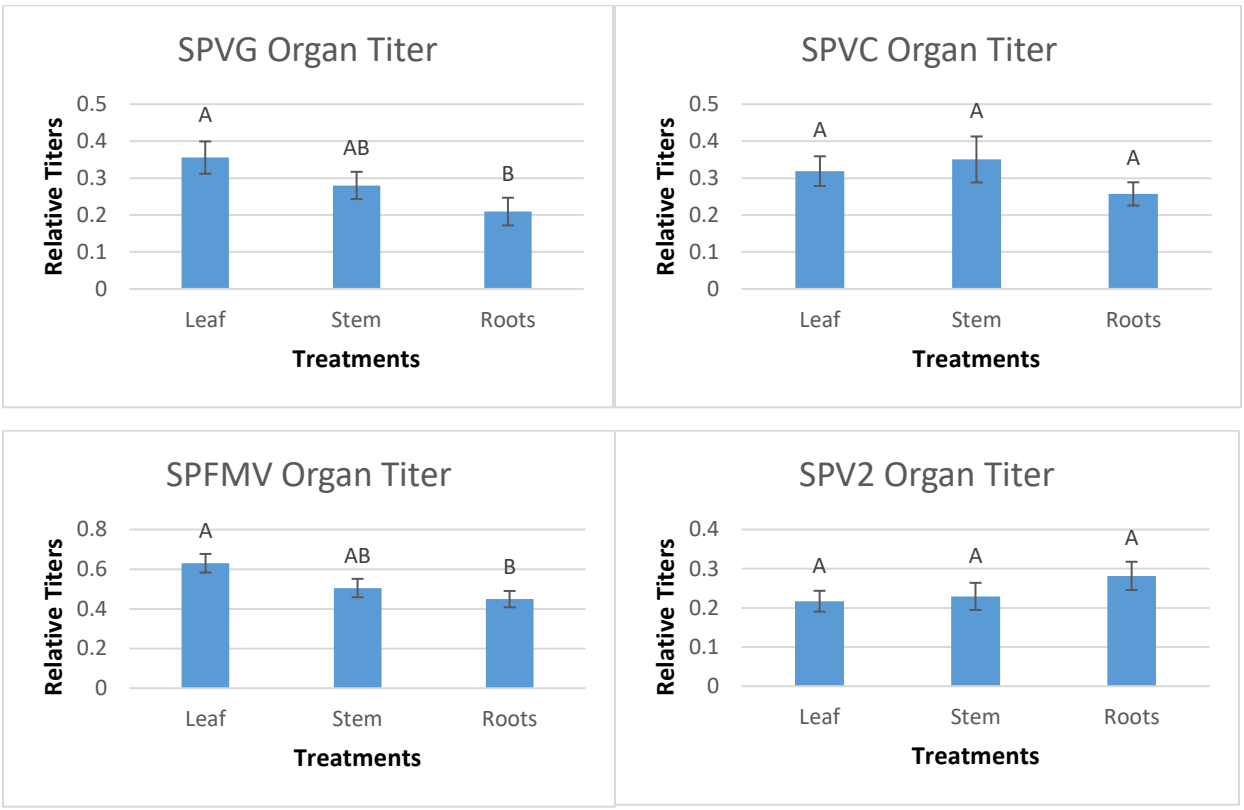

Figure 2.7. Relative quantification titers of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' in the different organs (leaf, root and stem). Analyzed data is a combination of the different treatments of singly infected plants, plants artificially inoculated with all four potyviruses (Poty Combo) and plants naturally infected during seven generations of propagation in the field (B14 G7 Natural). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

Relative quantification titers were also analyzed at three times based on growing degree-day (GDD) estimations of the phenological stages SR1 (week 1), SR2 (week 3) and SR3 (week 5). For SPVG, the third week had higher relative quantification titers compared to the first or fifth week. There were no significant differences among sampling times for SPVC, SPFMV and SPV2 (Fig. 2.8).

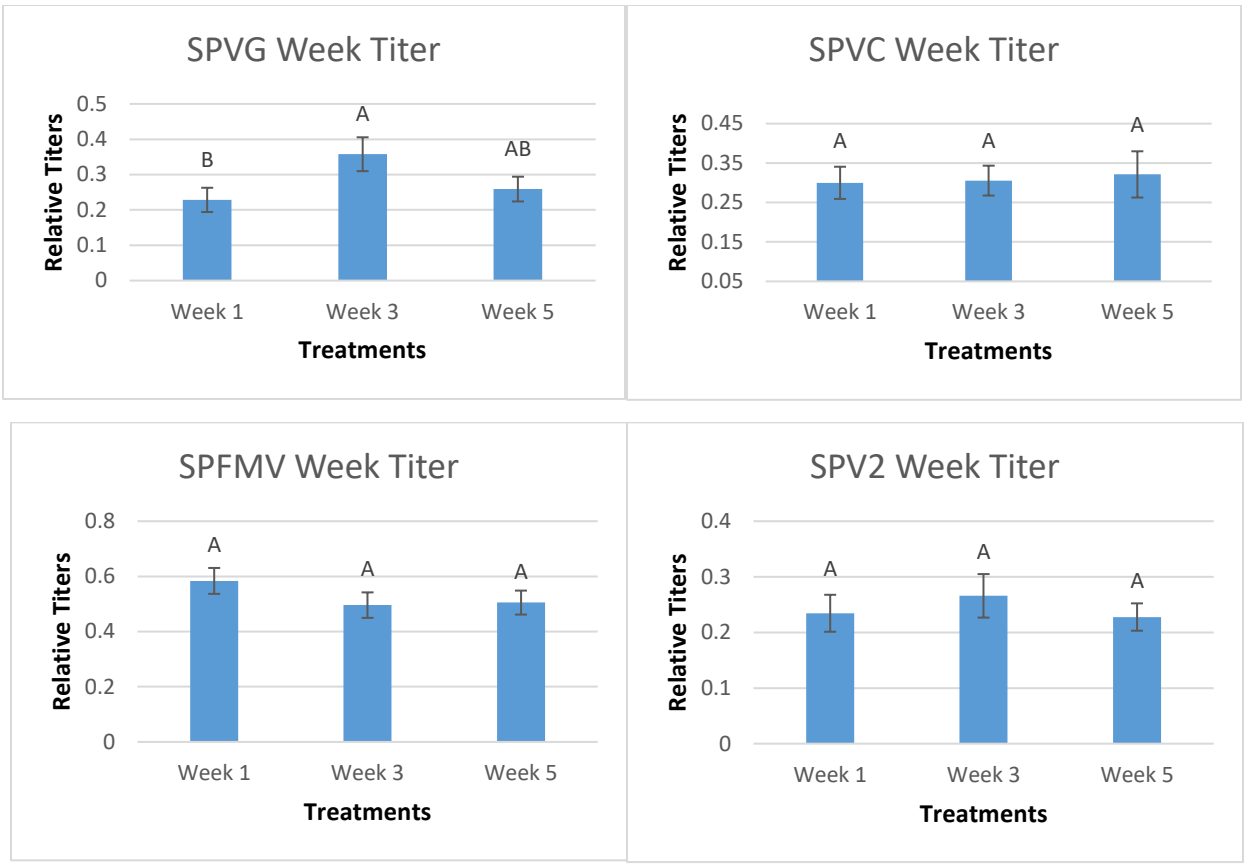

Figure 2.8. Relative quantification titers of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' of the different storage root development times (one, three and five week after planting). Analyzed data is a combination of the different treatments of singly infected plants, plants artificially inoculated with all four potyviruses (Poty Combo) and plants naturally infected during seven generations of propagation in the field (B14 G7 Natural). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

The different interactions were also analyzed. For the organ and development times, SPVG statistical differences were determined in leaves of the third week compared to root of the first week and stem of the fifth week. In SPVC and SPV2 there were no statistical differences. In SPFMV leaves of the first week had statistical differences compared with roots of the third week and stems of week five (Fig. 2.9).

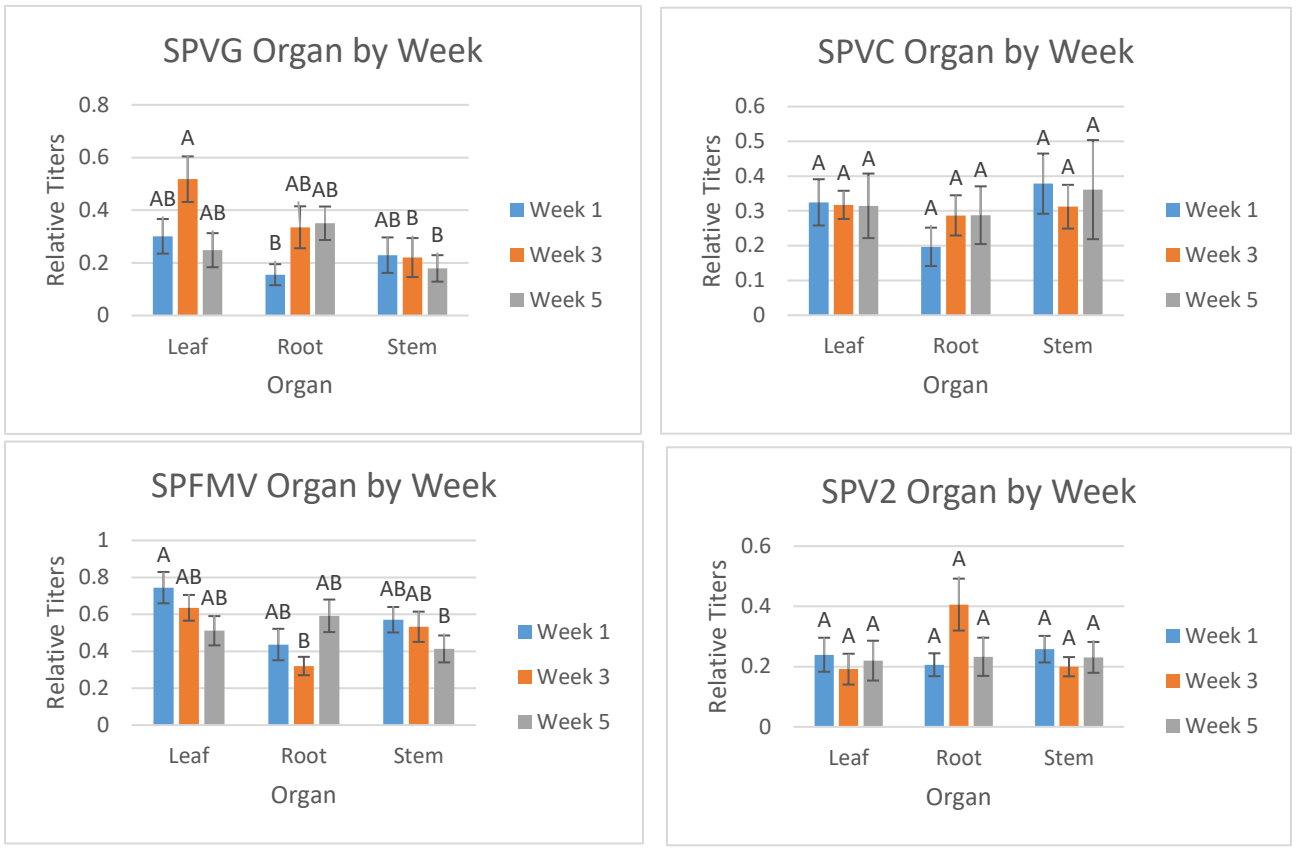

Figure 2.9. Relative quantification titers of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' of the interactions between the different storage root development times (one, three and five week after planting) and the three different organs (leaf, stem and roots). Analyzed data is a combination of the different treatments of singly infected plants, plants artificially inoculated with all four potyviruses (4-way) and plants naturally infected during seven generations of propagation in the field (Natural). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

The interactions of the different treatments and development times showed differences for the third week of the 4-way inoculation compared to the first week of single infections and all three weeks of natural infected plants in SPVG. There were no statistical differences for SPVC and SPV2. For SPFMV, there were differences between the first week, the third week of single infected plants and the fifth week of natural infection compared to the third week of the 4-way interaction (Fig. 2.10).

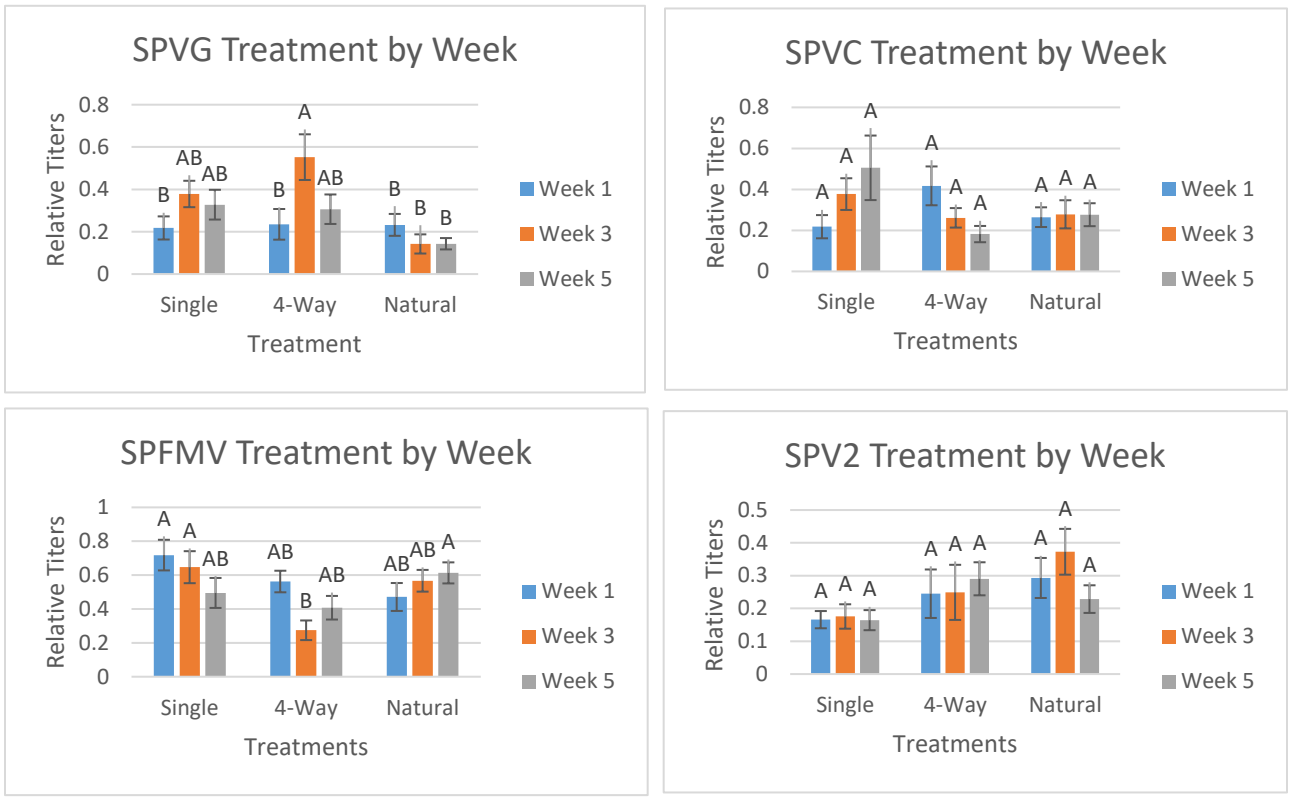

Figure 2.10. Relative quantification titers of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' of the singly infected plants, plants artificially inoculated with all four potyviruses (4-way) and plants naturally infected during seven generations of propagation in the field (Natural) interaction with different storage root development times (one, three and five week after planting). Analyzed data is a combination of the three different organs (leaf, stem and roots). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

SPVC did not show differences in the treatment and organ interactions. For SPVG, leaf organ of single and 4-way titers were different from stem organ of natural infections. SPFMV showed differences of leaf organ of single, natural infections and root organ of single infections compared to root organ of the 4-way inoculation. For SPV2 showed statistical differences of root organs of natural infections compared to stem organs of single infected plants (Fig. 2.11).

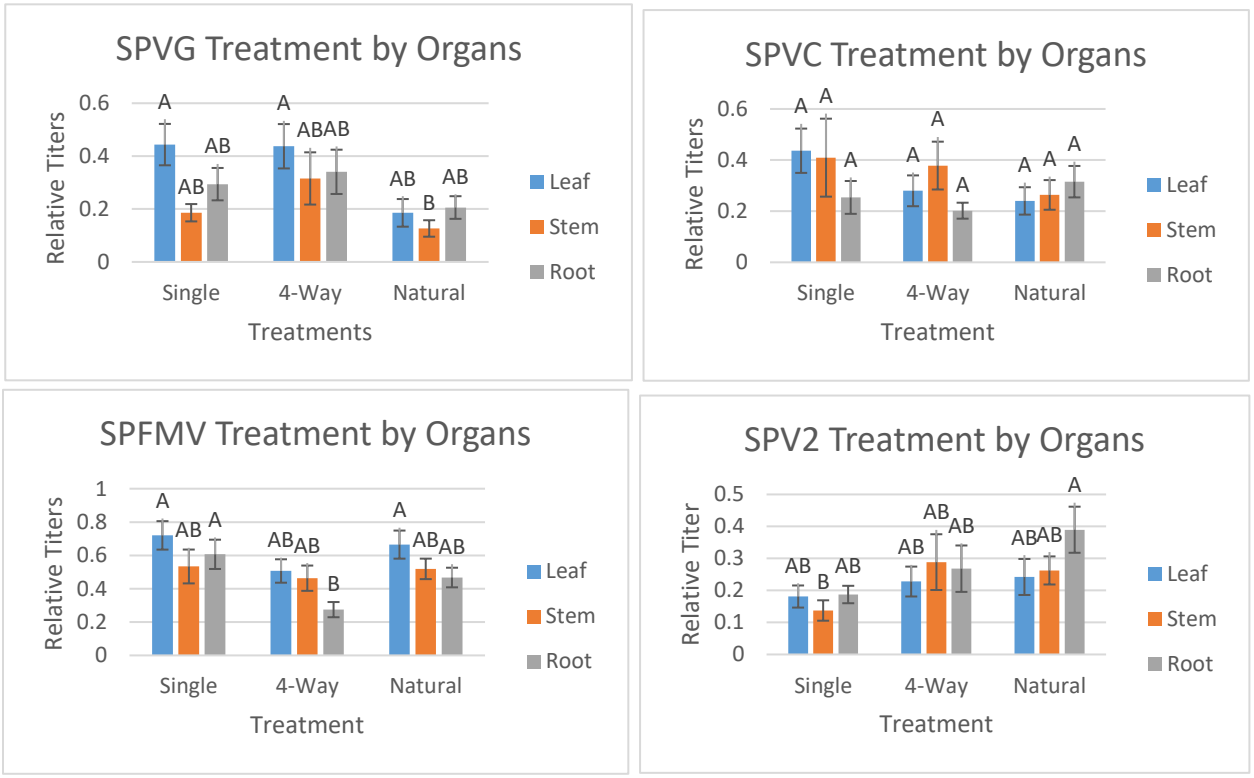

Figure 2.11. Relative quantification titers of SPVG, SPVC, SPFMV and SPV2 in sweetpotato 'Beauregard' of the singly infected plants, plants artificially inoculated with all four potyviruses (4-way) and plants naturally infected during seven generations of propagation in the field (Natural) interaction with the three different organs (leaf, stem and roots). Analyzed data is a combination of the different storage root development times (one, three and five week after planting). Bars with a common letter are not significantly different by ANOVA ($p<0.05$), Error bars = 1 standard deviation.

2.4 Discussion

Understanding the dynamics of virus replication within a plant is critical to developing practical approaches to detecting the viruses in plants and recognizing when and where vectors are most likely to acquire the virus and spread it to non-infected plants. In this study, greater titers of the four ubiquitous viruses present in Louisiana sweetpotato production fields (SPVG, SPVC, SPFMV and SPV2), accumulated in leaves during the third week after planting, which appears to be the ideal time to sample the plant for potyvirus infections.

To collect a sample that is representative for virus screening is a complex task in a plant like sweetpotato and different approaches were attempted to alleviate this problem. The morphology of the plant where the canopy can be very extensive, including over 600 leaves per plant at various stages of development or senescence (Kays, 1985; Firon *et al*., 2009) became a problem for diagnostic purposes. In the past, uneven SPFMV distribution in leaves was determined using ELISA tests (Green *et al*., 1988). However, at that time, SPVC was not

recognized as a different species (Untiveros *et al*., 2010) and perhaps these differences could be explained by the cross reactions, especially between SPFMV and SPVC, produced by the antibodies used using CP amino acid information (Souto *et al*., 2003). The extensive leaf organ reaches a further level of complexity based on the evidence that the distribution of SPFMV and other viruses at any point in time is often not uniform among parts of sweetpotato plants or other hosts of the morning glory family like *I. setosa* or *I. nil* (Gibb and Padovan, 1993; Kokkinos *et al*., 2006).

It is difficult to sample other parts of the plant, such as roots or stems, without destroying the plant, especially at early plant stages. In a more recent approach, a greater number of reads in roots were reported when compared to other organs under next generation sequence analysis (Gu *et al*., 2014). However, the data obtained did not consider the different phenological stages proposed in sweetpotato (Villordon *et al*., 2013) and next generation sequence data were analyzed at the harvest of the plant. The contrasting evidence could be explained by the observed differences of SPFMV and SPVC, since a decrease in titers is observed in leaf organs as time progresses but an inverse scenario is observed in roots. More importantly, at the bioinformatics level, the presence of defective DNA/RNA triggers siRNA production (Wu *et al*., 2010). This siRNA, which is used for the assembly of sweetpotato viruses, is commonly not distributed uniformly among the potyvirus genome that could confuse the assembly software when determining the contigs (Kreuze, 2014). This event could overestimate a significant amount of reads when our data suggests that SPVC titers do not have a significant difference for either time or organ.

It is also important to know if there are interactions among the four viruses that influence viral titers. The four potyviruses that are typically found in sweetpotato production in the U.S. infect sweetpotato in the field at different rates that leads to plants being commonly infected with different combinations of the viruses. While it is well documented that co-infection with SPCSV has profound effects on titer of potyviruses, symptoms they induce, and effects on crop yield (Kokkinos *et al*., 2006), it is not known how different species of potyvirus might interact with each other. In this study, single infections were statistically different for SPVG and SPV2 compared to their natural infections on their 4-way interaction, but SPVC and SPFMV had no statistical differences and remain stable. This could explain why, despite the interaction with other potyviruses, in field surveys it is more common to find SPFMV and SPVC than SPV2 and SPVG (Clark *et al*., 2002). Additionally, it is necessary to know if the viral titers of the artificial inoculations is similar to natural infections for future studies in the cultivar decline effect that appears to be the most important effect of these viruses on sweetpotato (Clark and Hoy, 2006). Data suggests that the artificial 4-way combination of viruses did not modify titers compared to naturally infected plants for the viruses used except SPVG. With this premise, the approach was used to determine if the recently distinguished SPVC is a missing component in understanding the cause of cultivar decline/yield reduction of sweetpotato in the U.S. (Herrera; Chapter 3). Results suggested that a factor is still neglected which could be involved in the reduction of viral titers of SPVG, but the same factor increases SPV2 under natural infections according to the data obtained in this study.

In previous experiments, the universal 18S gene has been used for potyvirus quantification (Kokkinos *et al*., 2006), which unfortunately is no longer available. In this experiment, the COX gene, previously determined to be a stable gene for sweetpotato gene expression under different abiotic stress (Park *et al*., 2012) appears to be useful for biological

agents like potyvirus relative quantification. This finding could serve to consider COX for other experiments of viral quantification like confirmation of virus expression following detection by next generation sequencing methods (Kostic *et al*., 2011; Zhang *et al*., 2014; Zheng *et al*., 2016). Recenlty, COX has been used as a housekeeping gene for quantitative multiplex PCR detection of SPVG, *Sweetpotato mild mottle virus* (SPMMV) and *Sweetpotato latent virus* (SPLV) (Lan *et al*., 2017).

Initial attempts to use the P1 region of the Potyviridae genome to develop specific primers for SPFMV and SPVC were unsuccessful despite the fact that this is a region of amino acid gene diversity (Untiveros *et al*., 2010). This probably relates to the variability within the gene (Adams *et al*., 2010; Untiveros *et al*., 2016; Mingot *et al*., 2014) or frequency of recombination (Revers *et al*., 2015). This problem was resolved using the CP region of SPFMV and SPVC. CP is a highly conserved region at the nucleotide level (Adams *et al*., 2005); however, the 5' end of the CP region provides enough mismatches for primer design between these two viruses. Primer design took advantage of the fact that mismatches between the 3' end of the primer and the template, reduce the ability of the oligonucleotide to prime (Crouse and Vincek, 1995). This design helped to amplify each virus without having to increase annealing temperature of the reaction, which also helped in the creation of Taqman probes using minor groove binding chemistries in a region of poor GC content. The CP region chosen appears to be conserved enough to differentiate between SPFMV and SPVC, which agrees with previous studies (Elvira-Gonzales *et al*., 2017; Lohmus *et al*., 2017; Voloudakis *et al*., 2004; Bejerman *et al*., 2016).

The fact that in this study leaves contained higher titers than other parts of the plant, correlates to the increased expression of genes related to photosynthesis following SPFMV infection. In a microarray analysis of gene expression of sweetpotato plants infected with SPFMV (Kokkinos *et al*., 2006), the plants infected with SPFMV, down regulated metallothionein-like type 1 protein (involved in cell rescue, defense and virulence; Golgi apparatus processing proteins for secretion) and 26S proteasome regulatory subunit S2 (RPN1) (involved in ATP regulation of ubiquinated proteins) and upregulated the L-arginine metabolizing enzyme plastocyanin (involved in copper-containing protein involved in electron transfer). The results also correspond well with the correlation of higher titers with increased aphid transmission of SPFMV during the third week after plating in the field in 2010, at the time of rapid vine growth (Wosula *et al*., 2012). All results combined suggests that protecting the sweetpotato plant canopy during the third week after planting could potentially reduce viral transmission by the different aphid populations in the field.

The utilization of qPCR is cumbersome and expensive for general virus detection. To improve the efficiency and reduce cost, improving each of the individual simplex real-time reverse transcription reactions into a single multiplex reaction will be needed for future experiments. In the past, the utilization of multiple fluorophores that can emit different wavelengths have been used to screen the presence of different viruses in heirloom sweetpotato cultivars (Ling *et al*., 2010), however, development of this assay also pre-dated elevation of SPVC to a distinct species. With the primers designed in this study, qPCR optimization modification with probes with different wavelengths could establish a cheaper assay that can detect the most common potyviruses in sweetpotato in the proposed organ and time by this experiment for a more efficient diagnostic assay.

CHAPTER 3: THE EFFECT OF SWEETPOTATO VIRUS C IN THE STORAGE ROOT NUMBER OF SWEETPOTATO (*IPOMOEA BATATAS*)

3.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is an important crop for food security due to the low agronomic inputs required to grow the crop, and its high nutritional value (Gibson *et al*., 2009). Among the different pathogens affecting sweetpotato, viruses have been shown to affect yields due to their accumulation during the continuous vegetative propagation of the crop. Plant viruses affect their hosts in several ways, but in U.S. sweetpotato production, potyviruses are associated with a phenomenon known as cultivar decline, which results in gradual reductions in crop productivity over years of cultivation (Clark *et al*., 2002). Four potyviruses are commonly found in field surveys in the U.S. (Clark and Hoy, 2006; Wosula *et al.*, 2012). *Sweet potato feathery mottle virus* (SPFMV) was the first sweetpotato virus fully characterized in 1978 (Moyer and Kennedy, 1978) and it was the only one reported in the United States until 2001. However, *Sweet potato virus G* (SPVG) and *Sweet potato virus 2* (SPV2; synonym Ipomoea vein mosaic virus), were subsequently characterized (Souto *et al.*, 2003). Recently, the former common strain of SPFMV-C was re-categorized as the distinct species, *Sweet potato virus C* (SPVC) based on amino acid sequence differences in the P1 region of SPFMV and SPVC (Untiveros *et al*., 2010). All four potyviruses are ubiquitous in Louisiana and commonly detected when surveyed. Most of the time, they are detected in combination rather than as single infections (Wosula *et al*., 2012).

Cultivars of sweetpotato currently grown in Louisiana have only shown relatively mild foliar symptoms when infected with the common potyviruses, suggesting they have a degree of resistance. However, this resistance is broken when potyviruses are co-infected with *Sweet potato chlorotic stunt virus* (SPCSV) in a synergistic interaction, resulting in the 'sweetpotato virus disease' (SPVD) (Karyeija *et al*., 1998). To analyze how this combination affects 'Beauregard' sweetpotato at the gene level in the plant, a microarray approach was used. SPFMV or SPCSV alone caused differential expression of only 3 to 14 genes, respectively, compared to virus-tested plants but when combined, 216 genes were expressed differently. Most of the genes were related to the photosynthetic pathway (McGregor *et al*., 2009). Although potyvirus symptoms are most commonly observed in leaves, the factors that affect root development, storage root initiation and enlargement are considered critical to improve global food security (Villordon *et al.*, 2014). To date, several factors appear to affect root formation in plants. Intrinsic factors such as ethylene and strigolactones (Ivanchenko *et al*., 2008; Koltai, 2011) and environmental variables such as substrate water, nutrient availability and plant viruses (Deak and Malami, 2005; Johnson *et al*., 1996; Kutz *et al*., 2002; Peltier *et al.*, 2011) have been tested in model systems. Water, nitrogen availability, and virus infections have been corroborated to decrease storage root formation in 'Beauregard' sweetpotato (Villordon *et al*., 2013; Villordon and Clark, 2014).

Despite all these efforts of molecular and applied studies to understand the factors that are involved in storage root production, the infection with viruses known until 2006 in the United States did not fully reproduce the magnitude of yield reduction of sweetpotato plants that were naturally infected with viruses over many years in field production (Clark and Hoy, 2006). Since SPVC was reclassified as a new species subsequently, the question arose as to whether it might account for the differences in yield between naturally infected plants and plants artificially

infected with SPFMV, SPVG, and SPV2. The objective of this experiment was to test if the inclusion of SPVC in the potyvirus complex reproduces the reduction in storage root number observed in naturally infected plants in sweetpotato 'Beauregard'. The interaction of the other three potyviruses and the recently described SPVC could lead to a better understanding of management and epidemiology of virus-induced decline in this important crop.

3.2 Materials and methods

3.2.1 Virus isolates

Sweetpotato plants, which showed potyvirus-like symptoms, were collected from the southeastern United States and separated by mechanical inoculations or single aphid transmission using different hosts (Souto *et al*., 2003; Table 3.1). Isolates were maintained in *I. nil* 'Scarlet O'Hara' (SOH) by periodic mechanical inoculations using leaf tissue ground with a mortar and pestle in 0.02M phosphate buffer, pH 7.2, amended with 0.1M sodium diethyl dithiocarbamate (DIECA) and rubbing the inoculum on Carborundum-dusted leaves. Their isolation was confirmed by a multiplex RT-PCR, which allows detection of SPVG, SPVC, SPFMV and SPV2 in the same reaction (Li *et al*., 2012). The SOH plants infected with different isolates were kept in Bugdorm rearing and observation cages (Bioequip products, CA) in the greenhouse facilities of Louisiana State University Agricultural Center, Baton Rouge, Louisiana.

Table 3.1. Potyvirus isolates from sweetpotato used in this study. Isolates were separated using differential host assay or single aphid probe transmissions. Isolates were mechanically transmitted into *Ipomoea nil* 'Scarlet O'Hara' and renewed every three weeks.

Isolate	Species	Location	Method used for separation
LSU-1	SPVG	Louisiana, U.S.	*Aphis gossypii* single probe
95-6	SPVC	North Carolina, U.S.	*Nicotiana benthamiana* mechanical inoculation
Ark-1	SPFMV	Arkansas, U.S.	*Chenopodium quinoa* single local lesion
CA-6	SPV2	California, U.S.	*C. quinoa* single local lesion

3.2.2 *Potyvirus* inoculation

Virus-tested plants of sweetpotato 'Beauregard' mericlone B-14 originated by meristem-tip culture (Carrol *et al*., 2004) were grafted with *I. setosa* seedlings. These seedlings were previously mechanically inoculated with SPVG (isolate LSU-1), SPVC (isolate 95-6), SPFMV (isolate ARK-1) and SPV2 (isolate CA-6). Two infected *I. setosa* scions were graft-inoculated to each sweetpotato plant for each isolate to create singly-infected plants. Four grafts, one each with a scion infected with SPFMV, SPVG, SPV2, and SPVC were made to virus-tested 'Beauregard' plants to create plants infected with all four potyviruses. After three weeks, leaves from plants with scions that survived grafting were collected in liquid nitrogen and stored at -80^{o}C until total RNA extraction with CTAB procedure (Li *et al*., 2008).

Total RNA was extracted from 100 mg leaf tissue ground in liquid nitrogen using a mortar and pestle, transferred to a FastPrep-24^{TM} tube containing beads for tissue disruption (MP Biomedicals; Eschwege, Germany), and mixed with 1ml of CTAB/beta-mercaptoethanol.

Samples were placed in a freezer at -20 ºC for 15 min. Tubes were homogenized using a Fastprep FP120 (Qbiogene, Inc.; North America) at 4.5 speed for 30 sec, cooled on ice for five min, and the homogenization step was repeated. Samples were incubated at 65 ºC for 15 min in a water bath and centrifuged at 5220 g in an Eppendorf 5415 microcentrifuge (Eppendorf, U.S.) for 5 min. 650 µl of the supernatants were mixed with 650 µl of 24:1 chloroform:isoamyl alcohol. Samples were mixed using a vortexer and centrifuged at 16,300 g for 10 min. 500 µl of the aqueous phase were mixed with 350 µl of isopropanol (2-propanol) and centrifuged again at high-speed (16,300 g) for 10 min. The supernatant was discarded and the pellet was washed twice with 500 µl of 70% ethanol after two high-speed (16,300 g) centrifugations of two min. Finally, the pellet was dried, resuspended in 50 µl of 20 mM Tris-HCl pH 8.0 and dissolved on ice for 15 min. To eliminate RNAses, Invitrogen RNase Out (40 units/µl) (ThermoFisher, Waltham, MA) was added at 1 µl per 50 µl of extract. To standardize the initial concentration of RNA, samples were measured by spectrophotometry (Nanodrop; Thermo Scientific) and adjusted to a concentration of 250 ng/µl, and 260/230 and 280/230 ratios both above 2.0. Samples were kept at -20ºC until PCR analysis.

3.2.3 Confirmation of *Potyvirus* infection

RNA from single infections, a four-way potyvirus combination and a sweetpotato 'Beauregard' propagated in the field and exposed to natural infection for seven generations (B14-G7); were tested by the multiplex-PCR which allows simultaneous detection of SPVG, SPVC, SPFMV and SPV2 in the same reaction (Li *et al*., 2012; Figure 3.1). The reaction of the multiplex RT-PCR consisted of 0.7 µl of sterile water, 10 µl of 2X reaction buffer (Invitrogen Superscript III; Thermofisher), 1.2 µl of Superscript RT/Taq enzyme (Invitrogen), 2.5 µl of SPVG forward primer (1.25 µM), 0.4 µl of SPVC forward primer (0.2µM), 2 µl of SPFMV forward primer (1 µM) and 0.2 µl of SPV2 forward primer (0.1 µM), 2 µl of SPFCF2R (1 µM) and 1 µl of template of total RNA for a reaction of 20 µl. The 2720 thermocycler (Applied Biosystems; Thermofisher) conditions consisted of preheating at 50 ºC, then a reverse transcription of 50 ºC for 30 min and 94 ºC for two min. The cDNA amplification consisted of 30 cycles of 94 ºC for 30 sec, 60 ºC for 30 sec and 65 ºC for one minute. The reaction was stopped with 72 ºC for 5 min.

At the same time, a strain-specific multiplex RT-PCR, which allows the detection of SPVC, SPFMV-RC (russet-crack) and SPFMV-O (ordinary), was used to compare 4-way inoculations against B14-G7 (Bejerman *et al*., 2016; Figure 3.2). The strain specific reaction consisted of 5.4 µl of sterile water, 10 µl of 2X reaction buffer (Invitrogen Superscript III; Thermofisher), 1 µl of Superscript RT/Taq enzyme (Invitrogen), 0.6 µl of each forward primer (0.3 µM) and 0.8 µl of reverse primer (0.4 uM) for a reaction of 20 µl. The 2720 thermocycler (Applied Biosystems; Thermofisher, U.S.) conditions consisted of a reverse transcription of 48 ºC for 50 min and 94 ºC for 4 min. The cDNA amplification consisted of 40 cycles of 94 ºC for 1 minute, 57 ºC for 1 minute and 68 ºC for 90 sec. The reaction was stopped with 72 ºC for 10 min. Both multiplex RT-PCR reactions were run in a 0.8% agarose electrophoresis for 90 min at 70V.

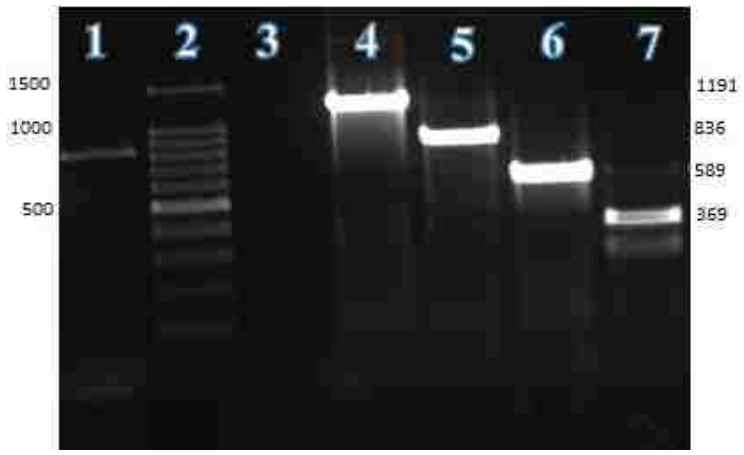

Figure 3.1. Agarose gel electrophoresis (0.8%) of the products from a multiplex RT-PCR reaction of total RNA extracts from the different treatments in this experiment. From left to right: Moyer-C (SPVC; positive control) (1), 100bp Bio-Rad Molecular Marker (2), No-template control (3), *Sweetpotato virus G* alone (SPVG; isolate LSU-1) (4), *Sweetpotato virus C* alone (SPVC; isolate 95-6) (5), *Sweetpotato feathery mottle virus* (SPFMV; isolate Ark-1) alone (6) and *Sweetpotato virus* 2 alone (SPV2; isolate CA-6) (7). Numbers on the left correspond to the molecular marker nucleotide size and the numbers on the right the expected fragment to be amplified by the different potyvirus isolate species used: SPVG (~1191bp), SPVC (~836bp), SPFMV (~589bp) and SPV2 (~369bp).

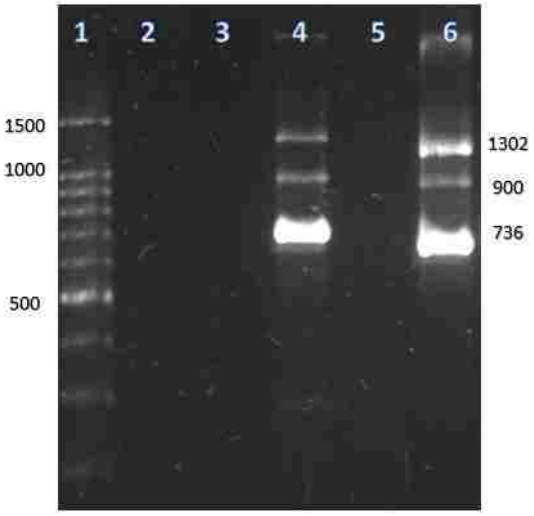

Figure 3.2. Agarose gel electrophoresis (0.8%) of the products from a multiplex RT-PCR that detects different strains of SPFMV (Bejerman *et al*., 2016). The reaction consists of total RNA extracts from the different treatments in this experiment. From left to right: 100bp Bio-Rad Molecular Marker (1), *Sweetpotato virus G* alone (SPVG) (2), *Sweetpotato virus 2* alone (SPV2) (3), 4-way combination treatment (*Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus 2* (SPV2) (4), Virus-tested B14 (5), B14-G7 (sweetpotato 'Beauregard' naturally infected plant propagated during seven generations in the field) (6). Fragments amplified corresponded to SPFMV-O strain (~1302bp), SPVC (~900bp) and SPFMV-RC strain (~736bp).

After grafting B14-G7 on *I. setosa*, leaves were tested using RT-PCR, qRT-PCR and NCM-ELISA, and found to be infected with SPVG, SPVC, SPFMV and SPV2. B14-G7 tested negative for *Sweet potato mild mottle virus, Sweet potato latent virus, Sweet potato chlorotic fleck virus, Sweet potato mild speckling virus, Sweet potato leaf curl virus, Sweet potato chlorotic stunt virus, Sweet potato collusive virus,* and *Cucumber mosaic virus*. However, the possibility that it was infected by viruses not yet recognized in sweetpotato cannot be eliminated.

Plants were grown under standard greenhouse conditions in 15-cm-diameter clay pots containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Jiffy-Mix® Plus (Jiffy Products of America Inc., Norwalk, OH) and 3.5g per pot of Osmocote® 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH). A weekly insecticide spray program was followed to control aphids and whiteflies.

3.2.4 Plant growing conditions

Vine cuttings with two nodes below the ground were grown in washed and autoclaved river sand in 10-cm diameter, 30cm high polyvinyl chloride (PVC) pots fitted with detachable plastic bottoms. Each plastic bottom had five drain holes (2mm in diameter). The diameter of sand particles varied from 0.05 to 0.9 mm. The moisture of the growing substrate was maintained at approx. 65 to 75% of field capacity (12% volumetric water content). Growth substrate moisture was measured with an ECH20 soil moisture sensor (Model EC-5, Decagon Devices Inc.). High intensity mercury vapor lamps were used to extend daylength to 14 hrs per day when necessary (Villordon *et al*., 2012; Villordon and Clark 2014). During the 1st, 3rd and 5th week, plants were fertilized with 200ml of Hoagland's solution (Hoagland and Arnon, 1950). A program of insecticide application, yellow sticky traps and sanitation was routinely used for insect control in the greenhouse (30.411380 N, 91.172807 W). The experiment was conducted three times during the months of July to December of 2016 with five replicate plants each time, for a total of 15 plants per treatment.

3.2.5 Data collection

Six weeks after transplanting, plants were washed carefully to avoid root damage using tap water, and then taken to the laboratory for data collection. Plants were then cut at the first main stem region above the soil and the roots were kept under DI water to allow precipitation of the grains of sand attached to the root system for eight hrs. Data collected include differences between different types of roots classified based on their diameters such as storage roots (>0.4cm), pencil root (0.2-0.4cm) and undifferentiated roots (<0.2cm). Measured variables included storage root number, pencil root number, storage root diameter, storage root length, undeferentiated root length, weight of storage roots and weight of total undeferentiated root mass, using a ruler and a digital balance. Data was analyzed by PROC ANOVA ($p<0.05$) in SAS version 9.4.

3.3 Results

Species of sweetpotato potyviruses were successfully separated using the different methods proposed by Souto *et al.* (2003) as determined by subsequent testing using Li *et al*'s. (2012) multiplex PCR (Fig. 3.1). However, the attempt to separate SPFMV strains from each other using single local lesions on either *Chenopodium quinoa* or *C. amaranticolor*, was

unsuccessful according to the SPFMV strain-specific multiplex PCR of Bejerman *et al*. (2016) (Fig. 3.2). The plants used in these experiments that were infected with SPFMV were found to be infected with both SPFMV-O and SPFMV-RC strains (Fig. 3.2).

Storage root number was the only yield variable to show a significant difference, in this case between naturally infected plants compared to SPV2 (Fig. 3.3). Despite the efforts to recreate the amount of reduction in storage root number observed in the naturally-infected plants under greenhouse-controlled conditions, the rest of the data showed high variability among treatments, and therefore, differences among treatments were not significantly different (Fig. 3.4 to 3.9).

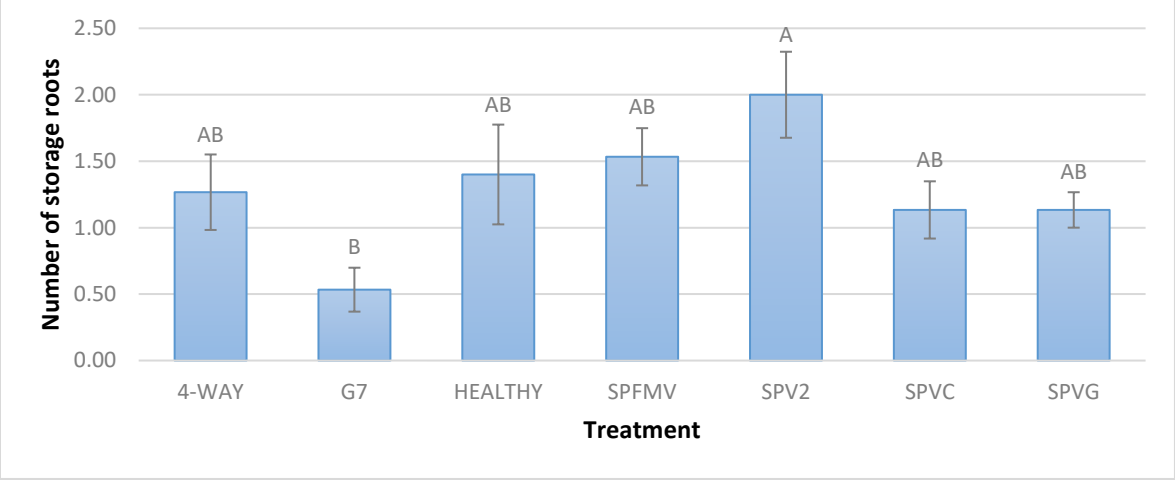

Figure 3.3. Total storage root number produced in a greenhouse by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant artificially inoculated and infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

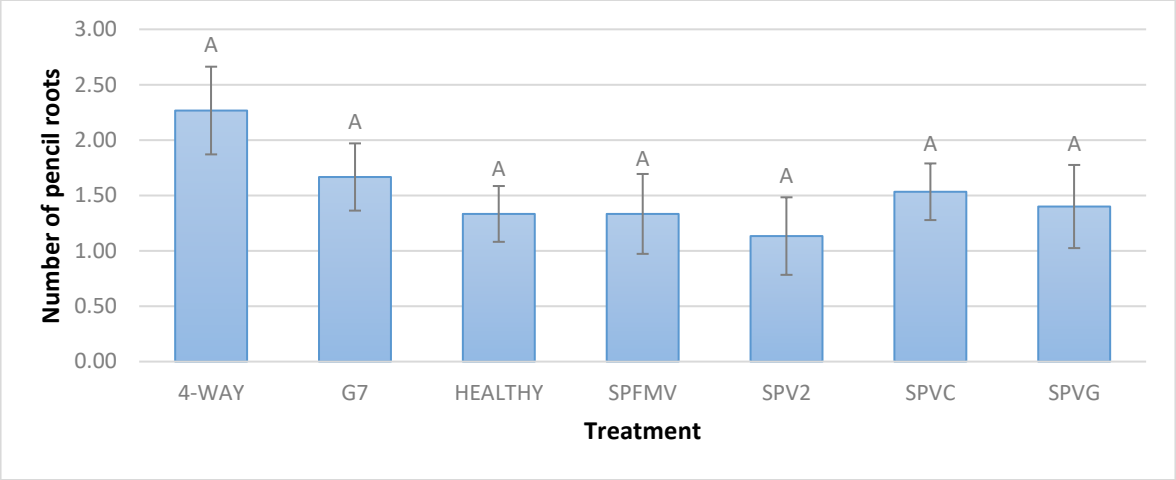

Figure 3.4. Pencil root number produced in a greenhouse by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), S*weetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which was infected with all four potyviruses (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

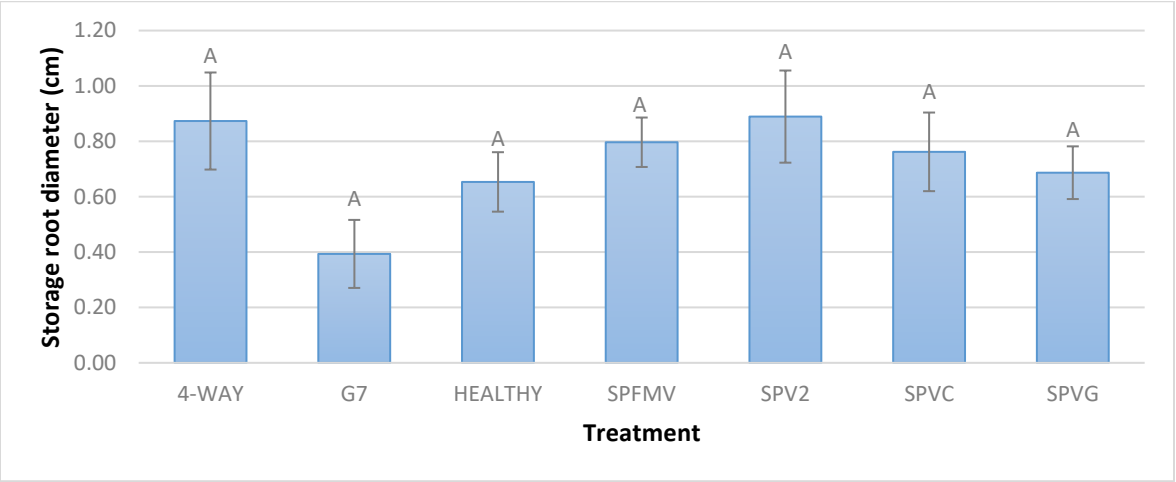

Figure 3.5. Diameter of storage roots (in cm) produced in a greenhouse by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

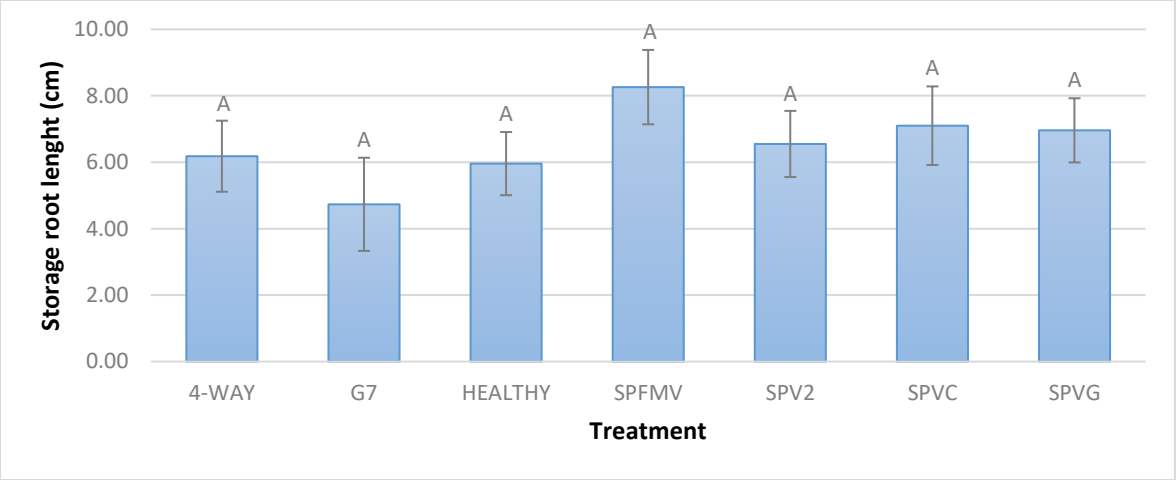

Figure 3.6. Length of storage roots (in cm) produced in a greenhouse by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

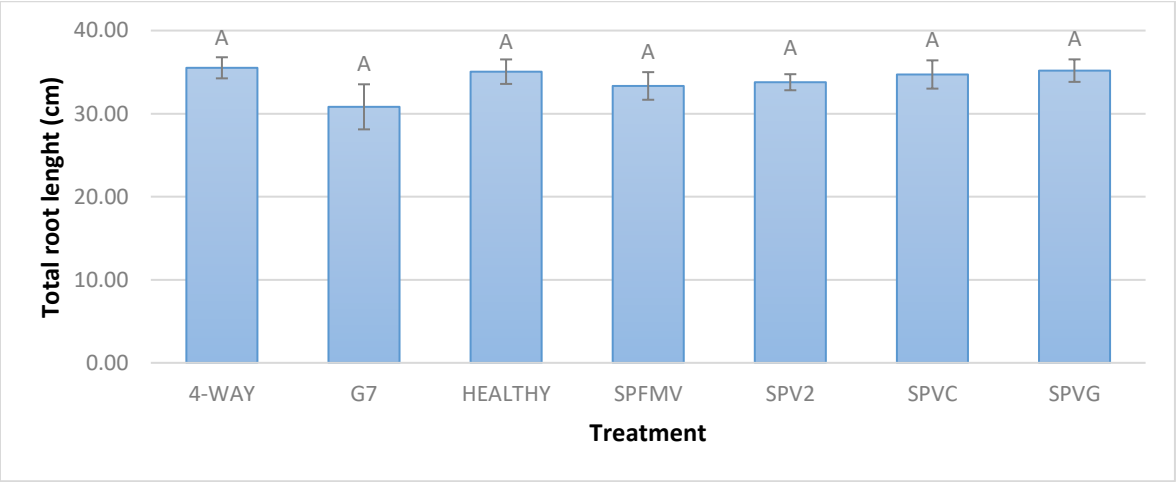

Figure 3.7. Length of undifferentiated roots (in cm) produced in a greenhouse by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

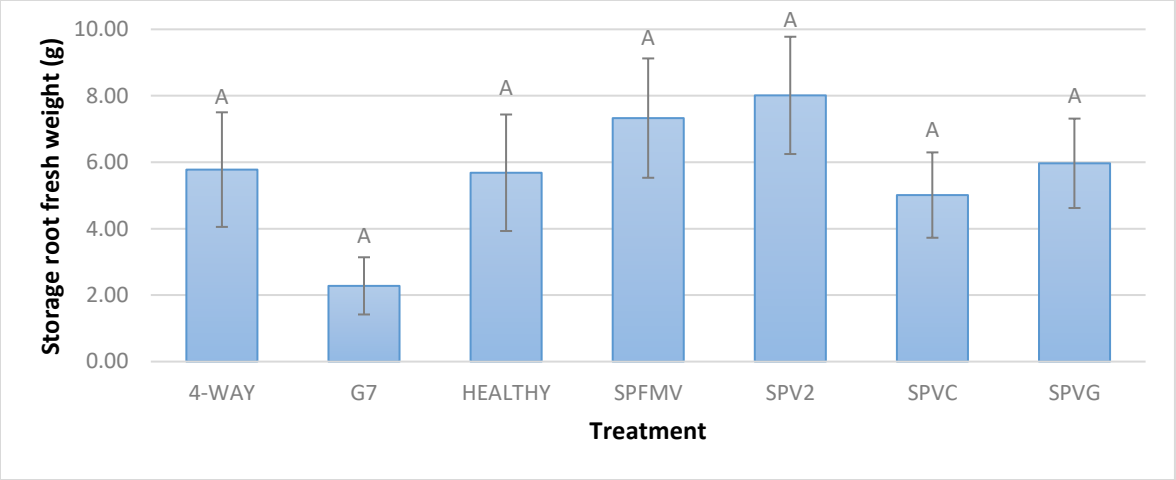

Figure 3.8. Fresh weight of storage roots (in grams) produced by 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), a plant infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

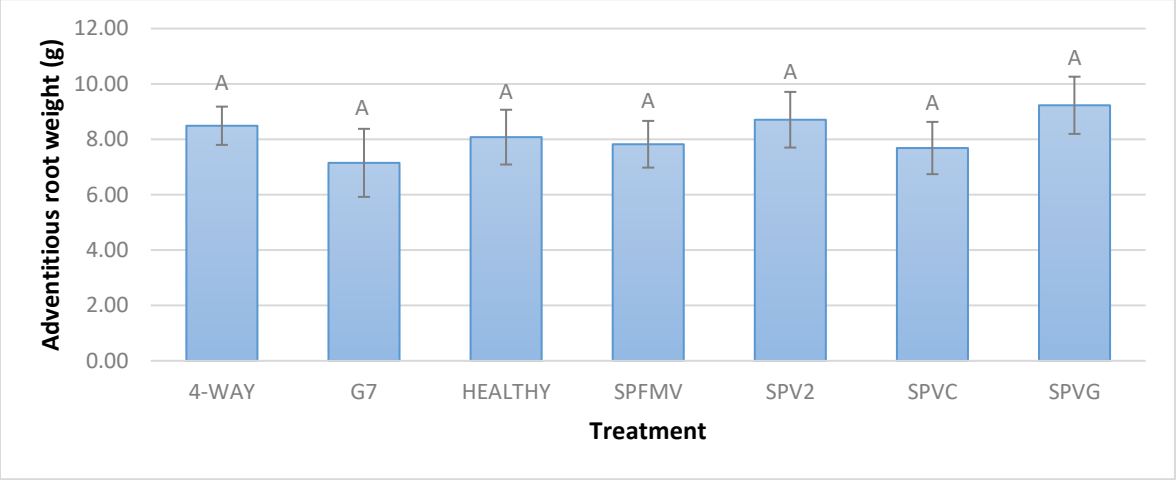

Figure 3.9. Undiferentiated root fresh weight (in grams) produced in greenhouse 'Beauregard' sweetpotato plants infected with different potyviruses. *Sweetpotato virus G* (SPVG), *Sweetpotato virus C* (SPVC), *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus 2* (SPV2), plants infected with all four potyvirus isolates (4-way), a naturally infected sweetpotato 'Beauregard' propagated during seven generations which had the four potyvirus species (G7) and a virus-tested sweetpotato 'Beauregard'. Bars with a common letter are not significantly different by ANOVA ($p<0.05$). Error bars = 1 standard deviation.

3.4 Discussion

Reduction of yield is the most common result of potyvirus infection in sweetpotato production (Clark *et al*., 2002). Previously, under field conditions, the amount of yield loss observed for plants artificially inoculated with SPFMV, SPVG, and SPV2 was as great as for plants naturally infected in the field after years of exposure (Clark and Hoy, 2006; Valverde *et al*., 2007). Since yield decline has been attributed to other factors like mutations or additional pathogens (Villordon and Labonte, 1995; Bryan *et al*., 2003), the discrepancy between yield reductions in naturally and artificially infected plants suggests additional factors should be considered. One of these factors is SPVC, which was described as a different species and appears to have a high replication rate in the sweetpotato plant (Untiveros *et al*., 2010; Gu *et al*., 2014). While attempting to determine if SPVC was the missing causal factor in the amount of yield reduction observed by naturally infected plants, it was observed that the infection from the four potyviruses is statistically similar to the plants in the field, based on storage root number (Fig. 3.1) but the rest of the variables analyzed do not recreate the trend where virus-tested plants yielded more than single infections and these more than mixed infections (Clark and Hoy, 2006), which suggests that SPVC alone is not the critical missing element. At the same time, data suggests that there are no statistical differences among other possible factors in root characteristics that could correlate to the observed differences in the field (Fig. 3.3 to 3.8). However, naturally infected plants have conspicuous symptoms of potyvirus infection, higher viral titers, and potyviruses are transmitted more frequently by different aphid species from these source plants compared to artificially inoculated plants (Kokkinos *et al*., 2006; Wosula *et al*., 2012). These phenomena were not replicated when SPVC was added into the potyvirus infection. In this study, symptoms appear to be more conspicuous on the naturally infected plants than on the plants artificially inoculated with isolates of SPFMV, SPVC, SPVG, and SPV2 combined. Relative viral titers also were greater in naturally infected plants (Herrera; Chapter 2).

Storage root formation is associated with the appearance of anomalous cambia around the central metaxylem cells, protoxylem arms and secondary xylem (Firon *et al*., 2009). Under optimal conditions, ‘Beauregard’ sweetpotato storage root initiation can be observed as early as 13 days after transplanting (Villordon *et al*., 2009). However, despite maintaining plants under greenhouse-controlled conditions, single infections or virus-tested plants had no statistical difference in storage root number compared to naturally infected plants, with the exception of SPV2-infected plants producing more roots than naturally infected plants. This result differs from previous field experiments where virus-tested plants yielded more than mixed infections under field conditions (Clark and Hoy, 2006). These differences could be attributed to the space restriction imposed by the PVC pipes. These space limitations may prevent unimpeded storage root differentiation (Villordon *et al*., 2017) and impede development of a root ecosystem in which plants with deeper and abundant roots improve soil structure, water and nutrient retention, and sustainable plant yields (Kell, D.; 2011). However, the total root mass did not suggest differences between artificial inoculations and naturally infected plants on the approach of using PVC pipes.

In ‘Beauregard’ sweetpotato, naturally infected plants and virus tested plants displayed statistical differences in adventitious root number when nitrogen was not applied compared to complete fertilization. However, the other variables analyzed (lateral root length, lateral root number or lateral root density) did not show a statistical difference among virus infections but

they did when the nitrogen variable was included. This suggested that in the interaction between nitrogen fertilization and virus inoculations, nitrogen is more involved in differences in root architecture than virus inoculations under greenhouse conditions (Villordon and Clark, 2014). That could explain why differences in storage root architecture were not detected, since viral infections alone appear to not cause statistical differences in the rest of variables measured except in storage root number. Despite the addition of Hoagland's solution, nutrient availability may have been limited by the use of sand that allows rapid movement of nutrients through the rooting zone (Villordon *et al*., 2012) compared to the most common type of soils in Louisiana – silt loam soils (Edmunds *et al*., 2008). Sand also differs in other soil parameters like organic matter and microbial populations that could affect recycling of nitrogen or phosphorus (Hooper and Vitousek, 1998). The addition of Hoagland's solution to the autoclaved sand, supplied minor nutrients that have not been studied in terms of their effects on roots and they are not usually applied in field cultivation. They may also have detrimental effects on plant development if supplied at toxic concentrations or if they are deficient (O'Sullivan *et al*., 1997). Despite the evidence, Hoagland's solution has been a standard for controlled experiments in different crops (Shipley and Meziane, 2002; Koca *et al*., 2007; Zhao *et al*., 2005).

One difficulty in studying the effects of single potyvirus infections in sweetpotato is the observation that 'reversion', or apparent loss of the virus, sometimes occurs in some cultivars (Gibson *et al*., 2013). Reversion was not detected in the viral inoculations of the four potyviruses which remained stable through the experiment. In particular, SPVC relative titers remained constant during the different storage root development stages and different plant organs analyzed (Herrera; Chapter 2). However, the fact that naturally infected plants could be infected with an unknown virus should be considered (Clark *et al*., 2012; Wosula *et al*., 2012). More than 30 viruses have been reported from sweetpotato, including the recently described Sweet potato pakakuy virus (SPPV), a virus composed of Sweetpotato badnavirus A and B (Mbanzibwa *et al*., 2014; Kreuze *et al*., 2009). The virus-tested plants used in this study did not show virus symptoms and were previously tested and found to be apparently free of other known sweetpotato viruses (*Sweet potato mild mottle virus, Sweet potato latent virus, Sweet potato chlorotic fleck virus, Sweet potato mild speckling virus, Sweet potato leaf curl virus, Sweet potato chlorotic stunt virus, Sweet potato collusive virus,* and *Cucumber mosaic virus*). The naturally infected plants have shown amplifications of badnavirus sequences under initial PCR screening of reverse transcriptase genes of both A and B regions, however, to date they remain poorly studied in terms of their effects in sweetpotato (Herrera, data not shown). A study suggested that SPPV is wide spread in sweetpotato plants in mixed infections with Sweet potato symptomless mastrevirus 1 (SPSMV-1) in Tanzania (Mbanzibwa *et al*., 2014) and that it is commonly found in sweetpotato landraces (Mbanzibwa *et al*., 2011). Since no means of horizontal transmission has been determined for SPPV, it is not yet clear whether it is truly a transmissible virus or if remnant DNA sequences are present in the host genome. Additional research is needed to clarify this critical aspect, and if it is determined to be a transmissible virus, to then determine its biological effects on sweetpotato alone and when co-infecting with other common viruses.

Plant hormones could mediate some of the effects observed in this study. Storage root initiation results from development of cambia around the protoxylem and secondary xylem elements (Villordon *et al*., 2009). The differentiation among root tissues has been associated with internal cues such as auxins, ethylene, abscisic acid, cytokinin and strigolactones

(Ivanchenko *et al*., 2008; Koltai, 2011). Possibly an unknown virus is associated with differential expression of a plant hormone involved in storage root formation. This is supported by a previous study in which SPVD infection resulted in apparent down regulation of gibberellin-regulated protein 5 (GASA5) (Kokkinos *et al*., 2006). Future studies using quantitative PCR of the GASA5 gene associated with stem growth and flowering (Zhang *et al*., 2009), and the amount of foliar and canopy production of naturally infected and virus-tested plants could lead to a better understanding of whether the viruses might modify the sweetpotato plant canopy.

Difference in viral titers between greenhouse and field experiments have been reported in sweetpotato plants infected with SPVG, SPV2 and SPFMV (Kokkinos *et al*., 2006). The ability to replicate faster or to stay at low titers to avoid competition in a virus population, has been demonstrated as a key advantage in viral survival (Elena *et al*., 2014). For example, different potyvirus species have different rates of spread in sweetpotato fields, which is possibly related to viral titer in the source plants that in turn affects acquisition by aphid vectors (Wosula *et al*., 2012). Thus, SPFMV is more commonly detected in the field than SPVG, SPV2, or SPVC (Wosula *et al*., 2012a and b). Four potyviruses replicating simultaneously in the same plant may require time for the each virus to reach a steady state titer, or the relative proportions of the viruses may vary over time as individual viruses go through cycles of increased or decreased rates of replication. Thus, naturally infected plants in which the viruses have been replicating for years may differ from plants where the four viruses were only recently introduced. Conceivably, plants may therefore perform differently even when infected with the same complement of viruses. Future studies of how viral titers differ during different generations of vegetatively propagated plants could help to understand the observed effects in the field or if one of them is prone to overcome the others.

SPFMV appears to be a more diverse species than the rest of the other potyviruses used in this study. Different strains of SPFMV have been reported around the world such as the ordinary strain, russet-crack strain (Yamasaki *et al*., 2009) and the East African strain (Gibson *et al*., 2009; Untiveros *et al*., 2008; Untiveros *et al*., 2010). This could be the reason why, despite the efforts to utilize isolates originated in Louisiana at the beginning of the study, SPFMV and SPV2 did not remain stable in the sweetpotato plant and had to be replaced with isolates with a different place of origin. The assumption that the naturally infected plants are mixed with isolates that are not closely related enough at the genome level to the isolates used in artificial inoculations could be the reason that storage root number is severely affected in naturally infected plants. Strain-specific PCR indicated that naturally infected plants were infected with the russet-crack and ordinary strains of SPFMV and SPVC, as were the artificial inoculations (Fig. 3.2). Additionally, phylogenetic analysis of several isolates from the United States place them in the same clade as isolates from other parts of the world (Herrera; Chapter 4), which suggests that at least in the United States there is not enough genomic variation, at least with the studied isolates, to place the isolates used in this study as a different strains. Despite the failed attempt to separate SPFMV using different *Chenopodium* species, future studies of the effects of the different SPFMV strains could help to elucidate which one is more detrimental to the plant.

The combinations of potyviruses used in this study did not fully reproduce the effects on storage root production observed with naturally infected ‘Beauregard’ plants, and it appears that SPVC alone is not the missing element. Further research is still needed to identify the missing factor(s) to reconstruct the complex that causes cultivar decline in ‘Beauregard’ and other

cultivars of sweetpotato. Even though root development and architecture are considered drivers of yield in sweetpotato, virus titers were greater in leaves (Herrera; Chapter 2). Previous research found that SPFMV and SPCSV differentially affect photosynthetic genes (Kokkinos *et al*., 2006). This suggests that future experiments should focus on the effect of potyviruses in leaves and their correlation with sweetpotato roots. For example, analyzing modifications in chlorophyll production since in tobacco plants infected with *Cucumber mosaic virus*, chlorophyll fluorescence lifetime of chlorotic leaves was significantly shorter than the healthy control leaves (Lei *et al*., 2017). Measurement of the amount of foliar tissue produced in the canopy using remote monitoring technologies might reveal whether sweetpotato viruses have similar effects as those seen in wheat infected with *Wheat streak mosaic virus*, which appear to reduce root and shoot mass production reducing water intake to the plant (Mirik *et al*., 2012; Price *et al*., 2010).

CHAPTER 4: MOLECULAR CHARACTERIZATION OF *SWEETPOTATO FEATHERY MOTTLE VIRUS* AND *SWEETPOTATO VIRUS C* IN LOUISIANA

4.1 Introduction

Sweetpotato [*Ipomoea batatas* L. (Lam); *Convolvulaceae*] is the 7th most important commodity in the world (FAO, 2012). There are several important diseases that affect the crop, but one of the greatest concerns is cultivar decline, which results from accumulation of pathogens and mutations during vegetative propagation (Bryan *et al*., 2003; Villordon and Labonte, 1995). The main contributors to cultivar decline in the United States are potyviruses, (Clark and Hoy, 2006). Potyviruses belong to the family *Potyviridae* and the genus *Potyvirus*, where *Potato virus Y* is the representative species of this group (ICTV, 2012).

The potyviruses associated with sweetpotato have filamentous particles approximately 850 nm long, and they are vectored in a non-persistent manner by many aphid species (Wosula *et al*., 2012). They have a genome size ranging from 10,731 to 10,800 nucleotides (nt) excluding the 3' poly (A) tail (Li *et al*., 2012). The polyprotein is translated entirely and then it is cleaved in conserved locations producing 10 mature proteins (Adams *et al*., 2005). Sweetpotato potyviruses also have a restricted host range, affecting primarily plants in the *Convolvulaceae*, the 'morning glory' family.

The genome of potyviruses consists of several genes, ordered from the 5' end to the 3' end. They start with a 5' untranslated region (UTR), a large open reading frame (ORF) and a 3' UTR region. The ORF consists of 10 functional proteins: the P1 (proteinase), cleaves the polyprotein and is involved in host recognition. HC-pro is involved in aphid transmission, as well as proteinase for polyprotein processing. P3 and 6K1 have unknown functions but 6K1 is possibly involved in polyprotein replication. CI, is involved in viral replication, and RNA helicase is involved in unwinding of dsRNA and membrane attachment. The 6K2 has an unknown function but is possibly involved in polyprotein genome replication. The NIa-VPg, which serves in virus replication as a primer. The NIa-pro is involved in major aspects of polyprotein processing including producing the VPg which acts as a primer of the initial polyprotein. The Nib is involved in genome replication as an RNA-dependent RNA polymerase. Finally, the coat protein (CP) which encapsidates and protects the RNA and is involved in aphid transmission and cell-to-cell movement (Shukla *et al*., 1994; Salvador *et al*., 2008). In sweetpotato potyviruses, an extra open reading frame named Pretty Interesting Sweet Potato Potyvirus ORF (PISPO) is involved in RNA silencing (Chung *et al*., 2008; Mingot *et al*., 2016).

In the United States, four potyviruses: *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), *Sweet potato virus 2* (SPV2), and *Sweet potato virus C* (SPVC) ; have been documented to date (Clark *et al*., 2012). This group of viruses are graft transmissible and can be transmitted mechanically under artificially controlled conditions, but are not transmitted mechanically in the field or by seed (Loebenstein *et al*., 2009). Prior to the utilization of sequence data, species and strains were differentiated using host range, symptomatology, and serology (Adams *et al.,* 2005). However, with the information of the CP nucleotide data, SPFMV can be divided into three representative strains: russet crack (RC), ordinary (O) and East African (EA) (Abad *et al*., 1992; Kwak *et al*., 2007). Because of the low homology of the previously named Common (C) strain of SPFMV at the CP (Kreuze *et al*., 2000; Ateka *et al*., 2007) and the amino acid (aa) differences in the P1 gene, it was reclassified

as the distinct species, SPVC, by the International Committee on Taxonomy of Viruses (Untiveros *et al*., 2010).

These four potyviruses are genetically conserved in the C-terminal half of their CP gene which has been used to differentiate species (Li *et al*., 2012). However, there has been some debate about using the CP region since it only represents 10% of the genome (Boss, 1992; Zettler, 1992). The evidence of sequence differences in the P1 region (Untiveros *et al*., 2010) led to the necessity to study the full genome sequence and their respective genes to increase the knowledge about genetic structure, diversity, dispersion and emergence (Kwak *et al*., 2015). Since most of the sweetpotato plants are commonly infected with mixtures of several potyviruses (Valverde *et al*., 2007), the emergence of new viral strains as a product of genetic recombination likely contributed to the emergence of new positive-sense RNA viruses (Chare and Holmes, 2006) and may even create some isolates that are no longer detectable by some qPCR assays (Ha *et al*., 2008; Lan *et al*., 2017).

The objective of this study was to determine complete genome sequences of five SPFMV and four SPVC isolates collected from sweetpotato plants representative of several sweetpotato production fields in the southern United States. The genetic structure and variability of isolates present in Louisiana were compared to other isolates present in the world to better understand the evolutionary relationship among the isolates.

4.2 Materials and methods

4.2.1 Isolate preparation

Virus isolates from sweetpotato were collected from different locations in the United States, either directly from sweetpotato plants or from *Ipomoea setosa* sentinel plants placed in sweetpotato fields (Table 4.1; Souto *et al*., 2003; Wosula *et al*., 2012; Moyer and Kennedy 1978). Sweetpotato plants were grafted with seed propagated scions of *I. setosa,* and then mechanical transmissions were made from symptomatic *I. setosa* leaves into *Ipomoea nil* 'Scarlet O'Hara' (*SOH*). To separate SPFMV isolates, mechanical inoculations were made into *Chenopodium quinoa* and single local lesions produced after approximately 10 days were mechanically reinoculated back to SOH where they were maintained. To separate SPVC isolates, mechanical inoculations of leaf tissue was made in *Nicotiana benthamiana* and then re-inoculated back into SOH. Mechanical inoculations were conducted with a chilled mortar and pestle using 0.02M phosphate buffer, pH 7.2, amended with 0.1M sodium diethyl dithiocarbamate (DIECA) and rubbing the inoculum on Carborundum-dusted leaves. (Souto *et al*., 2003). All isolates were kept in Bugdorm rearing and observation cages (Bioequip products, CA) and renewed every three weeks.

Isolate separation was confirmed by RT-PCR (Li *et al*., 2012) prior to CTAB total RNA extraction (Li *et al*., 2008). A 100 mg sample of leaf tissue previously ground in liquid nitrogen using a mortar and pestle was mixed with 1ml of CTAB/beta-mercaptoethanol. Samples were placed at -20^{o}C for 15 min. Tissue and extraction buffer was homogenized using a Fastprep FP120 (Qbiogene, Inc.; North America) at 4.5 speed for 30 sec, cooled on ice for five min and homogenized again. Samples were incubated at 65^{o}C for 15 min in a water bath and centrifuged at 5,220 g in an Eppendorf 5415 microcentrifuge (Eppendorf, U.S.) for 5 min. 650 µl of the supernatant was mixed with 650 µl of 24:1 chloroform:isoamyl alcohol. Samples were mixed

using a vortex mixer and centrifuged at max speed for 10 min. 500 µl of the aqueous phase was mixed with 350 µl of isopropanol (2-propanol) and centrifuged again at max speed for 10 min. The supernatant was discarded and the pellet was washed twice with 500 µl of 70% ethanol after two high-speed centrifugations of two min. Finally, the pellet was dried, resuspended in 50 µl of 20 mM Tris-HCl pH 8.0 and dissolved on ice for 15 min. To eliminate RNAses, Invitrogen RNase Out (40 units/µl) (ThermoFisher) was added at 1 µl per 50 µl of extract.

Table 4.1. Origin of sweetpotato potyvirus isolates of sweetpotato used in this study.

Isolate	Virus Sequenced	Location of origin	Host of origin
95-2 04R	SPFMV	New Mexico	Sweetpotato 'Beauregard'
TFSW-1 J		North Louisiana	*Ipomoea setosa* sentinel[a]
11-8		North Louisiana	Sweetpotato
95-2T		New Mexico	Sweetpotato 'Beauregard'
11-8		North Louisiana	*I. setosa* sentinel[a]
95-6	SPVC	North Carolina	Sweetpotato 'Beauregard'
11-5		North Louisiana	*I. setosa* sentinel[a]
SPVC PR3		Burden Center, Baton Rouge	Sweetpotato 'Beauregard'
Moyer-C		North Carolina	Sweetpotato 'Beauregard'

[a] *Ipomoea setosa* sentinel plants were placed in or adjacent to commercial sweetpotato fields and became naturally infected presumably as a result of aphid transmission from sweetpotato source plants in the field.

4.2.2 Isolate sequencing

Total RNA preparations were purified from SOH leaf samples infected with SPFMV or SPVC and supplied to Dr. Kai-shu Ling, a collaborator at USDA-ARS in Charleston, SC. Using the small RNA sequencing and assembly technology (Kreuze *et al*., 2009, Li *et al*., 2012), small RNA (sRNA) libraries were prepared following the T4 RNA ligase 1 adenylated adapters method as described by Chen *et al*., 2012. The bar-coded, small RNA libraries were pooled and sequenced using an Illumina HiSeq 2000 (Li *et al*., 2012). For virus identification, sRNA sequences were assembled and analyzed using the VirusDetect program (Zheng *et al*., 2017). Any sequence gaps were filled with RT-PCR flanking primers. A brief description of Illumina results are provided in Table 4.2.

Table 4.2. Summary of outputs from Deep sequencing sRNA on *Ipomoea nil* (SOH) leaf samples.

Library	Possible Viruses	Barcode	Total raw reads	Final clean read	
				reads	% of raw
KLL145 (Moyer-C)	SPFMV, SPVC	AAGCGC	1,628,525	1,059,251	65.04
KLL146 (95-6)	SPFMV, SPVC	AACAGA	2,679,219	516,084	19.26
KLL148 (11-5)	SPFMV, SPVC	GAACGT	2,306,331	1,674,988	72.63
KLL149 (SPVC PR3)	SPFMV, SPVC	GAATCA	2,271,380	1,426,038	62.78
KLL150 (95-2 04R)	SPFMV	GAGACT	1,931,074	1,280,120	66.29
KLL151 (95-2T)	SPFMV	AACGAC	12,083,368	9,017,879	74.63
KLL153 (11-1)	SPFMV	AACTCT	2,242,799	1,881,974	83.91
KLL154 (11-8)	SPFMV	AATACC	1,653,418	702,756	42.5
KLL155 (Healthy)	None	GAGGTC	1,803,531	1,000,804	55.49
KLL156 (TFSW1-J)	SPFMV	GAGTGG	1,477,150	1,080,203	73.13

4.2.3 Completion of viral genome sequences

Each potyvirus isolate was partially purified from infected SOH using polyethylene glycol (PEG) precipitation to determine the exact 5' terminal sequence of the VP-g linked viral RNA for potyviruses (Jones *et al*., 1980). Plant leaves and stems were chopped with a razor blade and weighed. The leaf tissue (2 g) was ground (1:3 w:w) with buffer containing 0.0065 M disodium tetraborate, 0.435 M boric acid, 0.2% ascorbic acid and 0.2% sodium sulphite at pH 7.8. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 5000 g for 20 min in a Beckman Coulter Avanti J-25 centrifuge. The supernatant was collected and

silver nitrate was added to 0.4%, mixed, and allowed to stand at room temperature for one hr. The mixture was centrifuged again at 5,000 g for 20 min. PEG was added to the supernatant to 4% (w:v) and mixed slowly at 4^{o}C for one hr. Samples were centrifuged again at 5,000 g for 20 min and the pellet was collected and re-suspended with buffer containing 0.065 M disodium tetraborate, 0.435 M boric acid, 0.5 M urea and 0.1% mercaptoethanol at pH 7.8. Samples were stirred for eight hrs at 4^{o}C and then centrifuged at 5,000 g for 20 min. The pellet was discarded and the supernatant was centrifuged at 64,000 g for 70 min. The supernatant was discarded and the pellet was re-suspended in buffer with 0.01 M tris-HCl buffer pH 8.0 and stirred for one hr at 4^{o}C.

The 5' terminal sequence was obtained through rapid amplification of cDNA ends (RACE) using a 5'/3' RACE kit (2nd generation ROCHE; Sigma, St Louis, MO, U.S.) following manufacturer instructions. Primers from the known contigs were designed to obtain fragments that can be sequenced by Sanger sequencing. After amplification of the correct PCR fragment and size tested by agarose gel electrophoresis, eight different PCR amplification samples per isolate were sequenced with their respective forward and reverse primers used in that PCR reaction using ABI3730XL Sanger sequencer (Macrogen, MD, U.S.). Fragments were assembled *de-novo* using Geneious (Biomatters Limited, NZ), checking for quality scores (QS) > 30.

Libraries were completed and assembled with DNASTAR (Lasergene 13) using a referenced based approach. A BLAST analysis (nBLAST, NCBI) of the complete genomic sequence and deduced polyprotein sequences (ORF finder, NCBI) available in GenBank was done to corroborate the completion of the sequences of each one of the isolates. Based on the molecular biology of potyviruses (Adams *et al*., 2010), a complete assembly was considered if the whole nucleotide sequence produced a single polyprotein and their respective mature proteins.

4.2.4. Sequence comparison

The complete nucleotide sequences and deduced amino acid sequences were aligned using MUSCLE algorithm and the percentage of sequence similarity of nucleotide and amino acid data was obtained using Sequence demarcation tool Version 1.2 (Muhire *et al*., 2014). Pairwise sequence comparison analysis with previously reported isolates of SPFMV and SPVC were analyzed (Table 4.3). Outgroups of SPVG, SPV2 and SPLV were used since they have similar genome composition as SPFMV and SPVC.

Table 4.3. Full Genome accession numbers of *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato virus C* (SPVC), *Sweetpotato virus G* (SPVG), *Sweetpotato virus 2* (SPV2), and *Sweetpotato latent virus* (SPLV) used in the phylogeny analysis. Isolates obtained in this study are shown in boldface. Louisiana State University (LSU)

Species	Isolate Name	Accession Number	Location	Citation
SPVC	**PR3**		**LSU**	**This study**
	95-6		**North Carolina**	**This study**
	11-5		**North LA**	**This study**
	Moyer C		**North LA**	**Moyer and Kennedy, 1978**
	Bungo	AB509453	Japan	Yamasaki *et al*. 2010
	Argentina	KF386015	Argentina	Bejerman *et al*. 2015
	IL	JX489166	Israel	Prakash *et al.* 2012
	China 1	KU877879	China	Not found
	C1	GU207957	Peru	Untiveros *et al*. 2010
SPFMV	**95-2T**		**Derived 95-2 04R**	**This study**
	95-2 04R		**New Mexico, US**	**This study**
	11-1		**North Louisiana**	**This study**
	TFSW-1 J		**North Louisiana**	**This study**
	11-8		**North Louisiana**	**This study**
	17-0	AB509454	Korea	Yamasaki *et al*. 2010
	Ordinary	AB465608	Japan	Yamasaki *et al*. 2010
	10-O	AB439206	Japan	Yamasaki *et al*. 2010
	O	KF386013	Argentina	Bejerman *et al*. 2015
	GJ122	KP115609	Korea	Kwak *et al*. 2015
	RUK73	KP729265	Uganda	Tugume *et al*. 2010
	Piu 3	FJ155666	Peru	Untiveros *et al*. 2010
	IS90	KP115610	Korea	Kwak *et al*. 2015
	CW137	KP115608	Korea	Kwak *et al*. 2015
	RC-Arg	KF386014	Argentina	Bejerman *et al*. 2015
	Severe	D86371	Japan	Yamasaki *et al*. 2010
SPVG	GWBG	JN613805	USA	Li *et al*. 2012
	Z01001	JN613806	South Korea	Li *et al*. 2012
SPV2	AUSCAN	KX017448	Australia	Maina *et al*. 2016
	GWB2	JN613807	USA	Li *et al*. 2012
SPLV	TW	KC443039	Taiwan	Wang *et al*. 2013
	HG181	KP115611	Korea	

4.2.5 Phylogenetic analysis

Nucleotide sequence alignments were estimated in MEGA7 (http://www.megasoftware.net/; Tamura *et al*., 2013) using the Muscle (Edgar, 2004) algorithm and MAFFT version 7 (Katoh and Standley, 2013) specifying a G-INS-I iterative refinement method and a 200PAM/K=2 scoring matrix or BLOSUM62. Alignments were considered for nucleotide and amino acid sequences for both the 5' UTR, the mature proteins produced by the polyprotein and the 3' UTR described in potyviruses (Adams *et al*., 2005).

The best-fit nucleotide substitution model was selected according to corrected Aikake's Information Criterion (AICc) with JModelTest 2.0 version 0.01.1 (Darriba and Posada, 2014) for for nucleotide analysis as well as ProtTest version 2.4 (Abascal *et al*., 2009) with AICc for the amino acid analysis. The best-fit model was chosen among a candidate set of 203 models according to AICc implemented in jModelTest and 66 models according to AICc in ProtTest. Maximum likelihood analyses were conducted in RAxML (Stamatakis, 2016) for protein data (raxml-PTHREADS -n tre -s infile -x 1234 -N 1000 -k -p 1234 -f a –m bestmodel) and Garli v2.01 (Zwickl, 2006) for nucleotide data (Table 4.4).

Each tree was constructed using the resources at the Louisiana State University high-performance computing center (http://www.hpc.lsu.edu). The maximum likelihood tree was generated by stepwise addition with 100 search replicates. Bootstrap proportions were estimated from a minimum of 1,000 pseudo replicate datasets, with the highest likelihood tree from two replicate searches per pseudo replicate dataset retained. Bootstrap proportions were calculated and mapped onto the maximum-likelihood phylogenetic trees using SumTrees in the Dendropy v3.12.0 phylogenetic computing library (Sukumaran and Holder, 2010).

Trees were visualized using the graphical representations of phylograms in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Full genomes of SPFMV, SPVC and six outgroups of SPVG, SPV2 and *Sweetpotato latent virus* (SPLV) were used in the analysis (Table 4.3).

4.2.6 Recombination analysis

SPFMV and SPVC full-length genome sequences (25 sequences, Table 4.3), were analyzed. Previously aligned sequences were uploaded to RDP4 v4.55 software (Martin *et al*., 2015) with default settings. Sequences were analyzed using the following algorithms: rdp (Martin and Rybicki, 2000), maxchi (Smith, 1992), geneconv (Padidam *et al*., 1999), SiScan (Gibbs *et al*., 2000), chimaera (Posada and Crandall, 2001), bootscan (Salminen *et al*., 1995), and 3seq (Boni *et al*., 2007). The P value was set as 0.05 and results for the isolates are summarized (Table 4.7).

Table 4.4. Best-fit models of sequence evolution based on ProtTest v2.4 (amino acid, 66 models tested) and JModelTest v0.01.1 (nucleotide, 203 models tested). Models were selected using the AICc criterion. Evolutionary models: JTT (Jones *et al*., 1992); LG (Le and Gascuel, 2008); GTR (Generalized time-reversible; Tavare, 1986); TIM2 (transitional model; Posada, 2003); HKY (Hasegawa *et al*., 1985). Rate of nucleotide change: I (proportion of invariable sites); G (gamma distributed rate variation among sites); F (unequal frequency model) (Darriba and Posada, 2014).

Potyvirus Viral Gene/Region	Protein Model (ProtTest)	Nucleotide Model (JModelTest)
5' UTR	-----	HKY +G
3' UTR	-----	HKY +G
Complete sequence	JTT +I +G	GTR +I +G +F
P1	JTT +I +G	GRT +G +F
HC-Pro	LG +G	GTR +I +G +F
P3	LG +G	GTR +I +G +F
6K1	JTT	HKY +G
CI	LG +G	GRT +G +F
6K2	LG	HKY +G
NIa-VPg	LG +G	TIM2 +G
NIa-Pro	LG +G	GRT +G +F
NIb	LG +G	GTR +I +G +F
CP	JTT +I +G	TIM2 +I +G

4.3 Results

The full-length genome sequences of five SPFMV and four SPVC were obtained from sweetpotato or *I. setosa* plants separated at species level using mechanical inoculations in *C. quinoa* or *N. benthamiana* (Souto *et al*., 2003). These isolates were representative of sweetpotato production fields in the United States (Table 4.3). Their complete genome ranged from 10,793 to 10,830 nt (3481 aa) for SPVC isolates and 10,819 to 10,820 nt (3493aa) for SPFMV isolates. Genome organization was typical of previously reported potyviruses in sweetpotato and the obtained sequences produced the potential 10 proteins. 5' UTR ranged from 125 to 160 nt (SPVC) and 117 nt (SPFMV). P1 ranged from 654 aa (SPVC) and 664 aa (SPFMV). The sizes of HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, and NIb were 458, 352, 52, 643, 53, 192, 253, 521 aa respectively. The CP aa size was 313 aa (SPVC) and 315 aa (SPFMV). Finally, the 3' UTR was 222 nt (SPVC) and ranged from 221 to 222 nt (SPFMV). Additionally, all isolates presented the previously reported Pretty Interesting Potyviridae ORF (PIPO) located in the P3 region (Chung *et al*., 2008) and the G_2A_6 motif, which belongs to the Pretty Interesting Sweetpotato Potyvirus ORF (PISPO) at the P1 region (Clark *et al*., 2012). Both PIPO and PISPO are produced by ribosomal frameshift and have been described to be involved in RNAi silencing processes (Chung *et al*., 2008; Olspert *et al*., 2016; Untiveros *et al*., 2016).

Pairwise sequence comparison of complete nucleotide and amino acid sequences associated the four sequences of SPVC (Moyer-C, 95-6, PR3 and 11-5) as similar to the isolates

Israel, Bungo and Peru with sequence similarities ranging from 94 to 99%. One of the SPFMV isolates (95-2T) was highly similar to the ordinary strains of SPFMV with 96% sequence similarity and the other four isolates (11-1, 11-8, TFSW-1 J, 95-04R) were similar to russet-crack strains ranging from 90 to 98 % sequence similarity (Table 4.5; numbers: 1, 9, 10, 11, 14 - SPFMV and 19, 21, 22, 24 -SPVC). Similar association results were obtained from the amino acid pairwise sequence comparisons of the different potential proteins deduced from the completed sequences of the isolates used in the present study (Table 4.6).

The complete nucleotide and amino acid sequence phylogenetic analysis using previously reported isolates on NCBI (Table 4.3) indicated similar association to pairwise sequence comparisons locating the SPVC and SPFMV isolates as monophyletic to previously reported SPVC/SPFMV isolates (Fig. 4.1 to 4.12). Additionally, SPFMV phylogeny has been reported with two-within-virus species phylogroup classification (Jones and Kehoe, 2016). The first is based on differences of biological characteristics or region of the world where each isolate originated and the second using a neutral nomenclature to avoid potentially misleading names based on biology or geography. The major phylogroup A comprised two minor phylogroups EA (I) and O (II) strains of SPFMV and major phylogroup B, the RC strains of SPFMV. Based on the phylogroup classification, simmilar tree topologies between nucleotide and amino acid data were observed in trees from genes of P1, HC-pro, CI, Vpg, NIa and NIb but not from P3, 6K1, 6K2 and CP (Fig. 4.2 to 4.12). Greater number of subtitutions were estimated in trees from the CP nucleotide data compared to amino acid data in isolate 11-1 of SPFMV (Table 4.11).

To examine whether recombination occurred in the sequenced potyviruses, 24 full-length sequences (16 from SPFMV and 9 sequences from SPVC) were analyzed in RDP4 using seven of the default algorithms (Table 4.7). In total 30 recombination events were detected, however only 16 of them were detected by more than 3 algorithms and out of those 16, for recombination events # 6, 9, 11 and 13 both major and minor parental sequences were determined. These results place the rest of the reported events as ‘tentative’ since they were supported by less than three methods or one of the parents is unknown. For the isolates completed in this study, a majority of the recombination events were reported in the P1 and CP regions and were reported commonly for isolates of SPFMV 95-2T, 11-1, 11-8 and SPVC isolates 11-5, PR3, 95-6.

Table 4.5. Pairwise sequence comparison of complete nucleotide sequences (% identity). *Sweetpotato feathery mottle virus* (SPFVM), *Sweetpotato virus C* (SPVC), *Sweetpotato virus G* (SPVG), *Sweetpotato virus 2* (SPV2), *Sweetpotato latent virus* (SPLV). O: ordinary strain, EA: East-African strain, RC: russet-crack strain.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	SPFMV_952T_Louisiana	100																														
2	SPFMVO_170_Korea_AB509454	96	100																													
3	SPFMVO_Ordinary_Japan_AB465608	96	99	100																												
4	SPFMVO_O_Argentina_KF386013	96	99	99	100																											
5	SPPFMVO_10O_Japan_AB439206	98	98	97	97	100																										
6	SPFMVO_GJ122_Korea_KP115609	97	97	97	97	98	100																									
7	SPFMVEA_Ruk73_Uganda_KP729265	94	94	94	94	93	93	100																								
8	SPFMVEA_Piu3_Peru_FJ155666	94	94	94	94	93	93	96	100																							
9	SPFMV_118_Louisiana	90	88	88	88	89	89	88	89	100																						
10	SPFMV_111_Louisiana	88	87	87	87	88	88	87	87	98	100																					
11	SPFMV_TFSW1_J_Louisiana	90	88	88	88	89	89	89	89	99	98	100																				
12	SPFMVRC_Severe_Japan_D86371	90	88	88	88	90	90	89	89	99	98	99	100																			
13	SPFMVRC_Cw137_Korea_KP115608	90	88	88	88	89	89	89	89	98	97	98	99	100																		
14	1SPFMV_95204R_Louisiana	90	88	88	88	89	89	89	89	98	96	98	98	98	100																	
15	SPFMVRC_IS90_Korea_KP115610	90	88	88	88	89	90	89	89	98	97	98	99	98	98	100																
16	SPFMVRC_SPFMVRCARg_Argentina_KF386014	90	88	88	88	89	89	89	89	98	97	98	98	98	98	99	100															
17	SPVC_China1_China_KU877879	73	73	73	73	73	73	73	74	73	72	73	73	73	73	73	73	100														
18	SPVC_IL_Israel_JX489166	73	73	73	73	73	73	73	73	73	72	73	73	73	73	73	73	99	100													
19	SPVC_PR3_Louisiana	74	73	73	73	73	73	73	73	73	72	73	73	73	73	73	73	99	99	100												
20	SPVC_Bungo_Japan_AB509453	74	73	73	73	73	73	73	74	73	72	73	73	73	73	73	73	99	99	98	100											
21	SPVC_115_Louisiana	74	73	73	73	74	73	73	74	73	72	73	73	73	73	73	73	98	98	98	99	100										
22	SPVC_956_North_Carolina	74	73	73	73	73	73	73	74	73	72	73	73	73	73	73	73	98	98	98	99	99	100									
23	SPVC_SPVCArg_Argentina_KF386015	73	73	73	73	73	73	73	74	73	73	73	73	73	73	74	73	95	94	94	95	94	94	100								
24	SPVC_MOYER_C_Louisiana	74	73	73	73	73	73	73	74	73	73	73	73	73	73	74	73	94	94	94	94	94	94	98	100							
25	SPVC_C1_Peru_GU207957	73	73	73	73	73	73	73	73	73	73	73	73	73	73	73	73	93	92	92	93	92	93	93	93	100						
26	SPVG_GWBG_US_JN613805	68	68	68	68	68	68	68	68	68	67	68	68	68	68	68	68	67	67	67	67	67	67	67	67	68	100					
27	SPVG_Z01001_US_JN613806	68	68	68	68	68	68	68	68	68	67	68	68	68	68	68	68	67	67	67	67	67	67	67	67	68	99	100				
28	SPV2_AuScan_Australia_KX017448	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	67	67	67	67	67	67	67	67	67	71	71	100			
29	SPV2_GWB2_US_JN613807	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	67	67	67	67	67	67	67	67	67	71	71	98	100		
30	SPLV_TW_Taiwan_KC443039	63	63	63	63	62	63	63	63	63	63	63	63	63	63	63	62	63	62	62	62	62	62	63	63	63	62	62	63	63	100	
31	SPLV_HG181_Korea_KC443039	63	63	63	63	62	63	63	63	63	63	63	63	63	63	63	62	63	62	62	62	62	62	63	63	63	62	62	63	63	100	100

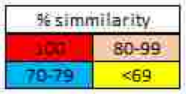

Table 4.6. Nucleotide and amino acid sequence identity (%) of the russet crack strain of SPFMV (95-04R) and other potyviruses reported to infect sweetpotato. *Sweetpotato feathery mottle virus* (SPFVM), *Sweetpotato virus C* (SPVC), *Sweetpotato virus G* (SPVG), *Sweetpotato virus 2* (SPV2), *Sweetpotato latent virus* (SPLV). O: ordinary strain, EA: East-African strain, RC: russet-crack strain.

	Full		5' UTR	P1	Hc-Pro	P3	6K1	CI	6K2	Vpg	Nia-Pro	NIb	CP	3' UTR
Virus Isolate	nt	aa	nt	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	nt
SPFMV_11-8_Louisiana	97	96	97	96	99	99	96	98	96	97	98	97	99	100
SPFMV_11-1_Louisiana	96	96	96	96	99	99	92	98	96	98	100	97	99	100
SPFMV_TFSW1_J_Louisiana	98	96	97	96	99	99	98	98	96	97	100	97	99	100
SPFMVRC_Severe_Japan_D86371	98	96	96	97	99	99	98	98	96	98	100	97	99	100
SPFMVRC_Cw137_Korea_KP115608	98	96	95	97	99	99	98	98	96	98	99	97	99	100
SPFMVRC_SPFMVRC-ARg_Argentina_KF386014	98	97	94	97	99	99	98	98	96	98	100	97	100	100
SPFMVRC_I590_Korea_KP115610	98	97	95	97	99	99	98	98	96	98	100	97	100	99
SPFMVEA_Ruk73_Uganda_KP729265	88	83	78	84	93	95	94	99	96	98	95	90	96	99
SPFMVEA_Piu3_Peru_FJ155666	88	82	76	82	93	95	96	99	98	99	95	90	96	99
SPFMVO_170_Korea_AB509454	90	83	80	84	93	96	94	99	96	98	96	88	96	97
SPFMVO_Ordinary_Japan_AB465608	89	83	80	84	93	95	94	99	96	98	96	87	96	97
SPFMVO_O_Argentina_KF386013	88	84	75	84	93	95	94	99	96	98	96	87	96	99
SPFMV_95-2T_Louisiana	89	89	96	89	93	96	96	99	98	99	96	88	97	99
SPPFMVO_10-O_Japan_AB439206	89	89	96	89	93	95	96	99	96	96	98	87	97	97
SPFMVO_GJ122_Korea_KP115609	89	89	95	89	93	96	96	98	94	98	98	88	98	98
SPVC_China1_China_KU877879	73	57	68	58	82	67	81	84	74	83	91	76	85	84
SPVC_IL_Israel_JX489166	73	56	68	57	82	68	81	84	72	83	91	76	85	83
SPVC_PR3_Louisiana	73	56	68	57	82	68	81	84	72	83	91	76	84	84
SPVC_Bungo_Japan_AB509453	73	56	72	57	82	67	81	84	74	83	91	76	84	84
SPVC_95-6_North_Carolina	73	56	71	57	82	67	79	84	74	83	91	76	85	84
SPVC_11-5_Louisiana	73	56	73	57	82	67	79	84	70	83	91	76	85	84
SPVC_SPVC-Arg_Argentina_KF386015	73	55	56	57	83	67	81	84	70	84	91	76	84	84
SPVC_MOYER_C_Louisiana	73	57	56	56	83	68	81	84	70	84	91	76	84	87
SPVC_C1_Peru_GU207957	73	56	69	55	82	67	79	85	70	83	91	76	83	83
SPVG_GWBG_US_JN613805	68	50	72	50	74	51	67	76	57	73	77	67	75	78
SPVG_Z01001_US_JN613806	68	50	72	50	74	51	67	76	57	73	77	67	75	78
SPV2_AuScan_Australia_KX017448	68	49	67	49	72	51	69	77	60	74	77	70	76	82
SPV2_GWB2_US_JN613807	68	49	68	49	72	51	69	76	57	74	77	70	73	82
SPLV_TW_Taiwan_KC443039	63	30	77	30	56	30	54	61	47	59	63	66	59	66
SPLV_HG181_Korea_KC443039	63	30	67	30	56	30	54	61	47	59	63	66	59	61

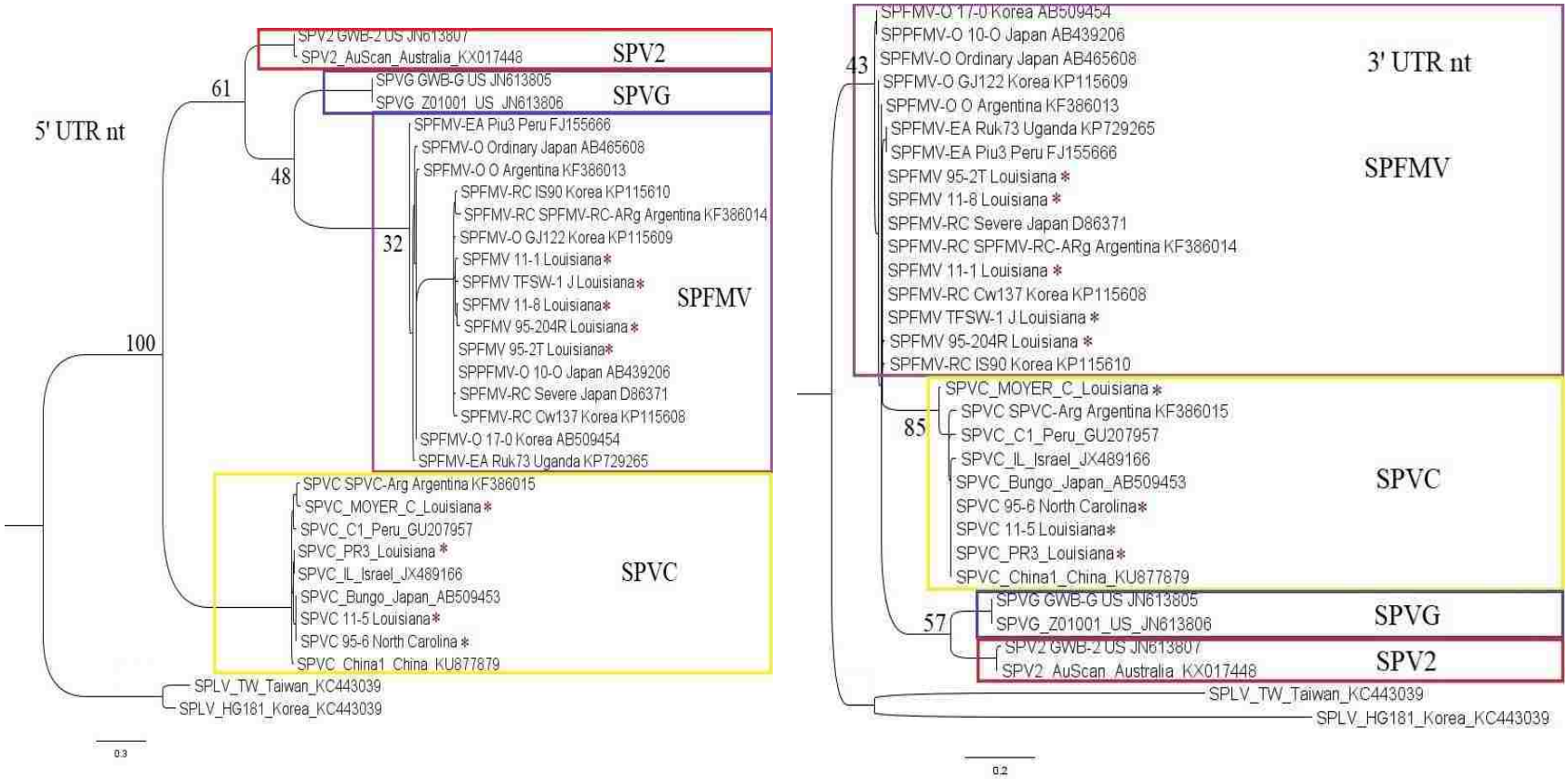

Figure 4.1. Maximum Likelihood Analysis of the 5' untranslated (UTR) (left) and 3' UTR (right) nucleotide (nt) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

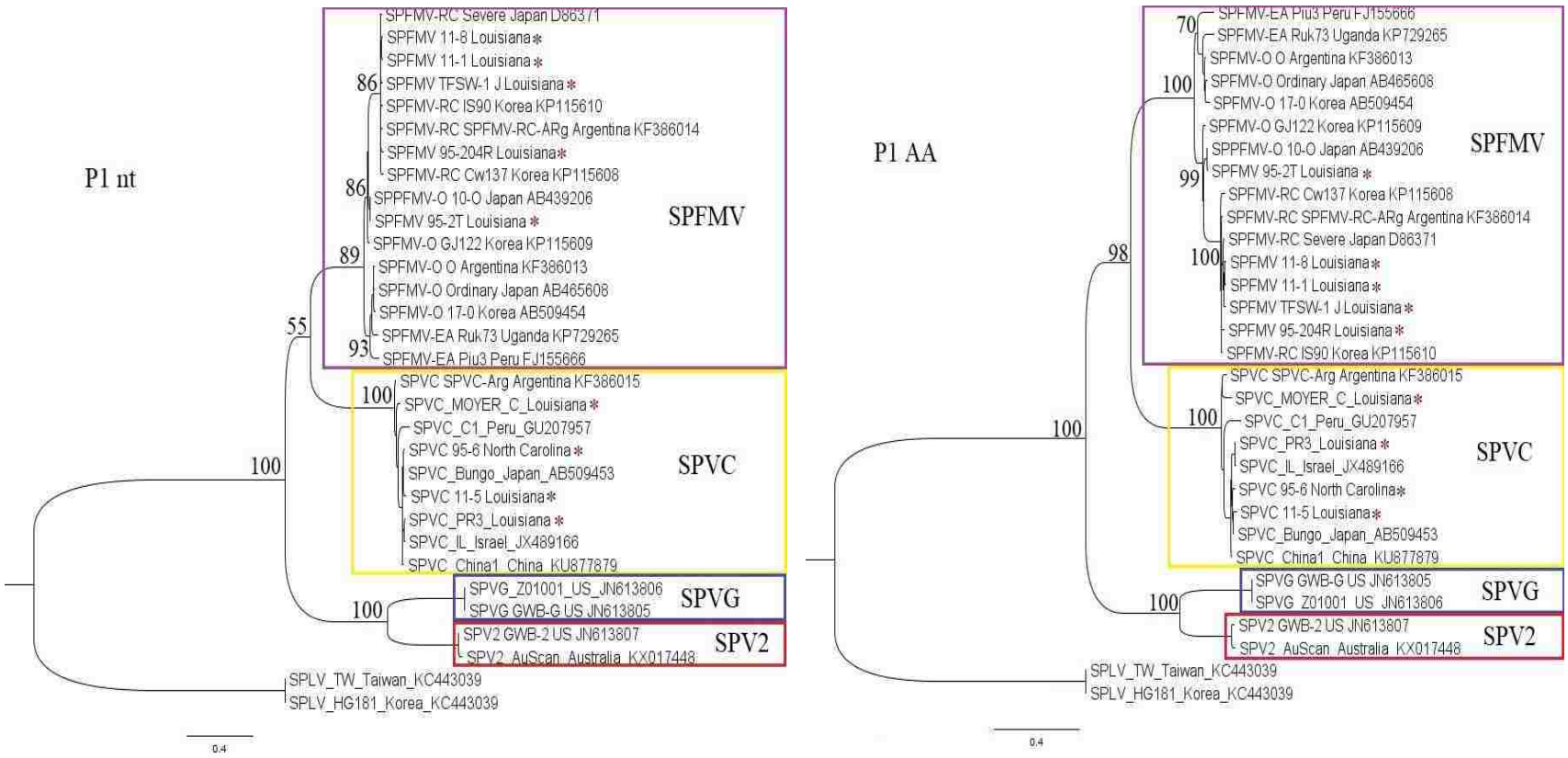

Figure 4.2. Maximum Likelihood Analysis of the P1 nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

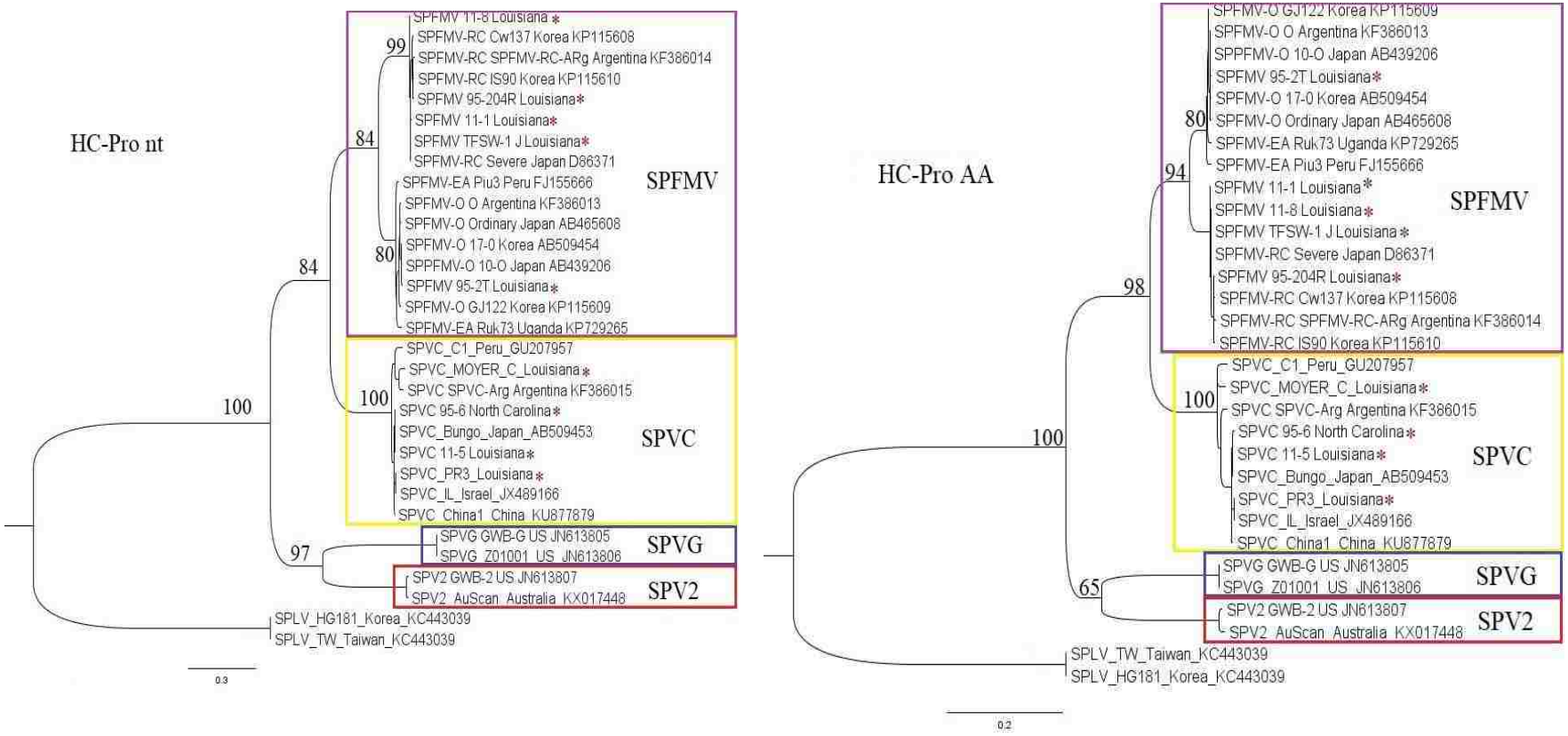

Figure 4.3. Maximum Likelihood Analysis of the HC-pro nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

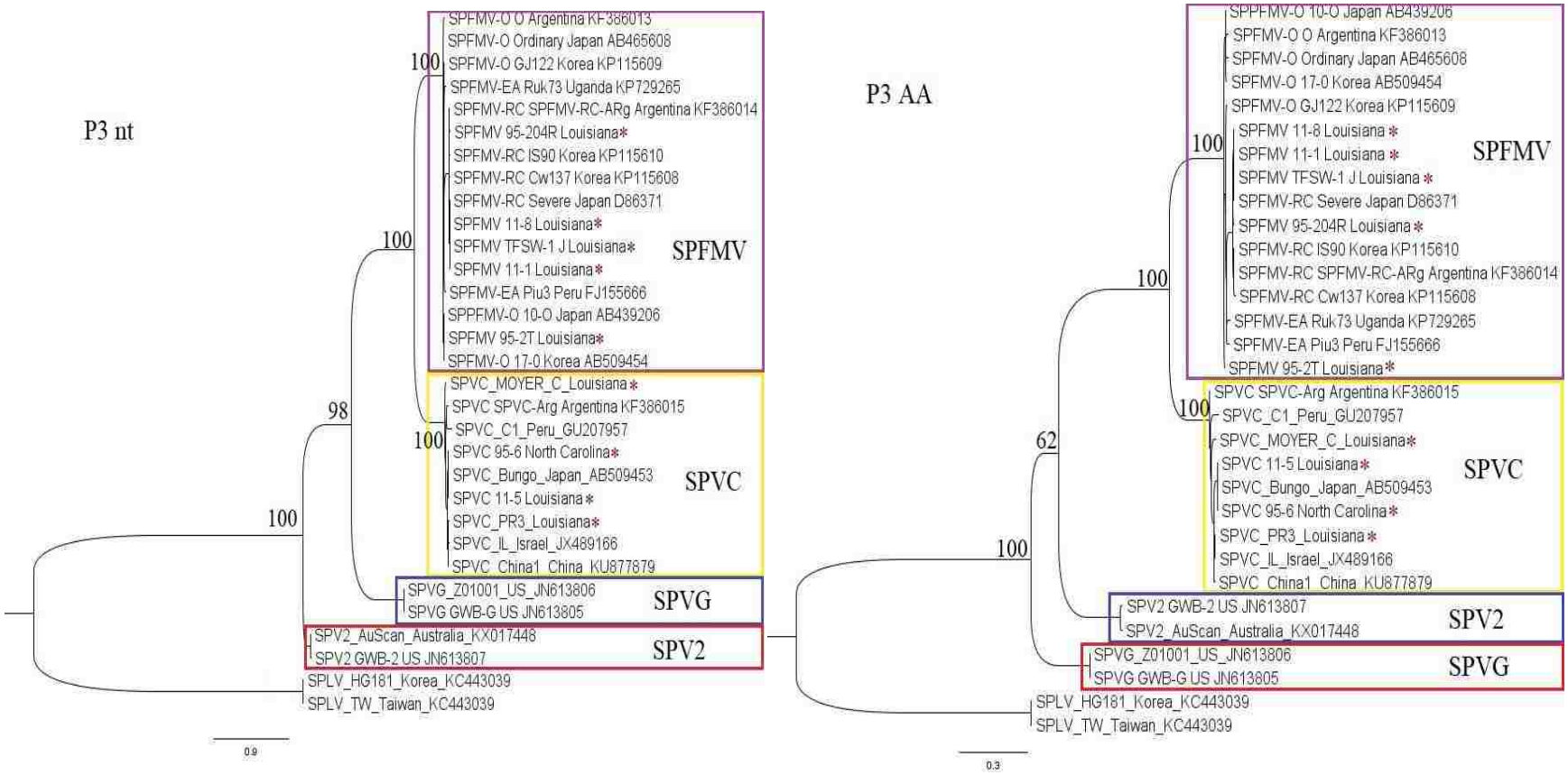

Figure 4.4. Maximum Likelihood Analysis of the P3 nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

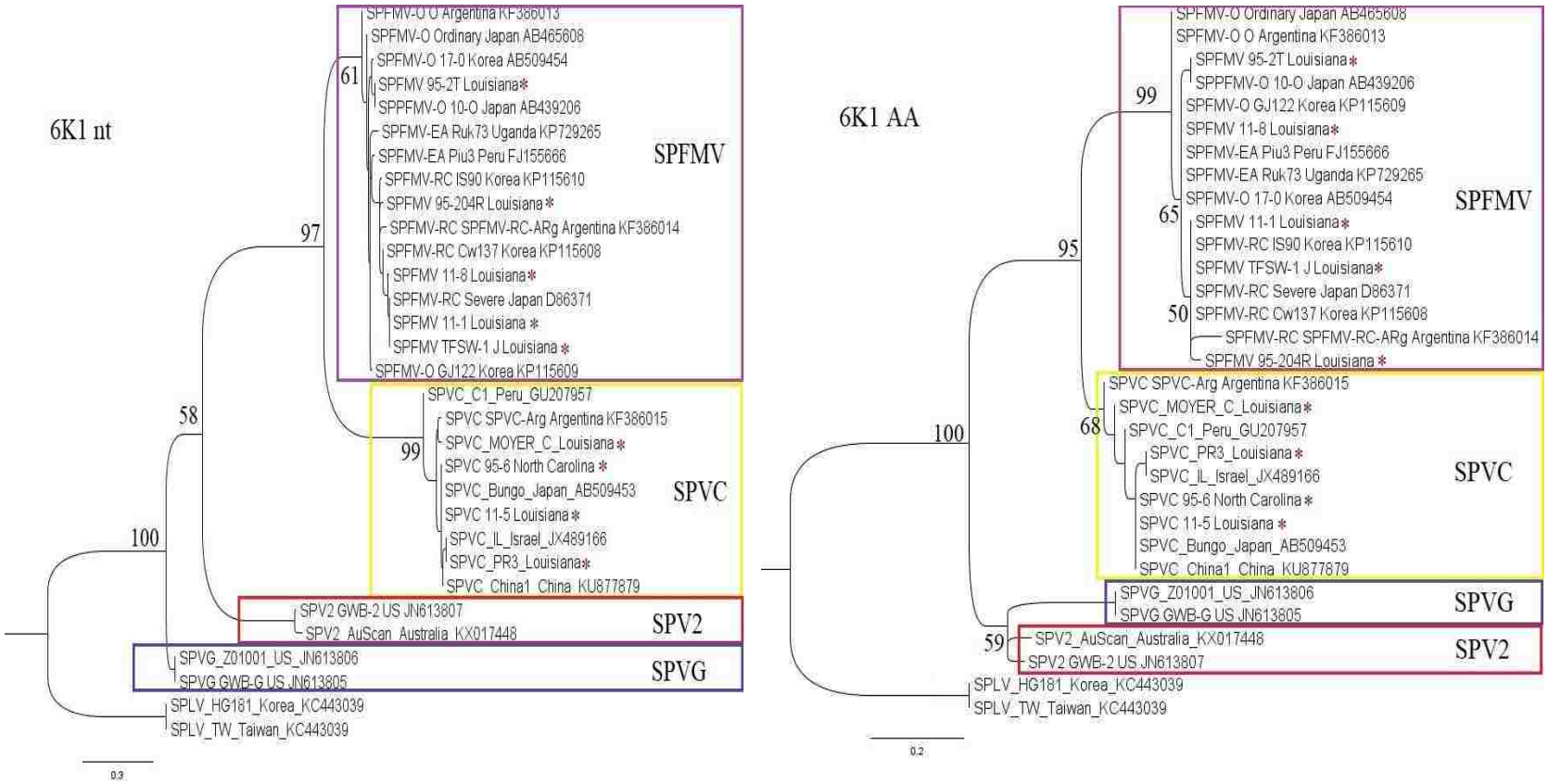

Figure 4.5. Maximum Likelihood Analysis of the 6K1 nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

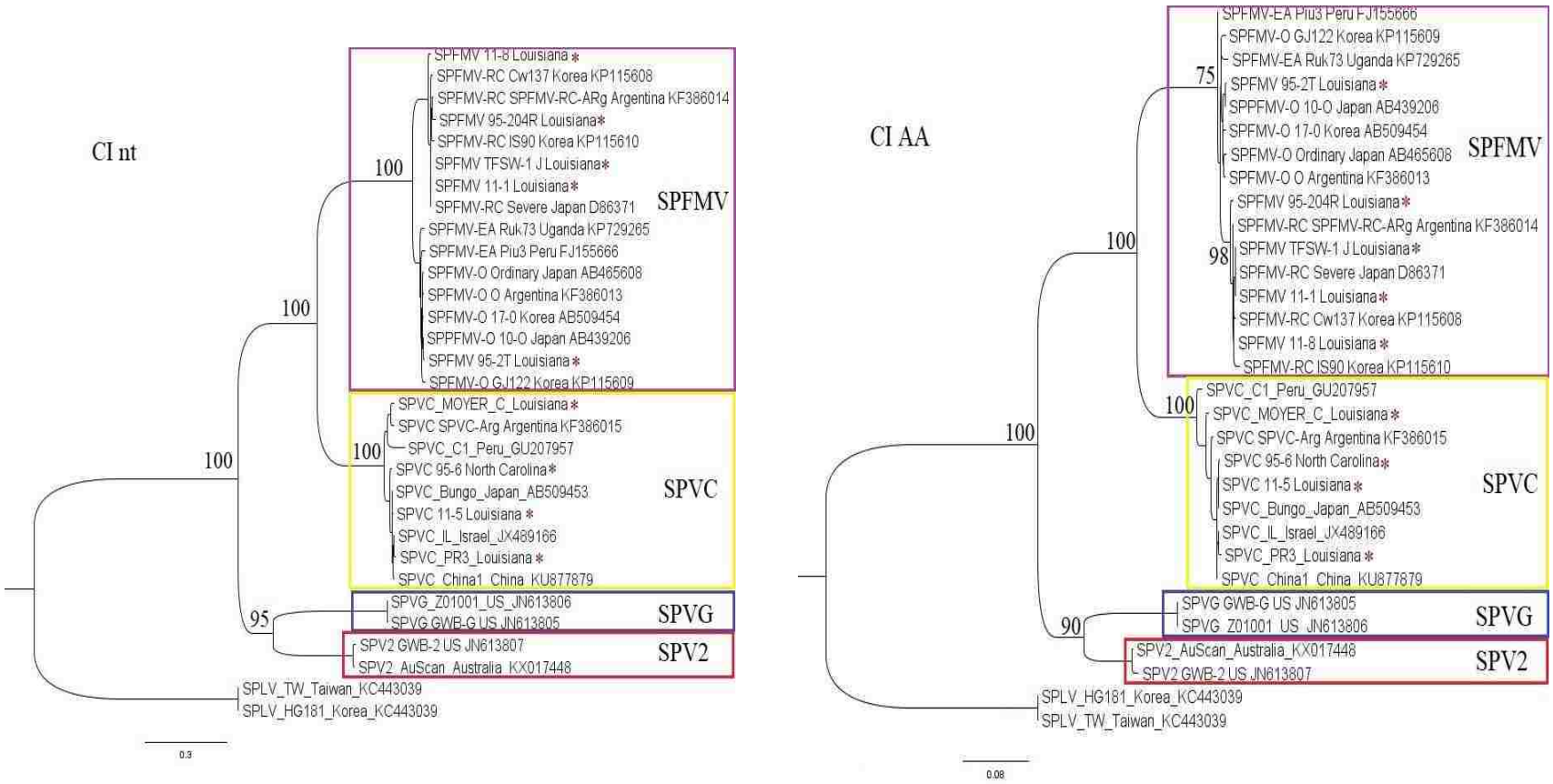

Figure 4.6. Maximum Likelihood Analysis of the CI nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

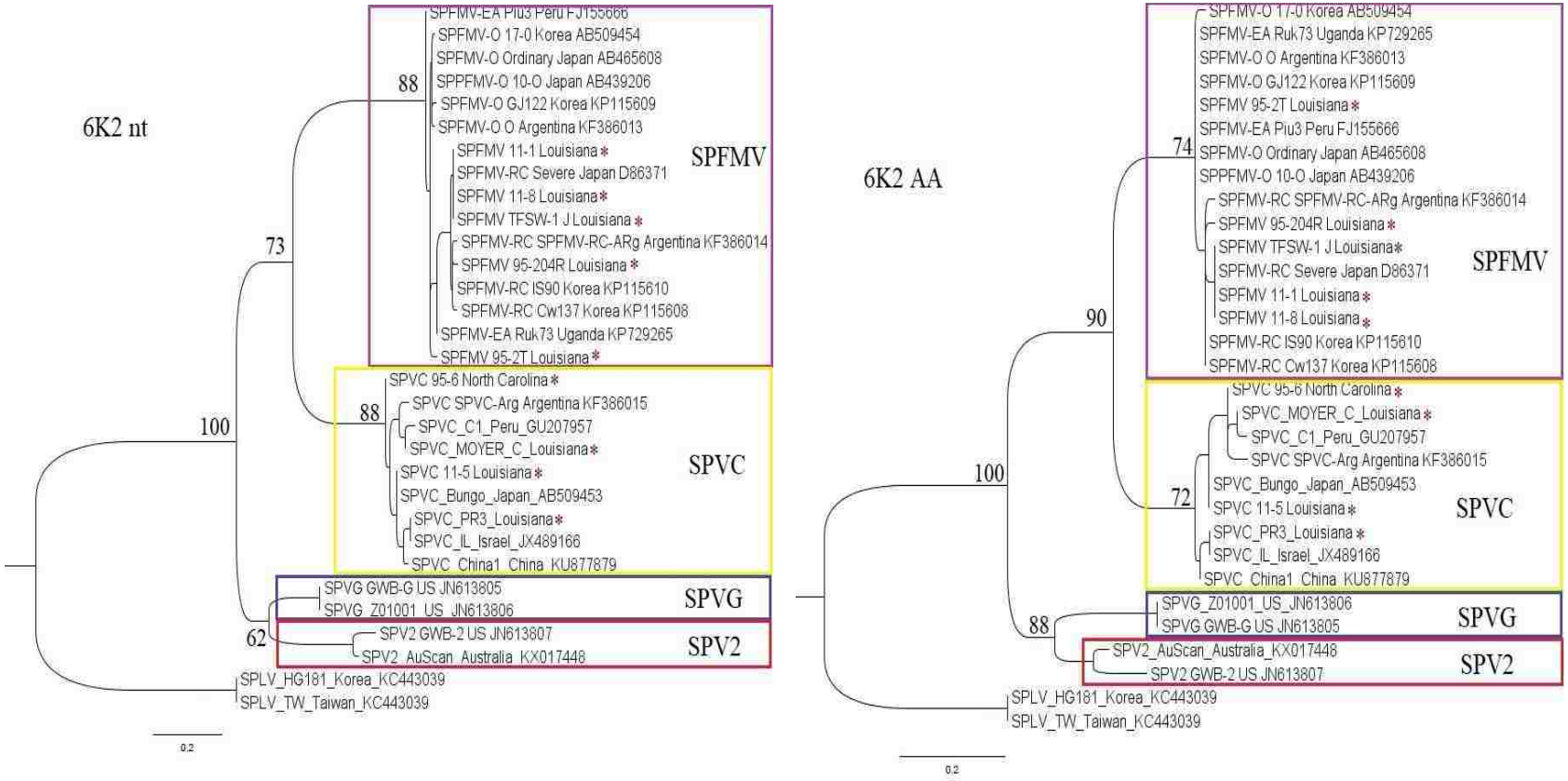

Figure 4.7. Maximum Likelihood Analysis of the 6K2 nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

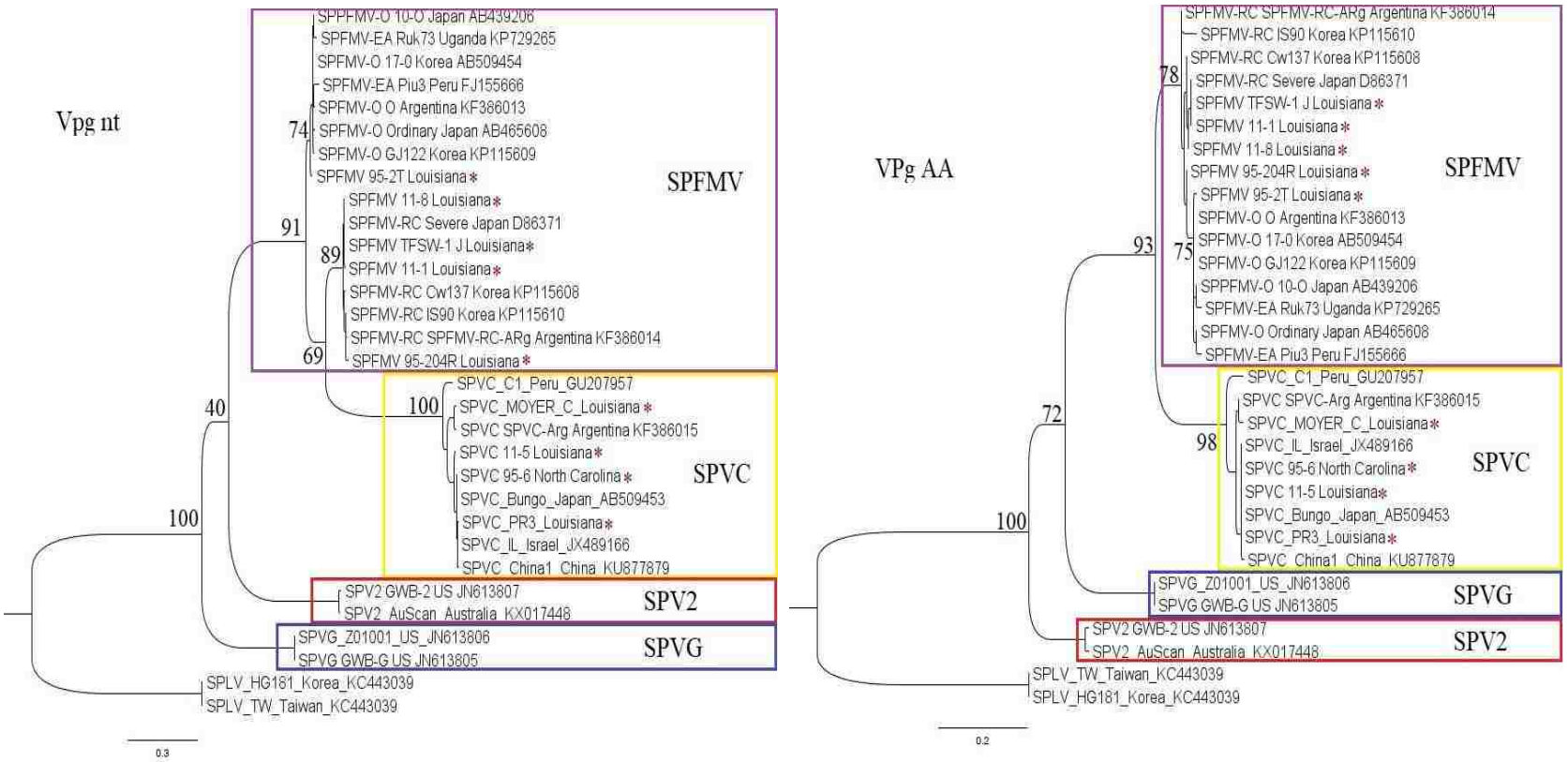

Figure 4.8. Maximum Likelihood Analysis of the Vpg nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

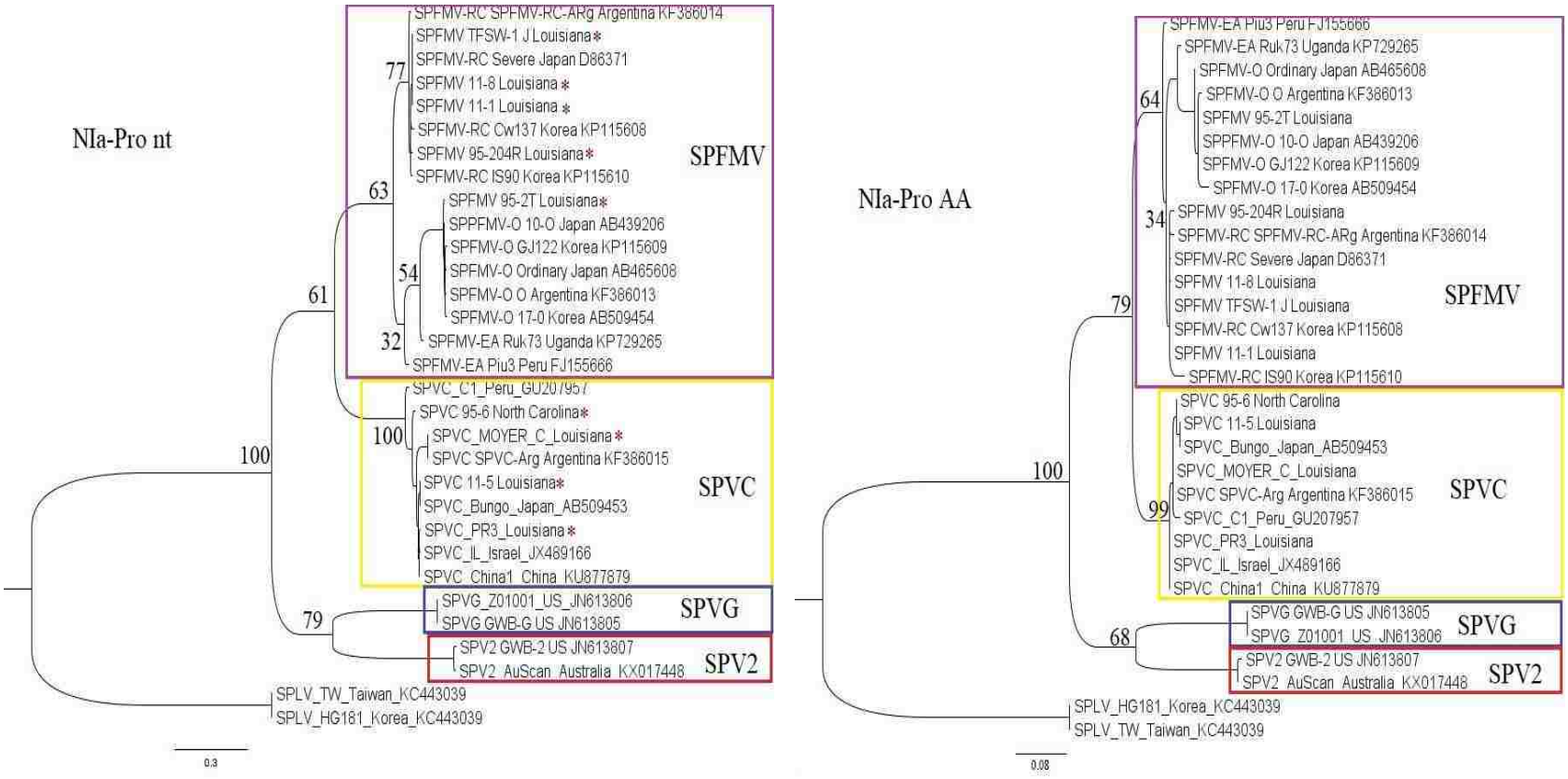

Figure 4.9. Maximum Likelihood Analysis of the NIa-Pro nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

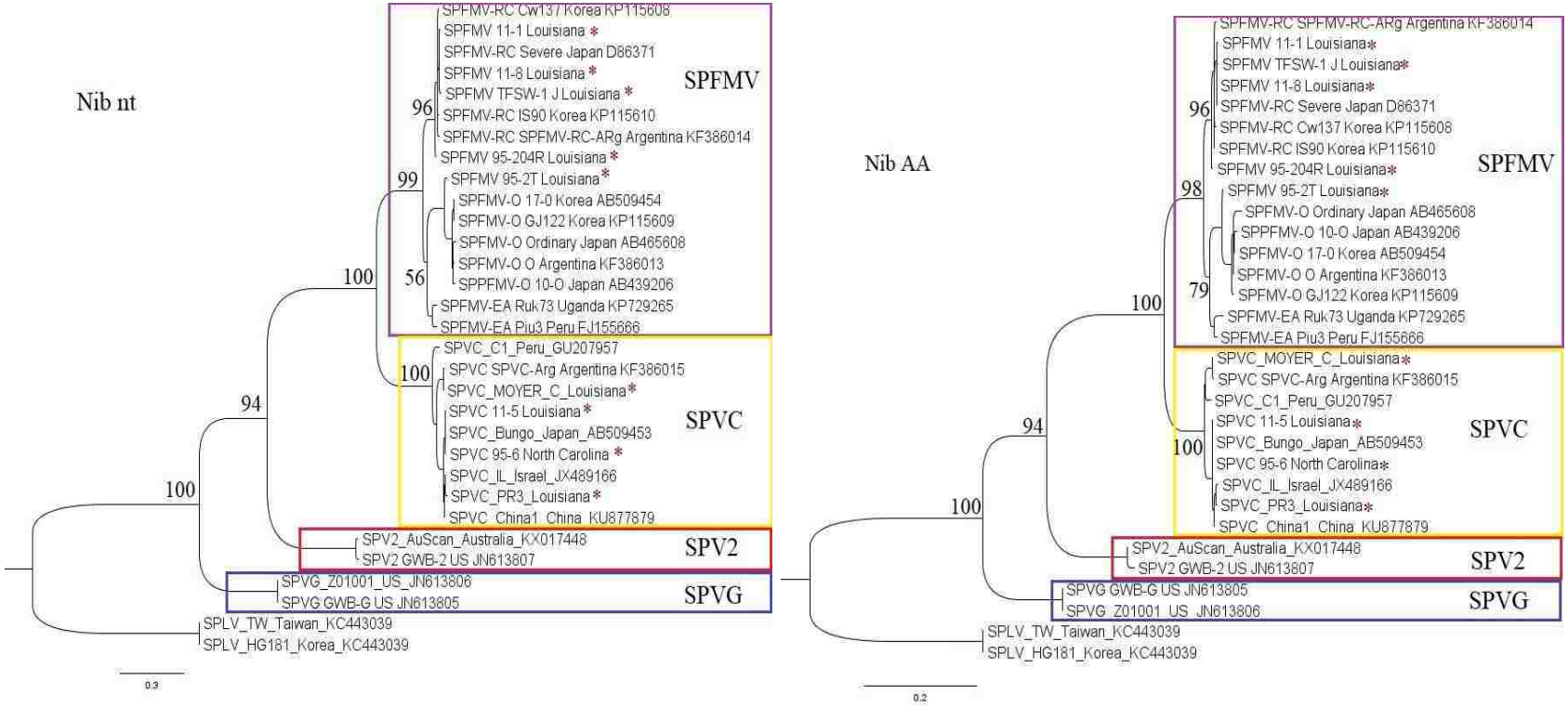

Figure 4.10. Maximum Likelihood Analysis of the NIb nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

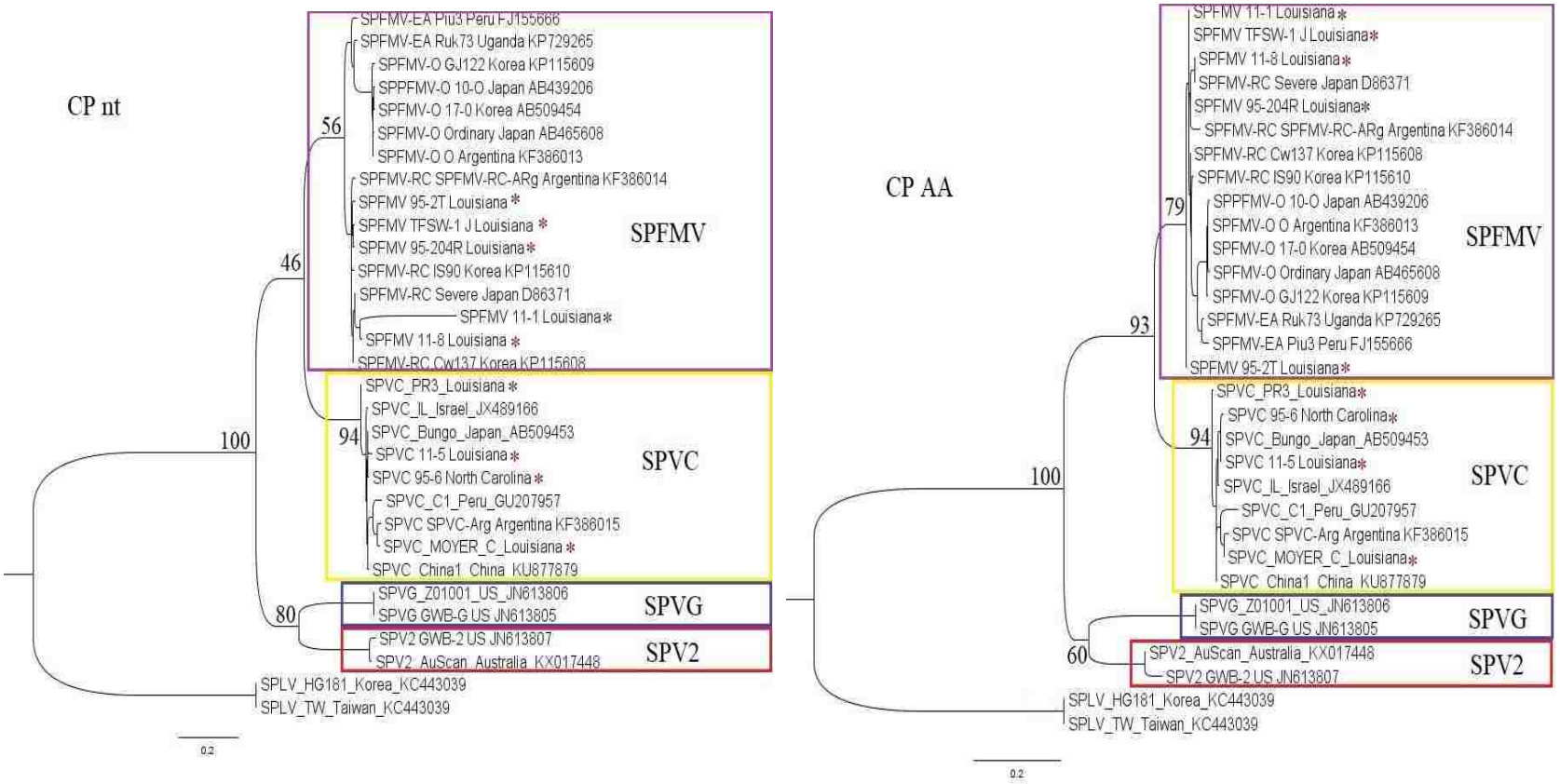

Figure 4.11. Maximum Likelihood Analysis of the CP nucleotide (nt, left) and amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

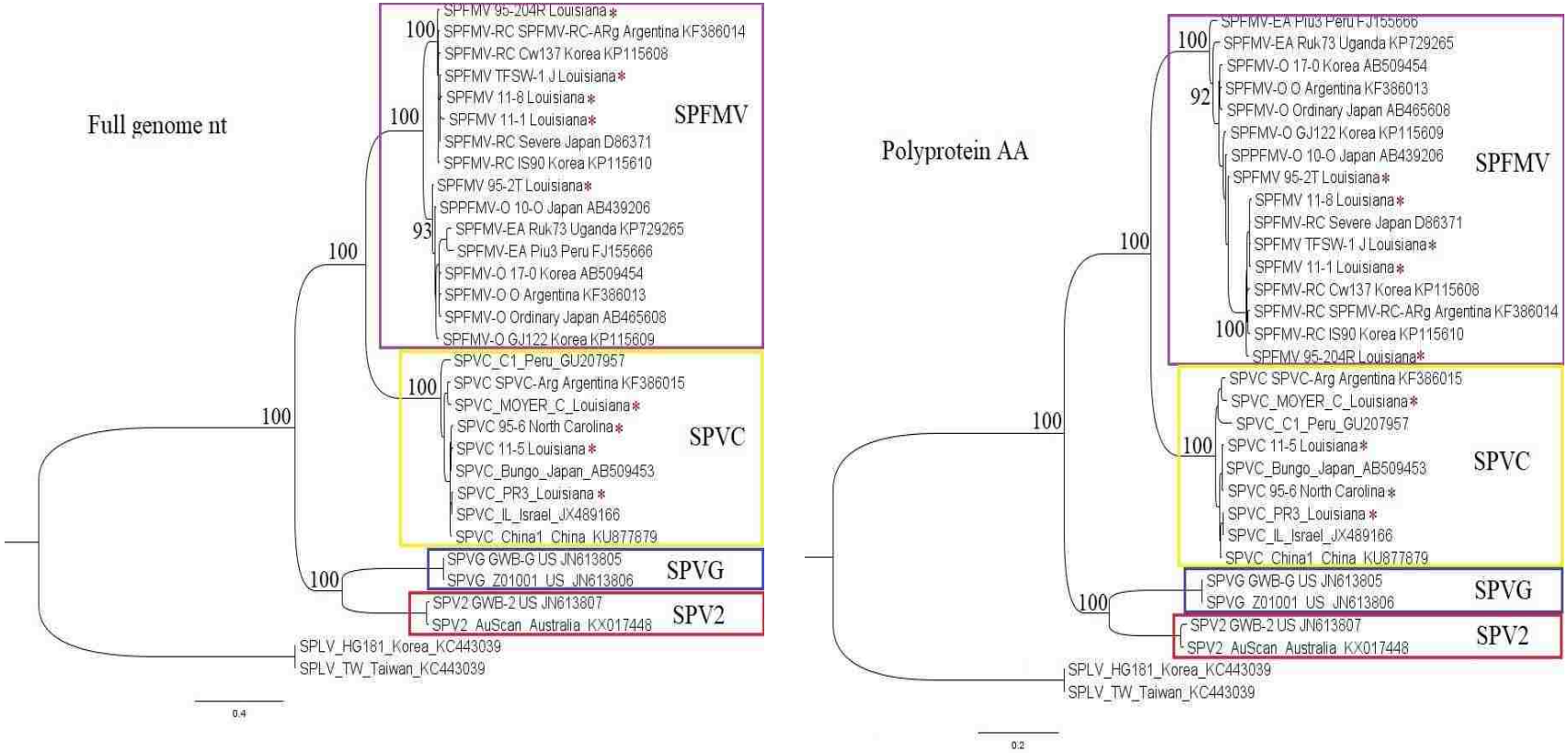

Figure 4.12. Maximum Likelihood Analysis of the complete nucleotide (nt, left) and polyprotein amino acid (AA, right) sequences of *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato virus C* (SPVC). Asterisks at the end of the name of sequences indicate the isolates obtained in this study. Numbers show bootstrap values (1000 replicates) in the major nodes. The bar below the tree represents estimated substitutions per site. Outgroups belong to *Sweetpotato Virus 2* (SPV2), *Sweetpotato virus G* (SPVG) and *Sweetpotato latent virus* (SPLV). Other abbreviations are O: ordinary strains, EA: East-African strains, RC: russet crack strains.

Table 4.7. Recombination events for SPFMV and SPVC isolates. Event number = ordered number of recombination events in all sequences. Site in genome: position of the recombination event in the sequence. Recombinant sequences: sequences that showed the recombination event. Major/Minor Parental sequence: the most likely parental isolate among those analyzed. Genes affected: potential genes affected with recombination breakpoints. Detection methods = R (RDP), G (GENECOV), B (Bootscan), M (Maxchi), C (Chimaera), S (SiSscan) and 3 (3seq). Algorithms that showed the highest statistical difference ($p<0.05$) are marked with an asterisk (*), (+) that the algorithm also reported statistical differences and those that do not with a NS.

		Site in Genome						Detection methods						
Virus	Event #	Begin	End	Recombinant Sequence (s)	Major Parental Sequence (s)	Minor Parental Sequence (s)	Genes Affected	R	G	B	M	C	S	3
SPFMV	1	1122	9665	SPFMV_95-2T_Louisiana	SPFMV-RC_Severe_Japan_D86371	Unknown	P1, CP	+	+	+	*	+	+	+
	2	9806	10705	SPFMV_11-1_Louisiana	SPFMV-RC_Severe_Japan_D86371	Unknown	CP	+	+	+	+	+	*	+
	4	7721	10675	SPFMV_95-2T_Louisiana	SPFMV-EA_Ruk73_Uganda_KP729265	Unknown	Nia-Pro, CP	+	+	+	+	*	+	+
	5	10186	36	SPFMV_11-8_Louisiana	SPFMV-RC_Severe_Japan_D86371	Unknown	CP, 3'UTR	+	+	+	*	+	+	NS
	6	7596	7821	SPFMV-EA_Piu3_Peru_FJ155666	SPFMV-O_O_Argentina_KF386013	SPFMV-RC_Cw137_Korea_KP115608	Nia-Pro	+	*	NS	+	+	NS	NS
	7	1122	8236	SPFMV_11-1_Louisiana	Unknown	SPFMV_TFSW-1_J_Louisiana	P1, CP	+	*	NS	+	*	NS	+
	8	9366	9665	SPFMV_TFSW-1_J_Louisiana	SPFMV_11-8_Louisiana	Unknown	CP	+	+	NS	+	*	NS	+
	9	9446	10600	SPFMV_95-2T_Louisiana	SPFMV-O_17-O_Korea_AB509454	SPFMV-EA_Piu3_Peru_FJ155666	CP	+	NS	NS	NS	+	NS	*
	10	944	1230	SPFMV-EA_Piu3_Peru_FJ155666	SPFMV-O_O_Argentina_KF386013	Unknown	P1	+	+	+	+	*	+	NS
	11	10496	10606	SPFMV_95-2T_Louisiana SPFMV_95-204R_Louisiana	SPFMV_11-8_Louisiana	SPPFMV-O_10-O_Japan_AB439206	CP	NS	*	NS	+	NS	+	+
	12	4628	6495	SPFMV_11-8_Louisiana	SPFMV-RC_Severe_Japan_D86371	Unknown	6K1	+	*	NS	+	+	NS	+
	13	9069	9175	SPFMV-O_Ordinary_Japan_AB465608	SPFMV_95-2T_Louisiana	SPFMV-RC_Severe_Japan_D86371	Nib	*	NS	NS	+	NS	NS	NS
	15	6708	7645	SPFMV_95-2T_Louisiana	SPPFMV-O_10-O_Japan_AB439206	Unknown	6K2	*	+	NS	NS	NS	NS	NS
	16	5082	6495	SPFMV_11-8_Louisiana	Unknown	SPFMV-RC_Severe_Japan_D86371	CI	NS	NS	NS	NS	NS	NS	*
SPVC	17	1490	1743	SPVC_11-5_Louisiana	SPVC_Bungo_Japan_AB509453	Unknown	P1	+	+	+	+	+	*	NS
	18	10052	867	SPVC_SPVC-Arg_Argentina_KF386015	SPVC_MOYER_C_Louisiana	SPVC_IL_Israel_JX489166	P1, CP	NS	NS	NS	+	*	NS	NS
	19	5776	8238	SPVC_95-6_North_Carolina	SPVC_Bungo_Japan_AB509453	Unknown	CI, Nib	+	*	+	+	+	+	+
	20	10242	10536	SPVC_11-5_Louisiana	SPVC_Bungo_Japan_AB509453	Unknown	CP	NS	*	+	NS	NS	NS	NS
	21	180	286	SPVC_95-6_North_Carolina	SPVC_Bungo_Japan_AB509453	Unknown	P1	NS	*	NS	+	+	+	+
	22	1614	2407	SPVC_MOYER_C_Louisiana	SPVC_SPVC-Arg_Argentina_KF386015	Unknown	P1	+	NS	*	+	+	NS	NS
	23	7425	7754	SPVC_95-6_North_Carolina	SPVC_Bungo_Japan_AB509453	SPVC_C1_Peru_GU207957	Nia-Pro	NS	NS	NS	+	*	NS	NS
	24	5905	5974	SPVC_95-6_North_Carolina	SPVC_Bungo_Japan_AB509453	SPVC_C1_Peru_GU207957	CI	+	+	NS	NS	NS	NS	NS
	25	1744	1992	SPVC_11-5_Louisiana	SPVC_95-6_North_Carolina	Unknown	P1	+	+	*	NS	NS	NS	+
	26	10225	10580	SPVC_PR3_Louisiana	SPVC_IL_Israel_JX489166	Unknown	CP	NS	+	+	+	*	NS	NS
	27	7030	7066	SPVC_11-5_Louisiana	SPVC_Bungo_Japan_AB509453	SPVC_SPVC-Arg_Argentina_KF386015	Nia-Vpg	NS	*	NS	NS	NS	NS	NS
	28	5622	6922	SPVC_MOYER_C_Louisiana	SPVC_IL_Israel_JX489166	SPVC_C1_Peru_GU207957	6K2, Nia-Vpg	NS	NS	NS	NS	*	NS	NS
	29	9286	9314	SPVC_PR3_Louisiana	SPVC_IL_Israel_JX489166	Unknown	Nib	NS	*	NS	NS	NS	NS	NS
	30	5852	5951	SPVC_PR3_Louisiana	SPVC_IL_Israel_JX489166	Unknown	CI	NS	NS	NS	NS	NS	NS	*

4.4 Discussion

In this study, molecular characterization of nine sweetpotato potyvirus isolates (five from SPFMV and four from SPVC) from representative sweetpotato production fields from the United States were completed. The genetic diversity using pairwise sequence comparisons and phylogenetic analysis was used as a tool to compare the genetic diversity of Louisiana SPFMV and SPVC isolates to previously reported sequences on NCBI. These isolates contained the potential major conserved proteins reported in Potyviruses (Adams *et al*., 2010) and the additional PIPO and PISPO produced by polymerase slippage (Chung *et al*., 2008; Olspert *et al*., 2016). These isolates did not have a high molecular variation compared to previously sequenced isolates, but six isolates did have recombination events mostly in the CP and P1 region of this group of viruses.

The ability to analyze the different genomic regions of these potyviruses allowed identifying genes of importance for different types of experiments. For example, due to its conservation, the CP nucleotide data has been used for identification and phylogenetic studies (Elvira-Gonzales *et al*., 2017; Lohmus *et al*., 2017; Voloudakis *et al*., 2004; Li *et al*., 2012). Other experiments included the determination of hot-spots for recombination events (Karasev and Stewart, 2013; Kwak *et al*., 2015) or the identification of new proteins like PIPO (Chung *et al*., 2008). Some proteins are unique to sweetpotato potyviruses like PISPO (Olspert *et al*., 2016, Untiveros *et al*., 2016). In terms of phylogeny of the different genes, regions such as the 5' UTR or 3' UTR apparently are not informative enough to differentiate strains of SPFMV (phylogroups A and B), probably due to recombination events (Kwak *et al*., 2015; Untiveros *et al*., 2010). Recombination events might have an effect on determining the accuracy of the dataset (Ruths and Nakhleh, 2005; Schierup and Hein, 2000) which would explain the monophyly of the different SPFMV isolates in this experiment at the 5' UTR. Viral regions such as Nib or CP appear to be the most informative in this group of viruses due to their conservation, which is understandable since both genes fulfill important functions for the virus such as RNA-dependent RNA-polymerase (RNA replicase in Nib) and encapsidation of the viral genome in the CP (Hong and Hunt, 1996; Revers and Garcia, 2015, Dolja *et al*., 1994).

Previous experiments reported that molecular variation at the CP nucleotide and P1 amino acid sequences classified SPFMV into three different strains: russet-crack (RC), ordinary (O) and East-African (EA); and allowed the reclassification of the common strain of SPFMV into the new species SPVC (Kreuze *et al*., 2000; Untiveros *et al*., 2010). The ability to analyze full nucleotide genome sequences allowed improving the viral taxonomy, possibly relating better to biological properties, identification of recombination events due to mixed infections in the sweetpotato plant and genetic connectivity between populations (Sakai *et al*., 1997; Yamasaki *et al*., 2010, Untiveros *et al*., 2010, Maina *et al*., 2017). The implementation of the sequences in this study could serve for future studies such as phylogenetic placement of new isolates (Kwak *et al*., 2015; Rännäli *et al*., 2009), annotation of contigs in next-generation sequencing analysis (Zheng *et al*., 2017) or diagnostics in the creation of primers for new and recombinant isolates (Bejerman *et al*., 2016).

In different sweetpotato surveys and experiments, plants showed mixed infections of several potyvirus species or strains in the same plant (Valverde *et al*., 2007; Kreuze *et al*., 2009; Guo *et al*., 2014). These mixed infections have shown to be the cause of the emergence of new viral strains due to genetic recombination (Chare and Holmes, 2006). In this experiment SPFMV isolates 11-1, 11-8, 95-2T and SPVC isolates PR3, 11-5 and 95-6 had recombination events primarily in the P1 and CP region but only 95-2T met the criteria to be called a true recombinant

since it had recombination events identified by more than three algorithms and both major and minor parents were identified. The P1 protein has been reported as as the most divergent region in length and amino acid sequences (Adams *et al*., 2005; Untiveros *et al*., 2010), and vulnerable to recombination (Nguyen *et al*., 2013; Valli *et al*., 2007; Seo *et al*., 2009). The CP region of the SPFMV-EA isolate Piu3 from Peru has been reported as a recombinant of SPFMV-O and SPFMV-RC and in the recombination analysis of *Potato virus Y* sequences (PVY), the CP has been identified as a hot-spot for the recombination junction #4 (Karasev and Stewart, 2013; Kwak *et al*., 2015). Other genes also had recombination events; however, based on the inability of the software to determine one of the parents or sensitivity of the algorithms, most of them were categorized as 'tentative'. The occurrence of such recombination events suggests the possibility that recombination could be a force in the emergence of new variants of sweetpotato potyviruses.

Traditionally, viral sequence completion has been accomplished using PCR fragment overlap to ensure that they belong to the same genome (Kwak *et al*., 2015; Sakai *et al*., 1997; Yamasaki *et al*., 2010, Untiveros *et al*., 2010). However, with the advent of next-generation sequencing techniques, viral completion has been archieved using techniques such as 454-pyrosequencing (Roche) or deep sequencing of siRNA (Illumina HiSeq Series) (Bejerman *et al*., 2016; Li *et al*., 2012; Mbanzibwa *et al*., 2014; Gu *et al*., 2014; Maina *et al*., 2017). The advantages of the utilization of next-generation sequencing methods is the ability to detect viruses that were not amenable to Sanger PCR fragment overlap sequencing. This method allowed identification of previously unknown viruses such as the Sweet potato pakakuy virus (SPPV), a virus composed of Sweetpotato badnavirus A and B (Mbanzibwa *et al*., 2014; Kreuze *et al*., 2009) or *Pepino mosaic virus* (PepMMV) infecting tomato (Li *et al*., 2012). In our experiment, next-generation sequencing complemented with the utilization of biological methods to separate SPFMV from SPVC (Souto *et al*., 2003) improved the annotation of contigs to references of SPFMV in SPVC isolates and vice versa which could serve for future experiments to avoid problems such as low quality of RNA in the next-generation sequencing process.

Co-infection of more than one sweetpotato virus in the same plant has been reported to affect the plant in different ways. For example, the co-infection of SPFMV and *Sweetpotato chlorotic stunt* (SPCSV) causes Sweet potato virus disease (SPVD), which severely decreases yield and increases synergistically the titers of these viruses in the plant (Kokkinos *et al*., 2006; Clark *et al*., 2012; Mingot *et al*., 2016). Another example of co-infection has been reported when plants have mixed infections with the Ordinary (SPFMV-O) and russet-crack (SPFMV-RC) strains of SPFMV. When SPFMV-O infects the plant it causes mild discoloration compared to SPFMV-RC that causes dark lesions on the storage roots. However, cross protection occurs in the plant when both strains infect the sweetpotato plant at the same time (Yamasaki *et al*., 2010). In this study, four sequences of SPFMV-RC were identified but these isolates did not cause russet-crack symptoms. Some reasons for this incongruency could be the ability of some sweetpotato varieties to have different degrees of infected plants naturally become healthy (reversion) (Gibson *et al*., 2014); co-infection of SPFMV-O and SPFMV-RC in the same plant (Bejerman *et al*., 2016); or the lack of congruity of the utilization of nomenclature using biological properties or origin of the isolate (Jones and Kehoe, 2016). The East-African strain of SPFMV (SPFMV-EA) has previously been considered restricted to this region, but now sequences (mostly from CP) have been reported from elsewhere (Tairo *et al*., 2005; Tugume *et al*., 2010). Additionally, SPFMV-RC isolates have been reported not to cause russet-crack symptoms in the storage roots associated with those isolates (Maina *et al*., 2017; Bejerman *et al*., 2016). This underlines the need for greater effort to associate

biological properties of sweetpotato potyviruses with their molecular properties and further indicates that the factors that trigger russet crack symptoms in sweetpotato require further investigation.

The widespread distribution of SPFMV and SPVC and their molecular variability around the world described in this and previous studies suggest the need to include rigorous programs for virus-tested sweetpotato. These programs include graft and PCR techniques to identify viral infections (Li *et al*., 2008; Ha *et al*., 2008; Li *et al*., 2012; Zheng *et al*., 2010; Wei and Nakhla, *personal communication*; Li *et al*., 2004; Ling *et al*., 2010; Kokkinos *et al.*, 2006) complemented with tissue culture techniques. Undestanding the molecular variation is essential to improve current methods to facilitate strategies in the control of sweetpotato potyviruses.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is an important crop for food security. Plant viruses affect yields in sweetpotato due to their accumulation in cultivars. The most common plant viruses which affect sweetpotato in the United States belong to the *Potyviridae* family. *Sweet potato feathery mottle virus* (SPFMV) was first described in 1978, being the only virus reported in the United States until 1998. With the improvement of molecular biology techniques, *Sweet potato virus G* (SPVG) and *Sweet potato virus 2* (SPV2), were characterized. Recently, the former common strain of SPFMV was changed to species status and renamed *Sweet potato virus C* (SPVC). These four viruses are similar at the nucleotide level, especially in the coat protein (CP) region, which has been used for classification, detection and identification. Even though these four viruses are commonly detected as mixed infections, their spread in the field, titers in the plant, and vector transmissibility are different.

The lack of sequence differences in the CP region between SPFMV and SPVC resulted in previous qPCR procedures that amplified both viruses, and thus a new approach was needed to quantify each species independently. With this premise, the first objective of this dissertation was to determine if storage roots at the 5th week after transplanting is the best organ and time to screen for these four viruses. New primers specific for SPFMV and SPVC were designed and evaluated along with a different housekeeping gene, *Cytochrome C Oxidase*, for relative quantification. When compared with root and stem organs, the greatest relative titers among the four potyviruses were found in leaf tissue at the 3rd week after transplanting.

Field experiments in which virus artificial inoculations did not replicate the amount of yield reduction observed on naturally infected plants led to further investigation of additional factors involved in the "yield decline effect". Additional factors such as water and nitrogen availability have been demonstrated to affect storage root production. Because previous experiments did not include SPVC in the combination of artificially inoculated viruses, the second objective was to test if the inclusion of the new species can replicate the observed yield reduction. Storage root production in the greenhouse among plants with different virus infections did not support the conclusion that SPVC was the missing element in accounting for "yield decline effect" and an additional factor(s) yet unknown may be involved.

In the absence of molecular information of isolates from the United States, nucleotide sequence information of the CP region has been used in most phylogenetic studies to describe species and strains of potyviruses. This focus on the CP region delayed the recognition of SPVC as a distinct species. The differences from SPFMV are located primarily in the amino acid sequences of the P1 region which triggered the interest of the molecular genetic variation among this group of viruses. The third objective was to test the molecular variation of isolates representative of the U.S. sweetpotato production fields was different from other isolates previously sequenced. Phylogenetic analysis and pairwise sequence comparison showed that the variation was not high but several recombination events were detected in the CP and P1 region.

The findings in this study indicate that there is a need to conduct research to determine what additional factors are involved in yield reduction, provide a cheaper system for quantifying titers of SPFMV and SPVC by multiplex qPCR, and improve the identification and management in the surveys of sweetpotato potyvirus isolates.

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