USE OF MOLECULAR AND BIOCHEMICAL METHODS TO DETERMINE CITRUS TRISTEZA VIRUS (CTV) VIRAL COMPONENTS AND RESISTANCE IN CANDIDATE ROOTSTOCKS TO REPLACE SOUR ORANGE

By

AZZA HOSNI IBRAHIM MOHAMED

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

To the memory of my parents and my brothers who passed away during my program

To my family whose encouragement kept me going

To my beloved husband Ahmad for his help and support

To my lovely daughter Aala whose love and smile lighten the long, dark tunnel for me and help me find a way where there is none apparent

To my best friend Haja Amal whose name means hope

ACKNOWLEDGMENTS

The completion of this dissertation would not have been possible without the encouragement, help and support of many people. First, I would like to express my sincere gratitude and appreciation to my major advisor Dr. Jude W. Grosser for his encouragement, valuable guidance and advice for both my personal and my professional life. I cannot thank him enough for his financial support, and for being very understanding to my condition as a graduate student. I especially thank him a lot for his time and effort in reviewing this manuscript. For what he did for me, I will be always in dept and grateful to him. His high ethical standards and respectful views for the others will always be memorable. I deeply thank Dr. Ronald H. Brlansky, for allowing me use the facilities in his lab and greenhouse and for his helpful suggestions and comments, and also for reviewing this manuscript. I would like to thank Dr. Fredrick Gmitter and Dr. Charles A. Powell for serving on my committee, giving me guidelines and advice for my research during my entire program, and reviewing this manuscript.

I am especially grateful to my former major professor Dr. Richard Lee for his financial support for the first one and half years of my program and his encouragement along all these years. I want to extend my gratitude to Debbie Howd, Diann Achor for their technical help. I am very thankful to our librarian at CREC Jennifer Dawson for her professional help. I want to thank Mrs Cecile Robertson for her endless help and kindness. Special thanks go to Dr. Edgardo Etxeberria for letting me use his lab and thanks to Pedro Gonzales for his help. Great thanks and appreciation go to Dr. Larry Duncan for his special support and dedication to graduate students at the CREC. I want to thank Dr. W.O. Dawson and his team for their assistance and providing me with the CTV isolates needed in this study. I thank Dr. Siddarame Gowda for his help. I want to express my gratitude to Gary Barth for his valuable discussion and support all the time and for proof reading this manuscript.

Some people who deserve special mention are Dr. Manjunath Keremane, Dr. Kajal Biswas and Dr. Avijit Roy for their help. I thank Dr. Fahiem El Borai for his time, help and encouragement that meant a lot to me. I thank all my friends (Eaman, Marwa, Faten, Naglaa, Ekta, Dalia, Safaa, Rashida, Samya, Luciana, Maysoon, Eaman, Abeer, Ling and Maysaa) for their encouragement and support. I also thank Becky McCoy for taking care of my plants in the greenhouse. I am grateful to Troy Gainey and the groove crew, especially Michael Clock, for taking care of my trees in the field. Without them, the work would be more delayed.

I want to thank Dr. Pete Timmer for his proof reading a great part of this manuscript. Special thanks go to Dr. Reza Ehsani for giving me a space in his lab to finish my writing. I want to extend my gratitude to all the sincere and unconditional friends that I have in my lab, Chuck, Milica, Manjul, Monica, Jamouna, Divya and Pamela for their help and support. Special thanks go to Ralph Story for his help with the field work and Julie Gmitter for her help in processing the samples. I also thank Allan Burrage, Zenaida Viloria, Gemma Pasquali, Anna Redondo, Mukkades Kayum, Kanjana Mahattanatawee and Marty Dekkers for their help and support. I thank the entire CREC community for their help and cooperation. I extend my appreciation to all the people in the personnel office at the CREC and the people at the Horticultural Sciences Department in Gainesville for their help and understanding to process the paper work in a timely manner.

Finally, I thank the Citrus Research and Education Center at University of Florida for its generous financial support during my graduate studies at the University of Florida. A special recognition goes to all my department members in Egypt for being understanding and giving me the permission to stay aboard to continue with my program.

My most sincere thanks and appreciation go to my family, for their understanding, support, believing in me and unconditional love they provided me throughout my life. I want to thank all my friends in Egypt and the USA either in Gainesville or in Lake Alfred, for their love, support and encouragement which kept me going and achieved my goal. Special thanks go to my friends Haja Amal and Tahereh for their pure love and their sincere support. Their continuous encouragement and concern are highly appreciated. I would like to express my sincere appreciation to my beloved husband Ahmad Omar for his love, support and his continuous help throughout my program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

USE OF MOLECULAR AND BIOCHEMICAL METHODS TO DETERMINE CITRUS TRISTEZA VIRUS (CTV) VIRAL COMPONENTS AND RESISTANCE IN CANDIDATE ROOTSTOCKS TO REPLACE SOUR ORANGE

By

Azza Hosni Ibrahim Mohamed

May 2009

Chair: Jude W. Grosser
Major: Horticultural Sciences

Citrus tristeza virus (CTV) is the causal agent of the most destructive viral disease of citrus and has a big impact on citrus production all over the world. CTV is a phloem-limited virus that belongs to *Closteroviridae* family. The virus causes a wide range of symptoms depending on the isolate and the host. Sour orange (*Citrus aurantium* L.) has been a widely used rootstock for citrus because of its desirable qualities including resistance to phytophthora diseases and citrus blight, wide adaptation, and ability to produce good yields of high quality fruits. Unfortunately, citrus scions on sour orange rootstock are highly susceptible to quick decline (QD) disease caused by CTV. This has lead to the reduction of sour orange rootstock in Florida and in other citrus areas. The current rootstocks in Florida are primarily trifoliate hybrids which are not adapted to high pH, calcareous soils. Several new rootstocks have been developed in attempts to replace sour orange rootstock. Previous efforts to screen new hybrid rootstock candidates in the greenhouse for resistance to tristeza-QD have been confounded by another CTV disease called seedling yellows that affect only juvenile plants. The main objective of the present study was to develop a new assay that bypasses the seedling yellows effect. Seventy- two selections, including parental pummelos, pre-selected sour-orange-like pummelo-mandarin rootstock hybrids, and

sour orange were top-worked onto 15-year old ‘Hamlin’ sweet orange trees known to carry the three CTV genotypes important in Florida (T30, T36 and VT). Virus infection was determined by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Over all, there was a significant difference in terms of shoot growth between the tested rootstock candidates and the sour orange that was stunted and showed strong disease symptoms. Movement of the various CTV genotypes from the ‘Hamlin’ interstock into the grafts was determined by molecular techniques including multiple molecular markers (MMM) analysis and heteroduplex mobility assay (HMA). Several CTV-induced quick decline resistant/tolerant selections, including some pummelo parents and new hybrids, were identified using quantitative real time PCR (qRT-PCR).

CHAPTER 1
INTRODUCTION

Citrus is one of the most widely grown and economically important fruit crops worldwide with an annual production of more than 100 million metric tons. Brazil has the highest citrus production followed by China and the United States of America (USA) (Table1-1) according to FAOSTAT (2007). United States production of citrus is worth about $21 billion annually with the state of Florida producing the majority of the USA's citrus. Citrus is a very valuable fruit in terms of nutrition as it is a good source of vitamin C, minerals and antioxidants. The center of origin of citrus is believed to be South-East Asia, 4000 years BC (Davies and Albrigo, 1994). Citrus is primarily produced within tropical and subtropical regions (within 40° North-South latitude). Mediterranean countries are considered the leaders for the international fresh fruit market. Egypt produces a significant amount of high quality citrus fruits, approximately 2.8 million tons in 2005, ranking eleventh in citrus production (Table 1-1).

World citrus production is being threatened by many viral, bacterial and fungal diseases. The most threatening diseases to citrus are citrus greening, citrus canker and citrus tristeza. Citrus tristeza caused by *citrus tristeza virus* (CTV), is the most destructive viral disease of citrus and has a big impact on citrus production all over the world. CTV is a single-stranded, positive-sense RNA virus in the genus *Closterovius,* family *Closteroviridae* (Bar-Joseph et al., 1989) and it is vectored by aphid species with the brown citrus aphid, *Toxoptera citricida,* being the most efficient vector. CTV is considered the largest known plant RNA virus with a genome about 20 kb long. Genome organization, mechanisms of gene expression, population complexity and sequence variation among different isolates combined with the host- pathogen interaction are all important factors controlling CTV biology and disease symptom development. For a better understanding of the pathogenecity of CTV, genetic analysis of the whole CTV genome is

desirable (Satyanarayana et al., 1999; Ayllon et al., 2001; Satyanarayana et al., 2002a; Satyanarayana et al., 2002b). Other factors attributing to the poor understanding of the disease mechanism are the virus restriction to phloem tissue, the low titer in virus-infected plants, and the population diversity. The genetic analysis of CTV genome has been advanced by the development of a full length infectious cDNA clone and a protoplast system for CTV replication (Price et al., 1996; Satyanarayana et al., 1999).

CTV isolates vary in their biological reactions on different hosts. Therefore, CTV causes a wide range of symptoms depending on the isolate and the host. Most field isolates are mixtures of different strains with one that usually appears to be dominant (major population). The most important disease caused by CTV is known as quick decline (QD), (McClean, 1950; Grant et al., 1951). On sour orange rootstock some CTV isolates cause an incompatibility at the budunion causing the tree to decline. Tree decline results in the necrosis and the death of the phloem at the budunion whereby sugars produced in leaves are blocked from being transported to the roots. Eventually, the feeder roots use up stored starch and start to die, leading to the ultimate death of the tree (Brlansky et al., 2008; Futch and Brlansky, 2008).

The introduction of the primary CTV vector, the brown citrus aphid, into Florida in 1995 (Halbert and Brown, 1996) resulted in the rapid spread of severe CTV isolates and therefore the CTV-decline isolates. This immediately jeopardized millions of commercial citrus trees planted on sour orange rootstock in Florida, since trees on sour orange are highly susceptible to citrus tristeza quick decline disease (Bar-Joseph et al., 1989). This has lead to reduction of sour orange (*Citrus aurantium* L.) rootstock. As a result, less desirable rootstocks are currently used (Bauer et al., 2005). Currently there is no rootstock that provides an adequate replacement for sour

orange for several reasons including problems with soil adaptation, fruit quality, horticultural problems and disease resistance.

Unfortunately, there is no effective method for controlling or eliminating CTV from citrus infected trees, especially in the field. When citrus trees are grown in the field in Florida, they are most likely to become CTV infected at some point of their life, even though planted virus free (Sieburth et al., 2005). Developing transgenic citrus with resistance to CTV is considered to be the best long-term approach for controlling CTV diseases. Molecular studies have revealed CTV resistant gene (s) in *Poncirus trifoliata*; but the transfer of this gene (s) into commercially important citrus scions and rootstock is a laborious and it will require more years of research to become reality (Deng et al., 2001b). Moreover, commercialization of transgenic citrus must still overcome regulatory hurdles and achieve consumer acceptance. Development of a replacement for sour orange that is resistant to QD and provides the acceptable horticulture qualities has become a primary breeding objective (Grosser et al., 2004a).

Several new citrus rootstock candidates have been developed using either conventional breeding or a somatic hybridization approach by the University of Florida and the Agricultural Research Service of the U.S. Department of Agricultural (USDA), (Bowman and Rouse, 2006). The citrus improvement program based on somatic hybridization has been led by Dr. Jude Grosser at the University of Florida-IFAS Citrus Research & Education Center with a primary goal of developing improved citrus rootstocks (Grosser et al., 2000; Grosser and Chandler, 2002). Sour orange has been shown by molecular markers to be a probable hybrid of mandarin and pummelo (Nicolosi et al., 2000). Therefore superior sour-orange-like rootstock hybrids have been produced by different combinations of pummelo and mandarin using the somatic hybridization technique, resulting in allotetraploid hybrids (Grosser et al., 2003). Hybrids

produced at the tetraploid level preserve the dominant traits of both parents and have the potential to control tree size via polyploidy (Grosser et al., 1995; Grosser et al., 1998; Grosser et al., 2000; Nicolosi et al., 2000; Grosser and Chandler, 2002). Moreover, somatic hybridization has the advantage of the immediate use of preselected pummelo seedlings as parents, whereas conventional breeding with the same material would require several years of waiting for the material to overcome juvenility to flower (Grosser et al., 2004a). Promising rootstocks must be evaluated for virus resistance and horticultural performance over a number of years before being released for commercial use.

The development of a good screen of the rootstock candidates for resistance to tristeza quick decline would facilitate the development of a replacement rootstock for sour orange. Moreover, sequencing and molecular characterization of the different CTV genotype complex should improve our understanding of the virus biology in these tested rootstock candidates to replace sour orange rootstock. Therefore, the main purpose of this study was to develop a more efficient screen of new candidate rootstocks for resistance to CTV-induced quick decline disease. In the past, the CTV-induced disease seedling yellows (SY) has confounded screening experiments conducted in the greenhouse (Garnsey S.M., unpublished data). Therefore, top-working of the new rootstock candidates to mature CTV-infected trees in the field was chosen as a means to bypass the seedling yellows problem in the greenhouse. Field tree virus infection was detected by serological techniques including tissue blot immunoassay (TBIA) and double antibody sandwich, indirect enzyme-linked immunosorbent assay (DASI-ELISA). Several molecular and biochemical methods were used to assay and study the movement of the virus from the infected interstock into the virus free grafted materials. These methods include multiple molecular markers (MMM) analysis and heteroduplex mobility assay (HMA). Quantitative real

time PCR (qRT-PCR) was used to provide a fast and a reliable assay to detect and quantify the virus titer in the source and the tested rootstocks after top-working (Ruiz-Ruiz et al., 2007). Based on the study done by Garnsey and Young (1975) that showed the depletion of starch content in the roots of CTV declining trees, starch, sucrose and total carbohydrates content were also studied in leaves and roots of these rootstock candidates. The measurements were done 12 months after inoculation with a QD inducing CTV isolate (T36) in a parallel greenhouse study.

Table 1-1. Total production of citrus fruit (Mt) (FAOSTAT, 2007).

Country	Production (Mt) Metric ton
Brazil	20,185
China	15,166
United States of America	10,410
Mexico	6,672
Spain	5,347
India	5,242
Iran	3,624
Italy	3,489
Argentina	3,036
Turkey	2,910
Egypt	2,800
South Africa	1,930
Morocco	1,245
Japan	1,207

CHAPTER 2
REVIEW OF LITERATURE

Disease History

"Tristeza" which means sadness in Spanish and Portuguese is one of the most devastating and economically important diseases in the citrus industry worldwide. The disease is caused by a phloem-limited, Closterovirus known as *citrus tristeza virus* (CTV) and occurs in most citrus production areas in the world. Although citrus tristeza is believed to have originated in Southeast Asia (Wallace, 1956), the disease was first recognized as a decline disease of citrus scions propagated on sour orange (*Citrus aurantium* L.) rootstock in South Africa in the 1910s (Webber, 1943). CTV is not transmitted by seeds, therefore, most of the early establishments of citrus, which were propagated only through seeds were CTV-free (McClean, 1957). Initial spread of the disease is believed to have been through the infected propagating materials. Another CTV decline disease causing devastating death of millions of citrus trees grafted on sour orange rootstock was reported in Argentina and Brazil during the1930s (Costa and Grant, 1951; Bar-Joseph et al., 1989). More than ten million trees have been lost in Spain from 1956 to late 1980s (Cambra et al., 1988). During the nineteenth century, Phytophthora root rot of sweet orange trees was the main concern and caused great losses of citrus. Therefore, the use of grafted trees onto the Phytophthora–tolerant sour orange (*Citrus aurantium* L.) rootstock became common (Klotz, 1978). However, problems associated with sour orange as a rootstock started to be recognized in Australia, South Africa and Java as incompatibility problems (Webber, 1925; Toxopeus, 1937).

The decline problem was first thought to be a graft incompatibility between rootstock and scion, a root disease, or a nutritional problem, but Meneghini (1946) transmitted the disease with aphids and confirmed the viral nature of the disease (Bar-Joseph et al., 1989; Lee and Rocha-

Pena, 1992). Besides quick decline, other diseases known to be associated with CTV infection include stem pitting (Da Graca et al., 1984) and the economically insignificant seedling yellows (Roistacher, 1982) . The latter disease has confounded the greenhouse screening of new rootstock candidates for quick decline resistance (Garnsey, unpublished data).

Quick decline disease was confirmed in the United States for the first time in California in 1939 (Fawcett and Wallace, 1946; Wallace, 1956) and become epidemic in Florida (Grant, 1952). CTV is believed to have been introduced into the United States first in California with Meyer lemon imported from China in 1908 and then introduced to Texas and Florida with the movement of Meyer lemon trees (Wallace and Drake, 1955).

Recently, Lee et al., (2002) reported an epidemic situation in the Bog Walk Valley, Jamaica, where the entire valley was undergoing a severe decline. Incidences and outbreaks of CTV isolates have been reported in many new citrus growing regions throughout the world (Davino et al., 2003; Papic et al., 2005).

Citrus Tristeza Virus Classification

Citrus tristeza virus is a member of genus *Closterovirus*, family *Closteroviridae* based on morphological, biological, molecular and phylogenetic analyses (Bar-Joseph et al., 1979a; Koonin and Dolija, 1993; Dolja et al., 1994). The *Closteroviridae* family contains more than 30 plant viruses with flexuous, filamentous virions and viruses have either a mono or bipartite genome and with positive-sense, single-stranded RNA (Bar-Joseph et al., 1989; Karasev, 2000). The Closteroviruses are most constantly found in the phloem and therefore are called phloem-limited (Esau, 1960). The Closteroviruses are transmitted by insects such as aphids, mealy bugs and whiteflies in a semi-persistent manner (Brunt et al., 1996). The virus particles of this group produce characteristic inclusion bodies in the infected cells (Bar-Joseph et al., 1979b).

CTV Host Range

Citrus tristeza virus (CTV) has a narrow natural host range and is essentially limited to the genus Citrus in the *Rutaceae*. Citrus tristeza virus infects most species, cultivars and hybrids of *Citrus* spp. Muller and Garnsey (1984). Some citrus relatives such as *Poncirus trifoliata* (L.) Raf., *Swinglea glutinosa* (Blanco) Merr., *Severinia buxifolia* (Poir.)Tenore and some pummelos [*C. grandis* (L.) Osb.] are reported to be resistant to CTV infection. Also, some hybrids between *P. trifoliata* and sweet orange or grapefruit have shown CTV resistance (Garnsey et al., 1987a; Garnsey et al., 1997). CTV has been inoculated into about 200 plant species outside the *Rutaceae*, but the virus only multiplies in some species of *Passiflora*, especially in *Passiflora gracilis* (Muller et al., 1974; Roistacher and Bar-Joseph, 1987a).

Morphological and Cytological Characteristics of CTV

The CTV genome is a single-stranded, positive-sense RNA virus about 20,000 nt in length. CTV virions are encapsidated with two coat proteins (CP), the 25-kDa major CP, that encapsidates about 95% of the genome, and the 27-kDa minor CP that encapsidates the remaining 5% of the 5' end of the genome (Febres et al., 1996; Satyanarayana et al., 2004). The viral particles are arranged in a "rattlesnake" structure (Agranovsky et al., 1995). Coat protein minor (CPm) accumulates in the host cell wall fraction (Febres et al., 1994). *Citrus tristeza virus* has long thread-like, flexuous particles about 2000 nm by 11 nm (Bar-Joseph et al., 1979a). The inclusion bodies are found in the phloem and phloem-associated cells (Schneider, 1959; Brlansky et al., 1988). The occurrence of the CTV inclusion bodies can be used as a method for rapid diagnosis of CTV (Brlansky and Lee, 1990). CTV produces distinct inclusion bodies that can be seen by light and electron microscopy (Garnsey et al., 1980; Brlansky, 1987; Brlansky et al., 1988). There are two types of the inclusion bodies presented as cross-banded patterns of aggregated virus particles and/or in aggregates of fibril-containing vesicles surrounded by

cytoplasmic membranes (Garnsey et al., 1980; Brlansky, 1987; Brlansky et al., 1988). Virus particles can easily be observed with the electron microscope (EM) in leaf-dip preparations from infected citrus plants (Figure 2-1), (Bar-Joseph et al., 1972).

CTV Symptoms

Citrus tristeza virus causes a range of symptoms depending on the host and the isolate. et al., 1994; Rocha-Pena et al., 1995). CTV symptoms range from symptomless or mild to death of trees on sour orange rootstock. The most important symptoms caused by different CTV isolates can be divided into five groups including mild vein clearing, seedling yellows (SY), stem pitting on grapefruit (SP-G) and on sweet orange (SP-O) and quick decline (QD). Mild vein clearing (Figure 2-2 A) symptoms in leaves are usually produced by some mild isolates even on the most sensitive host, Mexican lime (Bar-Joseph et al., 1989). The SY symptoms include severe chlorosis and stunting of sour orange (Figure 2-2 B), lemon and grapefruit seedlings (Roistacher, 1982). The SY symptoms can also be vein corking in Mexican lime (Figure 2-2 C). The SY symptoms are commonly observed in nurseries (Fraser, 1952) and greenhouses but they are not usually seen in the field (Roistacher, 1982). The SP disease is considered a serious problem caused by CTV because of the reduced tree vigor and the small fruits regardless of rootstock. Trees affected with CTV stem pitting strains do not decline severely, but have reduced fruit production and quality (Garnsey and Lee, 1988), (Figure2-2 G). The disease also induces leaf cupping, stunting, chlorosis, vein corking and pitting of scions especially grapefruit and sweet orange (Figure 2-2 D, E, and F) (Lee et al., 1994; Rocha-Pena et al., 1995). Sometimes, the longitudinal pits on the trunk are more pronounced producing a ropey appearance along with a reduction in fruit number and size (Figure 2-2 G). The histology of stem pitting caused by an Australian CTV isolate was studied in sweet orange using light and electron microscopy (Brlansky et al., 2002). Pits in the wood often contain a yellow gum, as shown by the scanning

electron microscopy and irregular growth of the phloem occurs in the area of these xylem pits (Brlansky et al., 2002). The QD symptoms are more severe and can occur on sweet orange, grapefruit and mandarin trees grafted onto sour orange rootstock (*Citrus aurantium L.*). The brown citrus aphid (BCA), *Toxoptera citricida* (Kirkadly) is considered the primary factor for the spread and increase of CTV quick decline isolates. The cause of the decline problem was unknown for many years and was thought possibly to be a graft incompatibility. The QD phenomenon develops from virus-induced phloem necrosis in the bark of the rootstock at the graft union that prevents the movement of carbohydrates from the canopy to the root system and causes the roots to starve. Depletion of starch in the root system causes the roots to degenerate and inhibits formation of new fibrous roots and trees decline rapidly (Figure 2-1 H) (Garnsey et al., 1987a; Lee et al., 1994; Rocha-Pena et al., 1995). A standardized panel of host range plants has been established by Garnsey et al., (1987b) to study the biological characteristics of different CTV isolates. The indicator plants include Mexican lime as a universal indicator for all the CTV isolates, sour orange for SY, Duncan grapefruit for SP-G, Madam Vinous for SP-O, and sweet orange grafted onto sour orange for QD (Garnsey et al., 1987a).

Transmission of CTV

Citrus tristeza virus is easily graft-transmitted among the compatible *Citrus* species (Roistacher, 1976) . The virus has been mechanically transmitted by stem-slash inoculation with partially purified preparations (Muller and Garnsey, 1984). In 1946, Meneghini was able to transmit tristeza using infected aphids and to prove the viral nature of tristeza. CTV is transmitted by many aphid species (Blackman and Eastop, 1984; Viggiani, 1988). The most important species of aphids that can transmit CTV in nature include *T. citricida*, *Aphis gossypii*, *A. spiraecola*, and *T. aurantii* (Roistacher and Bar-Joseph, 1987b; Brunt et al., 1990). *Toxoptera citricida,* the brown citrus aphid (BCA), is the most efficient vector of CTV, transmitting the

virus in a semi-persistent manner. Efficient transmission of CTV requires 30 min to 24 h of acquisition feeding (Sasaki, 1974; Roistacher and Bar-Joseph, 1987a)**.**

The aphid usually retains the ability to transmit the virus for one to three days after acquisition (Yokomi et al., 1994). Brown citrus aphid was first reported in 1946 in Brazil. Transmissibility of CTV by aphid is affected by donor and receptor host species and environmental conditions (Bar-Joseph et al., 1977) and the virus strain (Roistacher and Bar-Joseph, 1984). Over the years, BCA has been responsible for the natural spread of CTV in most citrus-growing areas including South America (Costa and Grant, 1951), Australia, and Asia (Tanaka, 1969) and South Africa (McClean, 1975). The BCA moved to Central America and the Caribbean Basin in the 1990s, (Yokomi et al., 1994; Rocha-Pena et al., 1995) and was reported in Florida in 1995 (Halbert and Brown, 1996).

Virus, Vector, and Plant Interactions

CTV transmission efficiency is affected by the species of aphid, the donor, the receptor plant, and the CTV isolate. Sweet orange is more suitable for acquisition and more sensitive to infection than grapefruit or lemon seedlings (Bar-Joseph et al., 1989). There is also a noticeable decrease in transmission from plants maintained at higher temperatures (Bar-Joseph and Lee, 1989). Red grapefruit cultivars present a problem with cross protection strategies due to the slow distribution of protecting CTV isolates throughout the plant (Lee et al., 1987; Broadbent et al., 1995). Pigmented grapefruits are more sensitive to stem pitting symptoms than non-pigmented grapefruit (Marais and Breytenbach, 1996). Moreover, grapefruits have been shown to influence the strain composition of CTV isolates (Van Vuuren and van der Vyver, 2000).

Detection of CTV

Several techniques have been developed for the detection and differentiation of CTV isolates. Garnsey et al., (1987b) succeeded in establishing a set of host range standards for

indexing purposes with the Mexican lime as the universal indicator for CTV. Although this is a reliable approach, it is expensive and time consuming for large-scale experiments. The presence or absence of inclusion bodies can be used as a method for rapid diagnosis of CTV (Brlansky and Lee, 1990). *Citrus tristeza virus* produces two types of inclusion bodies that can be seen by Azure A staining or *in situ* immunofluorescence with light microscopy (Garnsey et al., 1980; Brlansky, 1987; Brlansky et al., 1988). Several serological techniques have been used to detect CTV since the 1970s (Bar-Joseph et al., 1979b; Garnsey et al., 1993). Improvement in virus purification allowed for the development of specific antibodies used in the serologically specific electron microscopy (SSEM) technique (Brlansky et al., 1984).

Enzyme-linked Immunosorbent Assay (ELISA) and direct tissue blot immunoassay (DTBIA) using polyclonal and monoclonal antisera are used commonly to detect CTV infection (Cambra et al., 1991; Cambra et al., 2002)**.** The specific monoclonal antibody MCA -13 was developed against the T36 isolate of CTV, which differentiates mild from severe CTV isolates in Florida (Permar et al., 1990). In the Florida bud wood certification program, MCA-13 positive trees cannot be used for propagation. In spite of this, some CTV isolates have been reported to cause decline on sour orange and yet are MCA- 13 negative (Hilf and Garnsey, 2002). Brown citrus aphid (BCA) has been reported to separate the mixtures of CTV genotypes from field isolates and severe sub-isolates hidden among the mild isolates have been detected from different CTV isolates (Brlansky et al., 2003). In addition, some other techniques such as polymerase chain reaction by two-step reverse-transcription RT-PCR have been used for the detection of CTV in host plants (Cevik, 1995; Metha et al., 1997; Hilf and Garnsey, 2000; Huang et al., 2004) and in aphids (Cevik, 1995; Metha et al., 1997; Hilf and Garnsey, 2000; Huang et al., 2004). Immunocapture (IC)-RT-PCR (Cambra et al., 2000; Cambra et al., 2002); and multiplex RT-

PCR (Roy et al., 2005) are also used to detect CTV. The Multiple Molecular Markers (MMM) method based on the amplification of molecular markers using sequence specific primers designed for the non-conserved regions of T36,VT, T30 and T3 isolates (Hilf and Garnsey, 2000) is also used in the detection and differentiation of CTV isolates. The single strand conformation polymorphism (SSCP) method is based on the difference in the mobility of ssDNA fragments on polyacrylamide gels due to their conformation under the electrophoresis conditions which depend on the nucleotide sequence. This technique is used to characterize population variants in CTV from different regions of the genome (Rubio et al., 1996; Rubio et al., 2000). Besides sequencing, several other methods including restriction fragment length polymorphism (RFLP) (Gillings et al., 1993)and hybridization with strain-specific probes (SSP) (Cevik, 1995) have been used to study the sequence variation of the CTV genome. The heteroduplex mobility assay (HMA) is another technique to estimate the genotype variation in human and plant viruses (Cai et al., 1991; Delwart et al., 1993; Lin et al., 2000; Berry and C., 2001). This method was applied to detect the unknown genotypes in mixtures of CTV isolates (Biswas et al., 2004). Conventional PCR techniques can detect low virus titer; however, they are not quantitative. On the other hand, the real-time PCR method allows rapid detection of target-specific amplicons and accurate quantification at the same time. Moreover, real-time qRT-PCR has been reported for the detection for viruses in different insect vectors (Boonham et al., 2002; Fabre et al., 2003; Olmos et al., 2005) as well as from different woody plants (Marbot et al., 2003; Schneider et al., 2004; Varga and James, 2005; Osman and Rowhani, 2006; Varga and James, 2006; Osman et al., 2007). There are some recent reports about using quantitative real time PCR to detect and quantify CTV (Ruiz-Ruiz et al., 2007; Saponari et al., 2008).

Genome Organization of CTV

Citrus tristeza virus is the largest known plant virus with a positive-sense RNA genome containing 19,296 to 19,302 nt, depending on the isolate (Karasev et al., 1995; Mawassi et al., 1996; Vives et al., 1999; Yang et al., 1999; Suastika et al., 2001). The large size of the CTV genome, the genome organization, the number and functions of the different genes, and the population complexity besides mechanisms of gene(s) expression are important molecular and biochemical aspects of CTV. The effect of these factors individually and/or combined with the disease development have been investigated (Satyanarayana et al., 1999).

Based on sequence analysis, the CTV genome is organized into 12 open reading frames (ORF) with the potential to code for 19 protein products (Pappu et al., 1994; Karasev et al., 1995). CTV genomic RNA has an untranslated region of 107 nt at 5'end of the genome (highly variable) and 3' UTR of 273 nt (highly conserved among CTV isolates) (Pappu et al., 1994; Karasev et al., 1995).

The CTV genome can be divided into four modules: the core module, the chaperon module, the upstream module and the CP (coat protein) module. The core module contains the domains of RNA-dependent RNA polymerase, helicase and methyl transferase that are all associated with virus replication. The chaperone module includes one heat shock protein 70 homolog (HP70), one protein distantly related to heat shock protein HP90, and a small protein with membrane-binding domains. The upstream module contains a domain of two papain-like proteases. The CP module consists of the major coat protein (p25) and the minor coat protein (p27) genes and four 3' terminal ORFs (Figure 2-3). The heat-shock protein 70 homolog (HP70h) is postulated to have a cell-to-cell movement function. In CTV, the HP70h, p61, CP and CPm are also required for efficient virion assembly (Satyanarayana et al., 2000). CTV p20 (ORF 10 product) is found in infected protoplasts and in CTV inclusion bodies (Gowda et al., 2000).

Mexican lime plants transformed with the CTV p23 gene exhibit typical CTV symptoms of vein clearing (Ghorbel et al., 2001), suggesting that the p23 is a symptom determinant. P20 and p23 have also been reported to have post-transcriptional gene silencing (PTGS) suppressor activity (Lu et al., 2003; Reed et al., 2003). CTV contains some genes (p6 and p20) that play a role in the systemic infection of CTV (Satyanarayana et al., 2008).

Replication of CTV

Replication of CTV as a positive-sense RNA virus starts by producing genome -length negative sense or complimentary RNA strands from the genomic RNA that acts as a template for positive-sense RNA synthesis. RNA-dependent RNA polymerase (RdRp), helicase and methyl transferase are involved in the replication process and encoded by ORF1a and ORF 1b (Figure 2-3). The large complex genome, the phloem-limited nature of the virus and the low concentrations in the infected plants has hindered the progress toward understanding the replication strategy of CTV. The development of a full-length cDNA infectious clone (Satyanarayana et al., 1999) and protoplast system for CTV replication (Price et al., 1996; Navas-Castillo et al., 1997) have been used to determine the function of some of the replication-associated genes. This replicon provides a model system for manipulation and studying replication at the cellular level (Bar-Joseph et al., 2002). p23 has been shown to be involved in the asymmetrical accumulation of RNA (Satyanarayana et al., 2002b) and elucidated replication signals present in the 3' UTR for replication (Satyanarayana et al., 2002a). ORF 1a and 1b are necessary for the replication process (Satyanarayana et al., 1999). Moreover, Cis-acting sequences, present at the 3’ and 5’UTR of CTV genome have been proven to be required for replication (Satyanarayana et al., 1999; Ayllon et al., 2001).

CTV-infected plants usually contains defective RNA (D-RNA) that results from both genomic RNA termini with extensive internal deletions of up to 17 kb (Ayllon et al., 1999b).

Yang et al., (1997) reported the involvement of CTV ORF 11 subgenomic RNA (sgRNA) as building blocks in the recombination process leading to the generation of D-RNAs. These D-RNAs are thought to be created by the general recombination mechanisms (Nagy and Simon, 1997; Ayllon et al., 1999b).

CTV Gene Expression Strategies

Open reading frame (ORF 1a is expressed as a 349- kDa polyprotein and includes two papain-like proteases, helicase-like and methyl transeferase-like domains. ORF 1b encodes an RNA-dependent RNA polymerase (RdRp) via a +1 ribosomal frame shift (Karasev et al., 1995; Cevik, 2001). The 3' ORFs are expressed via positive and negative sense strands at the 3' co-terminal subgenomic RNAs (Karasev et al., 1995). Different 3' co-terminal sgRNAs are present as dsRNA in abundant quantities in infected plants. The sgRNAs for p20 and p23 are expressed at higher rates followed by the two CP (p25 and p27) gene sgRNAs (Hilf et al., 1995; Pappu et al., 1997). Overall, CTV produces a complex array of RNAs including a full-length complementary, negative-sense RNA that acts as a template for further transcription and single and/or double-stranded subgenomic RNAs (Hilf et al., 1995; Mawassi et al., 1995a) and positive-sense large molecular weight transcripts (LaMTs) and low molecular weight transcripts (LMTs) (Mawassi et al., 1995b; Che et al., 2001) (Che et al., 2001; Mawassi et al., 1995b). Approximately 35 RNA species have been shown to be produced during CTV replication (Petersen, 2003).

Genetic Diversity of CTV

Citrus tristeza virus isolates usually contains complex populations of distinct genotypes possibly due to multiple aphid transmissions, the perennial nature of the host, and vegetative propagation and genetic properties of the virus such as defective RNAs (D-RNAs) formation and recombination (Cevik, 2001). The complexity of CTV populations causes problems for diagnosis

and strain identification, therefore understanding the disease mechanisms and symptom development in different host plants is important. Several studies on the sequence variability among CTV isolates have been performed using the coat protein gene sequences (Cevik, 1995; Cevik et al., 1996a; Cevik et al., 1996b). Comparison of the CP sequences from several biologically and geographically CTV isolates showed that there is a minor sequence difference in the CP genes with different biological characteristics. This suggested that minor differences related to a specific biological activity may be involved in those biological characteristics of the CTV isolates (Cevik et al., 1996a).

CTV field isolates usually contain multiple genomic variants, which can be separated upon grafting to different host plants (Moreno et al., 1993) or aphid transmission (Tsai et al., 2000; Brlansky et al., 2003). Uneven distribution of the genomic RNA variants of CTV within the infected plant and the selectivity of aphid transmission change the population (d'Urso et al., 2000). Variable differential distribution of the genomic RNA variants in different plant parts may result in acquisition of different viral populations by aphids, depending on the vector probing site. Also, the high selectivity of individual aphids to CTV genotypes may change the population diversity of the variants (Moreno et al., 1993; d'Urso et al., 2000). Moreno et al., (1993) showed that sub-isolates obtained from mild CTV isolates by several host passages were more severe and expressed stem pitting. Also, Broadbent et al., (1996) reported that single aphid transmissions of Australian CTV isolates using BCA separated some of the sub-isolates. Population diversity has been studied using several techniques such as peptide maps of the coat protein, hybridizations with cDNA probes, dsRNA patterns, SSCP and multiple molecular markers in an attempt to differentiate CTV isolates and strains (Lee et al., 1988; Moreno and Guerri, 1997; Hilf and Garnsey, 2000; Niblett et al., 2000). Graft and aphid transmissions have been reported

responsible for the haplotype (sequence variants) distribution and frequency using (SSCP) analysis of two genes, p18 and p21 (Ayllon et al., 1999a).

The complete sequences of several CTV isolates have been reported: T36 (19,296 nt) and T30 (19,259 nt) from Florida (Pappu et al., 1994; Karasev et al., 1995; Albiach-Marti et al., 2000), VT isolate (19,226 nt) from Israel (Mawassi et al., 1996), T385 (19,259 nt) from Spain (Vives et al., 1999), SY568 (19,249 nt) from California (Yang et al., 1999), and Nuaga isolate (19,302 nt) from Japan (Suastika et al., 2001). The genomic organization in all the sequenced isolates of CTV was similar, but the genomic sequences were significantly different (Mawassi et al., 1996; Vives et al., 1999). CTV field isolates usually contain mixtures of different populations and may contain multiple defective RNAs (D-RNAs) (Mawassi et al., 1995a; Mawassi et al., 1995b) . From this mixture, strains of CTV having distinct properties can be selected resulting in change of the viral strains in different parts of the infected plants (Hilf et al., 1999). It is not known whether symptom development is due to the predominant strain or to the viral population, the combination of genomic RNA and defective RNA or other factors (Albiach-Marti et al., 2000). Since some CTV strains are more efficiently transmitted by certain aphid species, the structure of a population may change by time. Overall, CTV is one of the most diverse and highly complex plant RNA viruses. The multiple genotypes found in field samples, the numerous RNA species present in infected tissue, and the unknown function of most of its genes leave many questions about the virus biology, the infection process and the disease mechanisms. In this study, the multiple molecular markers (MMM) and the heteroduplex mobility assay were used to study the genetic diversity of the CTV isolate and sub-isolates in mature local sweet orange field trees and the movement of identified CTV isolates from the sweet orange interstock into new hybrid rootstock candidates top-worked onto these trees. Also, nucleotide sequence

analysis was also used to validate CTV strain differentiation and estimation of the molecular genetic variation (Rubio et al., 2001).

CTV Control

A number of management strategies have been developed for CTV control in order to minimize economic losses. These strategies are available for use based on the absence or presence of CTV in different citrus-growing areas (Bar-Joseph and Lee, 1989; Lee and Rocha-Pena, 1992). The strategies include quarantine and budwood certification to prevent the introduction of CTV, eradication programs to prevent the spread of the virus, the use of mild strain cross protection (MSCP), the use of CTV-tolerant rootstocks, breeding for CTV resistance, and genetic engineering (Bar-Joseph and Lee, 1989; Lee and Rocha-Pena, 1992).

Cross protection is the phenomenon in which a plant previously infected with a mild strain of the virus is protected against the infection by other more severe strains of the same virus or closely related viruses (Fulton, 1986). Different temperature regimes and field site conditions have to be tested for the mild strain before such CTV isolates are evaluated as a management strategy (Powell et al., 1992). Mild strain cross protection has been applied in several countries including Brazil, India, Australia, South Africa and Japan (Rocha-Pena et al., 1995). Without CTV cross-protection, grapefruit production would be uneconomic in South Africa due to stem pitting disease (Von Broembsen and Lee, 1988; Van Vuuren et al., 1993; Van Vuuren and da Graça, 2000). Cross protection has value only for stem pitting disease and has not proven effective against quick decline disease as evidenced by the breakdown of mild strain cross-protection in Florida (Lee et al., 1996).

Genetic Engineering for CTV Resistance

Recent advances in plant molecular biology and genetic engineering are providing new approaches and are opening new avenues for the generation and the evaluation of transgenic

plants for virus resistance outside of conventional breeding methods (Cevik, 2001). Genetic engineering allows the insertion of specific genes into the genome of currently successful cultivars, theoretically adding desirable traits without otherwise altering cultivar integrity. Genetic engineering has the potential for developing plants that have either host or pathogen-derived resistance against CTV infection. Virus resistance has been engineered in several plants by transferring genes or sequences from viruses and/or other sources (Fuchs and Gonsalves, 1997). The majority of transgenic plants engineered for virus resistance has been developed using sequences derived from plant viral genomes. Several citrus species have been transformed with either a functional or untranslatable coat protein (CP) gene of CTV (Moore et al., 1993; Gutiérrez et al., 1997; Domínguez et al., 2000; Ghorbel et al., 2000; Yang et al., 2000; Ghorbel et al., 2001; Dominguez et al., 2002; Herron et al., 2002; Febres et al., 2003; Batuman et al., 2006; Febres et al., 2008). The manipulation of non-structural genes, such as movement protein and replication-associated proteins such as RNA-dependent RNA polymerase (RdRp), is a promising strategy for developing virus resistance in transgenic plants (Beachy, 1994; Palukaitis and Zaitlin, 1997). Replicase-mediated and the RNA-mediated resistances were shown to be highly specific and effective only against the specific strain of the virus from which the transgenic sequences were obtained or against closely related strains of the same virus with a high degree of sequence homology (Audy et al., 1994; Zaitlin et al., 1994; Palukaitis and Zaitlin, 1997). Pathogen-derived resistance (PDR) has been found to be effective and reproducible in transgenic Mexican lime plants carrying the p25 CP gene of severe and mild isolates of CTV (Dominguez et al., 2002). Various degrees of resistance were reported (10-33%) whereas other transgenic plants showed a significant delay in virus accumulation and symptom development. Closteroviruses like CTV have been shown to suppress plant antiviral machinery at several

stages in the post-translational gene silencing (PTGS) pathway and might also have the capacity to silence other cellular nucleic acid 'invaders' (Herron, 2003). The CTV ORF 10 product, p20, has been demonstrated experimentally to have PTGS-suppressor function in *N. benthamiana* assays (Reed et al., 2003). Activity of these proteins is thought to occur after the Dicer-mediated dsRNA cleavage step in the PTGS pathway (Reed et al., 2003). Grapefruit (*Citrus Paradisi*) plants were transformed with several constructs derived from the CTV genome such as the RdRp construct containing the full length gene 1b, major coat protein (p25) and minor coat protein (p27), and then the transgenic plants were tested for their resistance to the virus. Most transgenic lines (27 lines) were susceptible, but a few (6 lines) were partially resistant and only one line, transformed with the 3' end of CTV, was resistant. The accumulation of siRNA has indicated that a PTGS mechanism is induced in these transgenic plants (Febres et al., 2008).

Natural Resistance and Breeding for CTV Resistance

There is no known genetic resistance in the genus *Citrus* that is effective against all CTV isolates, and CTV-infected citrus species and hybrids vary in their reaction from sensitive to tolerant (Muller and Garnsey, 1984; Mestre et al., 1997c). However, some citrus relatives, such as *P. trifoliata* (Tanaka et al., 1971; Hutchson, 1985; Kitajima et al., 1994), *Severinia buxifolia*, and *Swinglea glutinosa* (Muller et al., 1968; Salibe, 1977) are reported to be resistant or may be immune (meaning that they do not support virus replication) to CTV (Garnsey et al., 1987a). Of these three relatives, *P. trifoliata* is the only species that is routinely sexually compatible with citrus. Some hybrids between *P. trifoliata* and sweet orange or grapefruit are resistant to CTV infection. CTV is not able to replicate or cause symptoms in these hosts (Garnsey et al., 1987a).

The resistance found in *P. trifoliata* was conferred initially by a single dominant Mendelian gene designated *Ctv* (Gmitter et al., 1996; Fang et al., 1998). The development of CTV-resistant cultivars would provide the best long-term control but the integration of the CTV

resistance gene into new scion cultivars by conventional breeding will require several generations and much time to eliminate the undesirable fruit characteristics from *Poncirus* (Deng et al., 2001). Moreover, applying classical breeding is difficult because of the problems associated with citrus breeding including large plant size, inbreeding depression, polyembryony, heterozygosity, sterility, self- and cross-incompatibility and a long juvenility period (Soost and Roose, 1996). On the other hand, the progress that has been made toward mapping the location of *Ctv* gene (Deng et al., 1996; Gmitter et al., 1996; Fang et al., 1998; Deng et al., 2001b; Deng et al., 2001a; Fagoaga et al., 2005) makes cloning of the gene and using it to transform commercially important citrus cultivars a reality. The region containing this gene has been mapped, and markers flanking and co-segregating with *Ctv* have been developed (Fang et al., 1998). Further studies suggested that in *P. trifoliata* var 'Flying Dragon', there are at least two genes responsible for CTV resistance based on the short distance accumulation observed in some Ctv-Rr progeny segregant plants derived by self-pollination. Bulked segregant analysis of this population identified five RAPD markers linked to another locus called Ctm that is located in a different linkage group from the *Ctv* resistant gene (Mestre et al., 1997b). The fact that CTV can replicate in protoplasts of CTV-resistant plants (Albiach-Marti et al., 1999), has raised questions as to whether *Ctv* confers resistance by blocking virus replication or by interfering with virus loading or unloading from the phloem (Mestre et al., 1997a). Deng et al., (2000) identified 22 sequences similar to the nucleotide binding site-leucine rich repeat (NBS-LRR) class resistance gene in the citrus genome with one of the fragments being closely linked and another co-segregating with *Ctv* gene. Different bacterial artificial chromosome libraries have been developed and some BAC clones and BAC contigs containing resistance gene candidates have been characterized to further identify resistance genes to CTV (Deng et al., 2001a; Yang et al.,

2001). The *Ctv* locus was localized within a genomic region of approximately 180 kb (Deng et al., 2001a). Advanced studies on the resistance gene found in *P. trifoliata* revealed several resistant gene candidates for CTV. Five resistance genes (R1–R5) with complete ORFs have been identified and can be considered as candidates for *Ctv* (Yang et al., 2003). Refinement of genetic maps has delimited this gene to a 121-kb region composed of ten candidate *Ctv* resistance genes (Rai, 2006).

The Quick Decline Problem and Its Impact on Florida Citrus Industry

Citrus tristeza virus is one of the most severe pathogens affecting citrus worldwide. CTV is a major cause of the decline and eventually death of citrus trees on sour orange rootstock. Initially declining trees exhibit small leaves, heavy fruit set with small fruits and honeycombing on the inside face of the bark from the rootstock side of the budunion. The decline results from phloem necrosis at the budunion, preventing the transportation of starch and sugars to the roots and causing starch depletion in the roots. Then the death of the feeder roots leads to the ultimate death of the tree. Trees on sour orange rootstock are primarily affected by CTV-QD. Sweet oranges are more affected than grapefruit whereas lemons on sour orange rootstock, for example are not affected by CTV-QD (Brlansky et al., 2008; Futch and Brlansky, 2008). During the 1940s and 1950s more than nine million sweet orange trees on sour orange rootstock were destroyed by CTV-QD in Brazil and the Brazilian citrus industry was almost wiped out (Bove and Ayres, 2007). Tristeza was first reported in Florida in the 1950s and in 1980 it produced a great loss due to the quick decline problem caused by CTV (Futch and Brlansky, 2008). The total number of trees killed in South America was around 25 million and reached 100 million world-wide (Bove and Ayres, 2007). The introduction of the brown citrus aphid vector to Florida in 1995 has caused the spread of severe CTV strains including the quick decline-inducing isolates. Sour orange was the most important rootstock worldwide because it offers many desirable

horticultural traits, tolerance to Phytophthora diseases as well as to citrus blight, and its adaptation to virtually all soil conditions. Unfortunately sour orange is susceptible to CTV-QD disease (Stover and Castle, 2002). Therefore, sour orange rootstock use in new plantings has been virtually eliminated in Florida (Brown and Spreen, 2000). The remaining sour orange-rooted trees (approx. 15 million) in Florida are expected to die within the next decade due to QD (Grosser et al., 2004a). CTV is also threatening the citrus industry in other citrus growing areas such as Mexico and Texas since more than 95% of their citrus trees are on sour orange rootstock (Grosser et al., 2004a). Figure (2-4) shows the decline of sour orange rootstock usage and Figure (2-5) shows the increase in severe CTV infections [Citrus Budwood Registration Bureau (CBRB)], (Annual Report, 2003). As a result of the loss of sour orange, often less desirable rootstocks are currently used (Bauer et al., 2005).

The Current Rootstocks in Florida

The rootstocks commonly used in Florida often do not satisfy all selection criteria for citrus production in a specific location, because the top rootstocks are trifoliate hybrids which are not adapted to high pH, calcareous soils (Grosser and Chandler, 2000; Grosser et al., 2004a; Bauer et al., 2005) The ten top current rootstocks used in Florida are 'Swingle' citrumelo, 'Carrizo' citrange, 'Kuharske' citrange, 'Cleopatra' mandarin (Cleo), 'Volkamer' lemon, US-812, Sour Orange, Sun Chu Sha mandarin and US-802 (CBRB), (Annual Report, 2007). In addition, 'Benton' citrange, 'C-32' citrange, 'C-35' citrange, Cleopatra x Trifoliate (TF); (X639), Goutou, Kinkoji, 1584 (TF x Milam), US-852 (Changsha x TF), US-897 (Cleo x TF), Smooth Flat Seville and trifoliate orange rootstocks (Castle et al., 2006) are being used to a lesser extent. The attributes of some of the common rootstocks in Florida are summarized by Castle et al., (2006) and are presented in Table (2-1). Swingle was developed by crossing *C. paradisi* and *P. trifoliata*, and became widely planted starting in the late 1980s (Figure 2-4) as a CTV-resistant

productive rootstock with good yield and fruit quality (Fallahi et al., 1989; Castle et al., 1993). Swingle citrumelo rootstock has been the most popular commercial rootstock in Florida (Annual Report, 2007), however, Swingle was reported to perform poorly in high pH, calcareous soils in the flatwoods areas of Florida (Castle and Stover, 2001; Bauer et al., 2005). Carrizo (*Citrus sinensis x P. trifoliata*) rootstock is also CTV resistant, but susceptible to citrus blight (Castle, 1987; Castle and Tucker, 1998). Cleopatra mandarin (*C. reticulata*) rootstock is tolerant to CTV, but trees on this rootstock are often debilitated by Phytophthora diseases and blight (Bowman and Roman, 1999; Castle et al., 2006). In more challenging soils, the current top rootstocks, especially for sweet orange and grapefruit scions, have proven to be inadequate replacements for sour orange. Therefore, development of a replacement rootstock that can be used in high pH soils and has adequate disease resistance especially to CTV- QD has become a primary breeding objective (Grosser et al., 2004b).

Building QD-resistant Sour Orange-like Rootstocks Using Conventional Breeding and Somatic Hybridization

Citrus rootstock improvement is difficult and time consuming because the large number of traits needed including tolerance to diseases such as citrus tristeza virus, *Phytophthora* spp., citrus blight, Diaprepes, nematodes, and huanglongbing (citrus greening), and adaptation to challenging and/or high salinity soils while retaining the ability to produce high yielding trees with quality fruit. In addition, the ability to produce nucellar seeds and to control tree size must be combined in any successful new rootstock for citriculture in Florida (Grosser et al., 2003; Ananthakrishnan et al., 2006). Approaches such as conventional breeding and somatic hybridization are being used to develop new rootstocks in an attempt to provide the best rootstocks for citrus. A wide range of new citrus rootstock germplasm has been developed by the University of Florida and the Agricultural Research Service of the U.S. Department of

Agricultural (USDA-Natural Resources Conservation Service) (Bowman and Rouse, 2006). Approaches such as conventional breeding and somatic hybridization are being used to develop these new rootstocks in an attempt to provide the best rootstock for citrus, and some of the new advanced selections are currently being evaluated in different locations around the state (Grosser and Gmitter, 1990; Gmitter et al., 1992; Louzada et al., 1992; Grosser et al., 1994; Grosser et al., 1995; Grosser et al., 1996; Grosser et al., 1998; Bowman and Roman, 1999; Wutscher and Bowman, 1999; Bowman, 2000; Grosser and Chandler, 2000; Bowman and Garnsey, 2001; Bowman et al., 2002; Grosser and Chandler, 2002; Grosser et al., 2003; Grosser et al., 2004a; Medina-Urrutia et al., 2004; Ananthakrishnan et al., 2006; Bowman and Rouse, 2006; Bowman, 2007; Grosser et al., 2007a; Grosser et al., 2007b).

Using conventional breeding, the USDA has assessed a few thousand candidate 'super' sour orange hybrids and has identified to date 300 hybrids for further evaluation (Bowman 2007). US-812 is a newly released citrus rootstock from the USDA, developed by crossing Sunki mandarin (*C. reticulata*) and Benecke trifoliate orange (*P. trifoliata*). It is highly tolerant to CTV and citrus blight, gives good fruit quality with high yield, provides moderate tree size, and seems to have broader soil adaptation than other popular trifoliate hybrid rootstocks. This rootstock was released by the USDA in May 2001 (Bowman and Rouse, 2006).

Somatic Hybridization and Breeding at the Tetraploid Level with a Focus on Mandarin + Pummelo Combinations

Somatic hybridization is a powerful approach that can overcome the sexual barriers associated with conventional breeding (Saito et al., 1991; (Grosser and Gmitter, 1990; Saito et al., 1991). For the past several years, developing superior sour orange-like rootstock hybrids has been a primary goal of the citrus rootstock improvement program, a successful program based on somatic hybridization that has been led by Dr. Jude Grosser at the University of Florida, IFAS;

Citrus Research & Education Center. A primary focus of this program has been citrus rootstock improvement (Grosser et al., 2000; Grosser and Chandler, 2002).

The somatic hybridization approach has been used to produce allotetraploid hybrids and subsequently "tetrazygs" that are zygotic tetraploid hybrids produced from conventional crossing of allotetraploid somatic hybrids (Grosser and Gmitter, 1990; Grosser and Chandler, 2000; Grosser et al., 2003). Citrus rootstock breeding and selection at the tetraploid level is a very useful approach allowing the mixing of the genetic pool of three or four parents. Allotetraploid hybrids produced by somatic hybridization combine the intact nuclear genomes of the complementary parents in order to overcome a weakness in one parent by complementation (Grosser and Gmitter, 1990; Grosser and Chandler, 2000). Molecular marker studies indicated that sour orange is probably a hybrid of pummelo and mandarin (Nicolosi et al., 2000). Therefore, mandarin and pummelo parents were selected for desirable rootstock attributes and these were combined to develop mandarin + pummelo somatic hybrids (Grosser et al., 2004a; Ananthakrishnan et al., 2006; Grosser et al., 2007b; Chen et al., 2008) in attempt to develop an adequate replacement for sour orange. To date, more than 100 allotetraploid somatic hybrid combinations have been tested for their rootstock potential with several hybrid selections showing promise, as they have been screened and show a tolerance to the Diaprepes/Phytophthora complex (Grosser et al. 2003, 2007). Fruit collection from these hybrids (propagated by top-working) followed by seed germination showed that several tetraploid hybrids were able to produce nucellar seeds (Grosser et al., 2007b).

Several combinations of superior pummelo seedlings with [(Changsha and Amblycarpa) mandarins; 'Murcott' and 'W. Murcott' tangors, and 'Page' tangelo] were developed using somatic hybridization. Pummelo zygotic seedlings (*C. grandis),* selected from a greenhouse

screening for soil adaptation and Phytophthora resistance, were used as leaf parents in somatic hybridization experiments. Some of these pummelo selections also showed resistance/tolerance to CTV-induced quick decline after 2 years in the field. The mandarin-type parents were chosen for their performance in the protoplast system and general rootstock performance with wide soil adaptation (Grosser et al., 2003; Grosser et al., 2004a; Ananthakrishnan et al., 2006; Grosser et al., 2007b).

What Will These New Rootstock Candidates Provide?

Better rootstocks for citriculture should offer improved yield and fruit quality, better adaptation to different soil conditions, tolerance to diseases and tree size control (Wheaton et al., 1991). For example, new combinations of mandarins with pre-selected pummelos at the tetraploid level are expected to provide new sour-orange-like rootstocks with improved disease resistance and the ability to control tree size (Grosser et al., 2000). A recent study by Grosser et al., (unpublished data) on the effect of polyploidy on tree size on 4-7 year old sweet orange trees was conducted. The results for the tested somatic tetraploid hybrids, (based on % of Carrizo average canopy volume) – sweet orange scion showed a dramatic decrease in the size of the trees, ranging from 29-85% of Carrizo-size. The polyploid hybrids of two diploid rootstocks reduce the size of the sweet orange scion as compared to either of the diploid rootstocks alone. For example, using the Cleopatra mandarin (Cleo) + Carrizo somatic hybrid rootstock gave 61% which is lower than Cleo (100%) or Carrizo alone (100%); Cleo + Swingle gave 35% and Swingle alone was 78%. The same trend was seen with Milam+ Kinkoji which gave 42% where Kinkoji alone was 95% (Grosser et al., unpublished data). The small test trees were obtained on a somatic hybrid of sour orange + Benton citrange (29%). Using the conventional breeding and somatic hybridization techniques will make many rootstock options available in the future (Stover and Castle, 2002).

Dissertation Objectives

Previous efforts to screen new hybrid rootstocks in the greenhouse for resistance to tristeza quick decline (QD) have been confounded by seedling yellows. Also, several studies have shown that inoculation of sweet orange grafted on sour orange with CTV quick decline- inducing isolates does not induce decline in the greenhouse. Recently, a new procedure was used where sour orange was budded into the infected sweet orange (reciprocal budding) with different CTV isolates to screen for the ability of these isolates to cause decline (Pina et al., 2005).

The main objective of the present work was to develop a reliable assay in the field (onto non-juvenile trees) in order to bypass the seedling yellows problem caused by some CTV decline isolates (i.e.T36) in greenhouse assays (Garnsey, 1990). The top-working procedure was done by grafting buds of the new rootstock candidates onto 15-year- old field trees that showed a mixture of T30, T36, and VT genotypes of CTV. The goal was to screen new rootstocks to find a QD resistant potential replacement for sour orange and to study the citrus hybrid/CTV isolate interactions at the molecular level to learn more about tolerance/resistance mechanisms. Focus was on the evaluation of allotetraploid hybrids obtained primarily from somatic hybridization, 'tetrazygs' hybrids from crosses of somatic hybrids, and a few selected open-pollinated, tetraploid seedlings from a selected mandarin + pummelo somatic hybrid female (Table 3-1).

The specific goals were the following:-

- Serological studies of CTV isolates to determine the virus titer in the source and rootstock candidates; trees produced by top-working.
- Molecular characterization of CTV isolates by using multiple molecular markers methods (MMM) on the source tree and the grafted rootstock candidates.
- Molecular characterization of CTV isolates by using the heteroduplex mobility assay to determine which CTV genotypes moved from the sweet orange interstock into the grafted materials.

- Detection of citrus tristeza virus (CTV) by using quantitative real time PCR (qRT-PCR) to determine the level of resistance or tolerance in the new rootstock candidates.

- Biochemical studies on CTV-infected rootstock candidates inoculated in the greenhouse with quick decline-inducing isolates to determine the effect of CTV infection on total carbohydrate content in the leaves and the roots based on the previous study by Garnsey and Young (1975) on the starch reserves in roots from citrus trees affected by tristeza quick decline isolates.

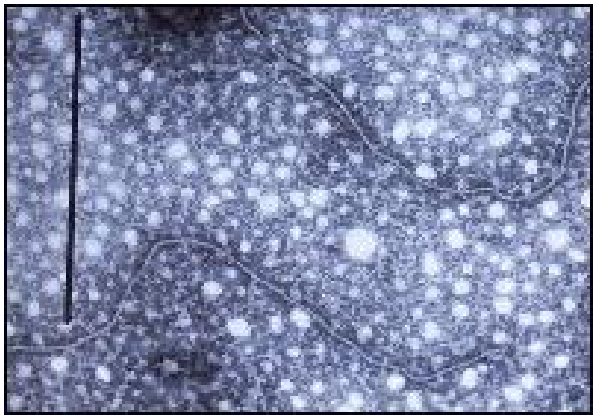

Figure 2-1. Citrus tristeza virus as seen with a transmission electron microscope (TEM) after positive staining. The bar equals 55 nm. CTV is a long flexuous rod about 11 X 2,000 nm. Photo downloaded from http://edis.ifas.ufl.edu/CH089 website (P.D. Roberts, R.J. McGovern, R.F. Lee and C.L. Niblett).

Figure 2-2. Symptoms caused by *Citrus tristeza virus*. A) Vein-clearing symptoms in the leaf of a Mexican lime seedling (Lee, R.F.). B) Seedling yellows reaction on sour orange seedlings in the greenhouse (Roistacher, C.N.). C) Vein corking symptoms on leaves of a Mexican lime seedling inoculated with a very severe seedling-yellows tristeza isolate (Roistacher, C.N.). D) Stem pitting on grapefruit due to CTV virus in Venezuela (Lee, R.F.). E) Stem pitting on Pera sweet orange, occurring in Brazil (Lee, R.F.). F) Stem pitting causing a ropey appearance of a Marsh grapefruit trunk in South Africa (Lee, R.F.). G) Grapefruit collected from a Marsh grapefruit tree on rough lemon rootstock in Colombia affected by stem pitting strains of tristeza (Lee, R.F.). H) Sweet orange tree on sour orange rootstock with tristeza-induced quick decline (Lee, R.F.). Photograph in this figure were downloaded from http://www.ecoport.org . The supplier of the photograph is given in the parenthesis.

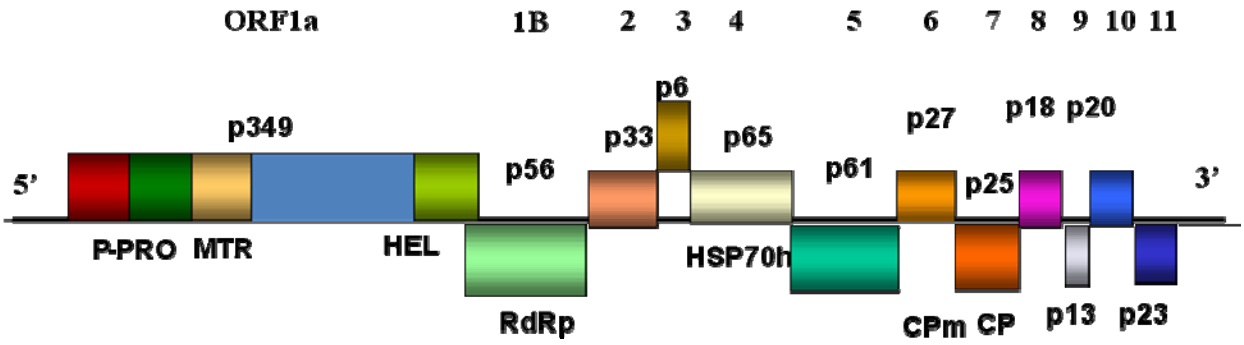

Figure 2-3. Citrus tristeza virus (CTV) genome shown the two papain-like proteases, the methyl transferase, Helicase RNA-dependent RNA polymerase (RdRp) and open reading frames (ORFs 1a, 1b, and 2-11). Diagram was adapted from (Satyanarayana et al., 1999).

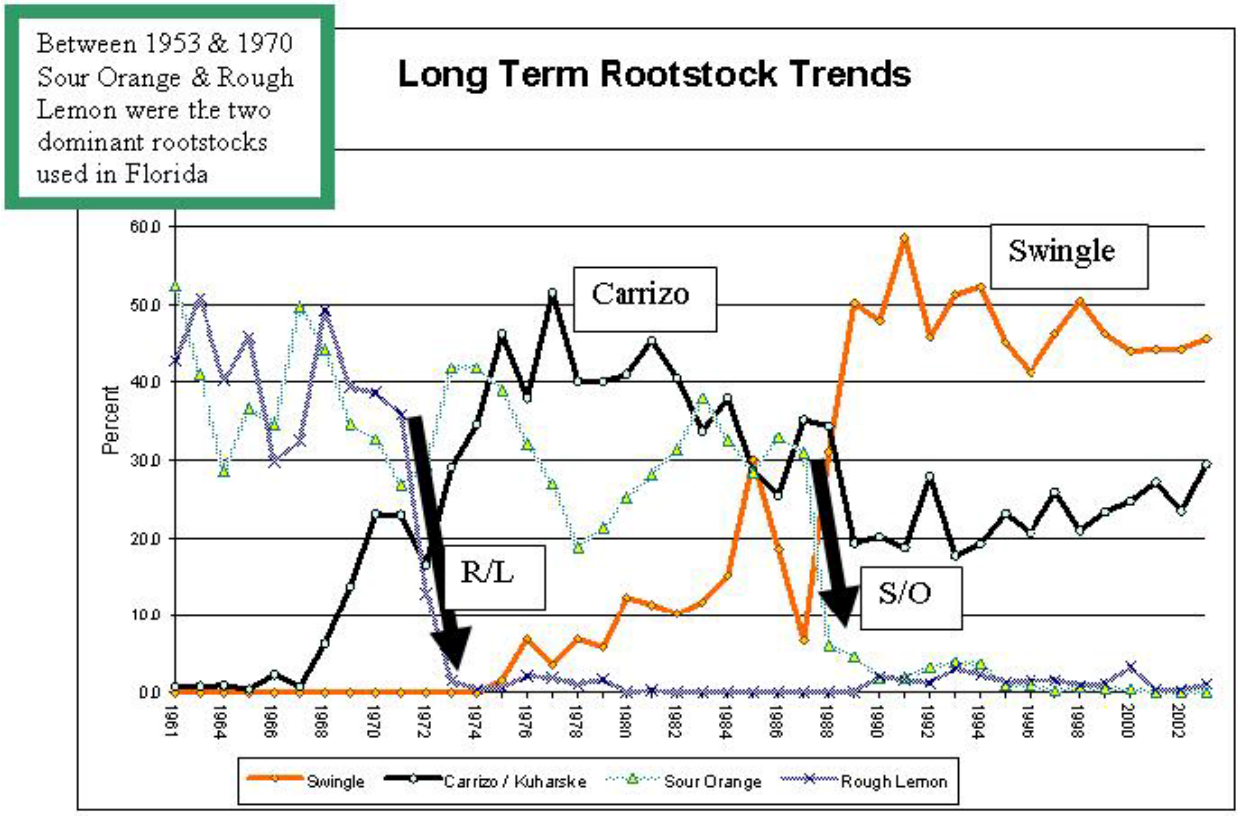

Figure 2-4. Long term rootstock trends CBRB, (Annual Report, 2003)

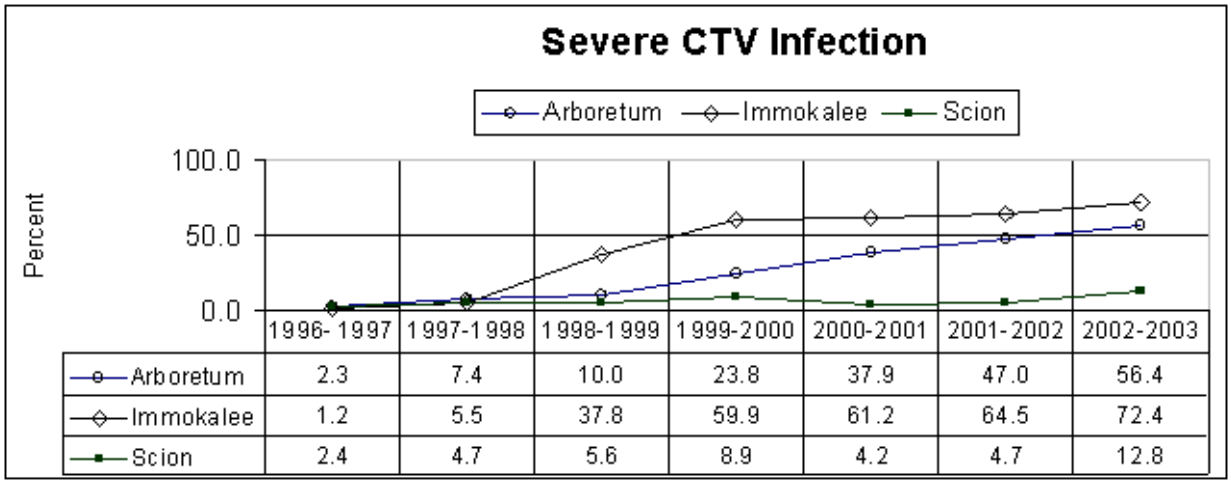

Figure 2-5. CTV infection trend with severe isolates.

Table 2-1. Characteristics of the top-ten citrus rootstocks of citrus in Florida adapted from (Castle et al., 2006).

Characteristics	Rootstock									
	Swingle Citrumelo	Carrizo citrange	Kuharske citrange	Kinkoji	Cleopatra mandarin	Volkamer lemon	US-812 (Sunki x Benecke TF)	Sour Orange	Sun Chu Sha mandarin	US-802 (Pummelo x TF)
Salinity	P	P	(P-I)	?	G	I	?	I	(I)	?
High pH	P	P	(P)	(I)	I	T	G	G	I+	(I)
Clay soil	P	P	?	(G)	G	I	(I)	G	G	(G)
Freezes	G	G	(G)	?	G	P	(G)	G	(G)	G
Tree size	I	Lg	Lg	I	Lg	Lg	I	I	Lg	Lg
Yield/tree	I	H	(H)	(I)	L-I	H	H	I	L-I	H
Juice quality	I	I-H	I	L-I	H	L	H	H	H	L-I
Blight	T	I	?	?	S-T*	S	G	G	?	G
Phytophthora nicotianae (foot and root rot)	T+	I	T	T	S	T	T	T**	S	T
P. palmivora/ root weevil complex	(S)	(S)	(S)	(S)	(S)	(S)	(S)	T	(S)	(T)
Burrowing nematode	S	T	T+	(S)	S	S	?	S	S	?
Citrus nematode	T	T	(T)	(S)	S	S	T	S	S	T
Xyloporosis	T	T	(T)	(T)	T	T	?	T	T	?
Exocortis	(T)	S	(S)	(T)	T	T	?	T	T	?
Tristeza	T	T	(T)	T	T	T	T	S	T	T

Key to symbols: G= good; H= high; I= intermediate; L=low; Lg = large; P=poor; S=susceptible; T=tolerant; () = expected rating.

S-T* means that while incidence of blight is low among trees, substantial losses can occur when the trees are 12 to 15 years old the infection is high in trees

T**= Sour orange has good foot rot tolerance but mediocre root rot tolerance.

CHAPTER 3
DEVELOPMENT OF A TOP-WORKING METHOD AND BIOCHEMICAL STUDIES TO EVALUATE ROOTSTOCK CANDIDATES FOR CITRUS TRISTEZA VIRUS (CTV) QUICK-DECLINE (QD) RESISTANCE IN EFFORTS TO REPLACE SOUR ORANGE

Introduction

Changing the cultivar of an existing tree is known as top-working. Top-working has been done in several crops such as pine trees (Bramlett and Burris, 1995); pears (XinZhong et al., 2005); apple trees (Blazek, 2002); walnut (Rezaee, 2008) and citrus (Button, 1975). Both rootstock and the interstock must be compatible with the new top, and compatibility of various citrus combinations was studied by Tanaka (1981). In citrus, the top-working of established citrus trees is sometimes desirable for a number of reasons. For example, it is advantageous to change to a different variety when the original selection is nonproductive, or of poor quality (Opitz, 1961). Trees threatened by virus disease may be saved by top-working to a tolerant scion (Platt and Opitz, 1973). Several procedures including T- budding and grafting can be used to top work citrus trees, but some of these procedures require considerable horticultural skills. Top-working trees usually become productive sooner than nursery trees because of the already well-established root system (Platt and Opitz, 1973).

In this study, top-working technique was applied as a new method to screen new citrus rootstock candidates developed for quick decline (QD) disease caused by citrus tristeza virus (CTV) resistance in an effort to find a replacement for sour orange. Previous efforts to screen new hybrid rootstock candidates in the greenhouse for resistance to quick decline have been confounded by another less important CTV disease called seedling yellows (Garnsey, S. M; unpublished data). Other researchers reported on the difficulty and the length of time in inducing QD symptoms in sweet orange grafted onto sour orange rootstock under greenhouse conditions (Pina et al., 2005). Therefore, top-working was used here in an effort to develop a reliable assay

for QD resistance. An added benefit of this approach is that the end result is a seed producing tree of any new rootstock candidate showing resistance to quick decline.

Materials and Methods

Top-working

Pre-selected rootstock candidates developed mainly via protoplast fusion (Table3-1) were top-worked using the hanging bud method (Fig. 3-1) onto 15 year old 'Hamlin'/Carrizo trees infected by three different strains of CTV common to Florida (T30, T36 and VT); the three CTV isolates important in Florida (T30, T36 and VT). Seventy- two selections, including parental pummelos and pre-selected sour-orange-like pummelo-mandarin rootstock hybrids produced *in vitro* via protoplast fusion (Figure 3-1) were used. The germplasm included in the present CTV study was divided into different categories including selected zygotic pummelos (somatic hybrid parents), somatic hybrids, tetrazygs (zygotic tetraploids from crosses of two somatic hybrids), diploid hybrids, and open pollinated tetraploids (Table 3-1).

Virus infection in the 'Hamlin' interstock was determined prior to top-working by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The 15-year old 'Hamlin' sweet orange trees were located in the North-40 research field, north of the Citrus Research and Education Center (CREC). The trees were pruned down to 4 scaffolds. The top-working procedure using the hanging bud method (Figure 3-1) was applied one month after the pruning to allow the tree to recover from the shock of the severe pruning. One branch of each tree was dedicated for sour orange (control), and then the three remaining scaffold branches were all grafted with one rootstock candidate selection. Summer was the best season for grafting, therefore grafting was done in June and July. The buds were wrapped using the grafting tape for 3-4 weeks then they were unwrapped carefully. If the buds were alive and appeared to be well callused in, the budded limbs were shortened or girdled to stimulate bud growth. As the shoots

grew, they were tied to the stumps of the girdled and defoliated branches. Trees were painted white to reduce unwanted sprouting from the 'Hamlin' interstock.

The regular maintenance of the field including irrigation, fertilization, pesticide treatments and weed control were performed by the CREC grove crew according to a routine schedule. The new graft was maintained, observations of disease symptom development and shoot growth were recorded periodically. The data for the shoot growth were analyzed by one-way ANOVA (analysis of variance) using SAS (2000). Mean (average) values were separated using the Least Significant Difference (LSD) separation of means at a probability level of 0.05.

Seedling Yellows (SY) Assay

A small experiment in the greenhouse was carried out to evaluate some of the tested rootstock candidates (sub-population of the top-worked field study selections), to study their SY reaction in the greenhouse. Seedlings of nine different somatic hybrid rootstock candidates (A+ HBJL-1, A+ HBJL-3, Page + HBJL-3, A + SN7, A+ HBJL-5, A+ Chandler #A1-11, A+ 4-4-99-6, A+ 4-3-99-2 and A+ 7-2-99-5) were used in this experiment along with sour orange seedlings as a control. Three replicates were inoculated for each rootstock candidate along with 3 healthy un-inoculated controls for each. The test seedlings were inoculated with the quick decline isolate (T36) in *citrus macrophylla* provided by Dr. Dawson's lab using the inverted- T budding method. The experiment was done in Dr. R.H. Brlansky's greenhouse and the plants were maintained as the rest of the plants in the greenhouse. After 2-3 weeks, the grafts were unwrapped then all plants were trimmed down to the same height to force new flush, and visually monitored for SY reactions on the new flush. After 8 months all the plants were tested for CTV by ELISA to confirm the CTV infection. Visual assessments of SY symptoms were done according to methods detailed by Garnsey et al., (1987b) and with the help of Cecile J.

Robertson. The severity score (0-3) was assigned to each plant where 0 = no symptoms, and it was assigned to the un-inoculated healthy control, whereas the sour orange was scored as a 3.

Chlorophyll a, chlorophyll b, and total chlorophyll content in the test rootstock candidates

Chlorophyll a, chlorophyll b and total chlorophyll were measured to determine the loss of the chlorophyll content due to the CTV T36 infection and relate this to the chlorosis in leaves as a symptom of the SY. The procedure was done according to Mackinney (1941). Ten leaves were randomly sampled from each plant where similar sized leaves were uniformly collected from the test rootstocks in the SY experiment. The leaves were ground in liquid nitrogen. Then, the extracts were prepared by crushing the plant material in 4 ml 80 % acetone. During this operation, the mortars were kept into the ice bath. The decanted supernatants obtained from each replicate (three) were recombined and the total volume was adjusted to 10 ml, and optical density (absorbance) evaluated at OD 663 nm and OD 645 nm in order to determine chlorophyll a and chlorophyll b respectively using UV-Vis spectrophotometer. The chlorophyll concentrations as g/L were obtained from the following equations:

Chlorophyll a (mg/g) = $\{(0.1127 \times OD_{663} \times d - 0.0259 \times OD_{645} \times d)\}/M$

Chlorophyll b (mg/g) = $\{(0.229 \times OD_{645} \times d - 0.0467 \times - OD_{630} \times d)/M$

Where d = dilutions (1) and M = sample weight (0.25 g).

The total chlorophyll concentration (mg/g) = Chlorophyll a + Chlorophyll b.

Starch assay and biochemical aspects of CTV-quick decline problem

To test the compatibility or the incompatibility between the top-worked rootstock candidates and the ‘Hamlin’ interstock, anatomy of the bud-union was examined to determine the presence of any necrosis in the bud-union area. Samples of the bud-union of sour orange or tested hybrids on infected sweet orange were taken and freeze sectioned using a microtone. Ten to twenty sections for each sample were stained then examined under the microscope and

compared to the negative control from the greenhouse (healthy sour orange on healthy 'Hamlin' sweet orange) and no necrotic cells were detected. The results showed no differences between sour orange and all test CTV-infected rootstock candidates. A possible explanation for this is that it may be too soon for the necrotic cells to have formed. Azure A staining also was used to stain sections of test rootstocks and sour orange from the field then the CTV inclusion bodies were counted in the infected tissue and there were no significant differences in the number of the CTV inclusion bodies in the infected tissue of sour orange, infected rootstock candidates or the interstock.

Starch content in the roots and the leaves as an indicator of CTV QD infection

The problem with the quick decline of sweet orange on sour orange is a budunion problem where necrosis occurs causing the death of phloem tissue, and thus sugars produced in leaves are blocked from being transported to the roots. Eventually, the feeder roots use up stored starch and start to die, leading to the ultimate death of the tree (Brlansky et al., 2008; Futch and Brlansky, 2008). Although the Spanish assay (Pina et al., 2005) to assess QD using reciprocal grafting (grafting sour orange on CTV-QD infected sweet orange) was working well to determine the QD affect in the greenhouse, no similar results on the Florida rootstocks were reported. After several useful discussions with Dr. R. H. Brlansky, it was still questionable if the reciprocal model would provide as accurate results as the standard ordinary sweet orange on sour orange graft. For better understanding, another experiment was conducted in the greenhouse by using selected test somatic hybrids (A + 4-3-99-2, A + 4-4-99-6, A + HBJL-1, A + HBJL-3, A + HBJL-5, A + Chandler #A1-11, Page + HBJL-3, A + SN7 and A + 7-2-99-5) as a rootstock along with sour orange as a control. Three replicates were inoculated for each test rootstock along with three healthy controls. The quick decline isolate T36 in *Citrus macrophylla* (kindly provided by Dr. Dawson's lab) was used for the inoculation of the sour orange and the tested rootstocks, then the

CTV infection was confirmed by MCA13 ELISA six months after inoculation. The pathogen free 'Hamlin'sweet orange provided by Dr. R. H. Brlansky was T-budded into these rootstocks. Starch content in the leaves and the roots was then measured, based on the previous study by Garnsey and Young (1975) who showed that starch reserves were depleted in roots from citrus trees affected by tristeza quick decline isolates.

Iodine staining using iodine solution (8.8 g KI + 2.2 g I_2 /L) was conducted according to Hong and Truc (2003) to test for starch accumulation in the roots of the infected seedlings from the greenhouse along with the healthy controls . Starch content in leaves and roots (mg/g dry weight) was done according to Nelson (1944) and Somogy (1952) colorimetric method (see Appendix a). The measurment was done at OD_{520} nm using Shimadzu UV-Visible spectrophotometer UV-160. Five standars of glucose were prepared: 0, 10, 20, 40, and 60 µg/ µL and were run along with the samples. The starch content (mg/g) = [glucose concentration from the spectrophotometer X sample volume (5 mL)] /dry weight (g). The data for the starch content were analyzed by one-way ANOVA (analysis of variance) using SAS (2000). Mean (average) values were separated using the Least Significant Difference (LSD) separation of means at a probability level of 0.05.

Results and Discussion

Top-working Experiment

Shoot growth

The shoot growth of the grafted materials on the 'Hamlin' interstocks was recorded every three months, and the presented data 18 months after top-working graft (Table 3-2). Overall, there were significant differences in the shoot length among all the test selections, especially in comparison with sour orange that was severely stunted. These field results were in agreement with a greenhouse study conducted by Pina et al., (2005). They stated that inoculation of sweet

orange grafted onto sour orange with QD- inducing isolates does not induce decline in the greenhouse. In order to overcome this problem, they developed a quick decline assay using reciprocal grafting in the greenhouse where sour orange was budded into the infected sweet orange with different CTV isolates to screen for the ability of these isolates to cause graft union necrosis and decline. The results showed that buds propagated on healthy seedlings or on those infected with a non-decline isolate grew normally; producing shoots at least 20 cm long after 2 months, whereas buds propagated on seedlings infected with QD-inducing isolates did not sprout or produced very weak shoots less than 5 cm. These results may be caused by failure to produce a normal budunion on seedlings infected with QD-inducing isolates. This procedure allows evaluation of decline ability in 6-8 months after inoculation under greenhouse conditions in Spain (Pina et al., 2005). In the present study, overall the highest shoot growth in the seventy-four tested germplasms was with the 5-1-99-2 pummelo seedling, whereas the somatic hybrid Amb + HBJL-4 showed the lowest shoot growth. In the parental Pummelo seedling category, the highest three shoot growth obtained were from seedlings 5-1-99-2 (268.67cm ±56. 50), HBJL-3 (254.00 cm ±.25.24) and 8-1-99-2B (252.33 cm ±34.0), (Table 3-2 and Figure 3-2). The lowest shoot growth in the pummelo seedlings were in 4-4-99-4, MG10 and SN3, with shoot growth 123.00 cm ±21.63, 116.67 cm ±16.50 and 140.00 cm ± 11.14 respectively (Table 3-2 and Figure 3-2). For the somatic hybrid category, the highest three shoot growth were obtained by Amb+ HBJL-1, Amb+ HBJL-3, and Amb+HBJL-2B with values 286.33 cm ±13.32, 280.33 cm ±23.54 and 278.67 cm ± 29.54, respectively (Table 3-2 and Figure 3-3). Whereas, the lowest shoot growth were obtained by somatic hybrids Amb+HBJL-4 (98.67cm ±18.01) and Amb + Chandler#69 (99.33 cm ± 8.02). In the tetrazyg category, the highest growth rate was obtained by Green 6 (265.00 cm ± 34.07). However the tetrazyg N+HBP x SO +RP-04-7 showed the

lowest shoot growth (108.00 cm ± 38.74), (Table 3-2 and Figure 3-4). For the diploid hybrid category, the highest shoot growth was for Volk x P (268.67 cm ±20.74) whereas the rootstock 46x20-04-12 showed the lowest shoot growth (108.33 cm ±19.86), (Table 3-2 and Figure 3-5). The open-pollinated tetraploid group included 2247-OP-A2 rootstock with the highest shoot growth 226.33 cm ±05.69, whereas the rootstock SORP-OP-02-8 showed the lowest shoot growth (146.00-06.56), (Table 3-2 and Figure 3-6). There was no significant difference between Marsh grapefruit and Ruby Red Grapefruit, 237.33 cm ± 14.84 and 224.67 cm ±48.01 respectively (Table 3-2 and Figure 3-7).

The sour orange shoot growth mean was 66.67 ± 2.52 and there were significant differences between the shoot growth of most of the test hybrid rootstocks and sour orange except A+7-2-99-5, A+HBJL-4, Amb+ Chandler #69, Murcott+ Chandler#80, Murcott+ Chandler#A-11, Amb+SN7 and Amb+MG-1. These results clearly showed that except for the few stunted rootstock candidates mentioned above, the top-worked rootstock candidates are growing well, indicating a normal formation of the budunion. It is important to note that there was a strong correlation between the shoot growth and the MCA13, ELISA data presented in Chapter (4). In general, most rootstock candidates in the category with no virus or with low CTV titer based on MCA13, ELISA, exhibited a high shoot growth, whereas the slightly tolerant and susceptible category hybrids with high CTV titer exhibited low shoot growth.

Disease symptoms

Stems of all the grafts were collected 12 months after grafting. The bark was peeled and no stem pitting symptoms were found, even after observation under the light microscope. Visual observation of stem pitting symptoms is an obvious indicator generally used to identify the presence of stem pitting isolates. In general, no seedling yellows-type symptoms were observed in any of the top-worked trees, including the sour orange grafts. This result indicates that top-

working overcame the seedling yellows (SY) effect that has previously caused problems with our greenhouse QD resistance assays. Vein clearing was noticed in all the sour orange and in two somatic hybrid rootstock candidates (Murcott+ Chandler # 80 and Amb +7-2-99-5). The only other clearly observed symptom was the stunted growth in all top-worked sour orange, the diploid pummelo seedling MG-10, and in the following somatic hybrid rootstock candidates: Amb+ 7-2-99-5, Amb+ Chandler # 69, Amb+MG-1, Amb+ HBJL-4, Murcott+ Chandler # 80, Murcott + Chandler # A1-11 and Amb+ SN7. These results indicate that these selections are highly susceptible to CTV infection.

Top-working advantage to fast fruiting

Another advantage of the top-working approach is to speed flowering and fruiting, allowing for a more rapid assessment of the test rootstock candidates for seed propagation. Some of the top-worked rootstock candidates including SRxSH-99-5, 4-3-99-2, 5-1-99-2, 4-4-99-4 and 7-2-99-2 are already bearing fruits (Figure 3-1). Many of the top-worked rootstock selections are growing well and are expected to fruit during the next year or two. As they fruit, seed will be extracted to determine seediness (excluding the parental pummelos). Microsatellite analysis will be conducted on germinated seedlings to determine if they are of zygotic or nucellar origin. Nucellar seedlings are very favorable since, the standard nursery propagation of rootstocks relies on nucellar seedlings for rootstock uniformity. Alternatively, good rootstock candidates producing zygotic seedlings could be propagated using a rooted cutting method.

General considerations for improving the top-working QD-resistance assay

- Choosing healthy, relatively young trees is critical for successful top-working.
- Grafting can be done to scaffold branches or a trunk. If the trunk is used, it minimizes the new sprouts from the interstock.
- You have to have a flowing sap for a successful graft.

- The hanging bud method provides a high efficient method for top-working.
- Girdle above the graft to enhance the bud growth.
- Painting the trees with whitewash from the ground level to just above the bud insertion to inhibit sprouting.
- Interstock sprouts must be removed in a timely fashion to ensure proper subsequent genotypic identification.
- The number of buds required per tree for successful top-working depends on the tree condition.
- The use of bright color spray paint facilitates the identification of grafted branches.
- Even under the best conditions, it was uncommon to have 100% bud- take in top-working, but 80-90% success was common, which we considered good.
- Bud shoots should be allowed to grow to about 15 inches and then pruned to nine inches for strengthening, with repeated pruning as needed.
- Vigorous shoots growing from new grafts are more susceptible to wind breakage, this can be minimized by the addition of physical supports.

  If available, it would be beneficial in future work if at least three replicate trees were used for each candidate rootstock selection. Whitewashing the trees prior to grafting is highly recommended. Leafminer damage on new flush was a significant problem. Careful management of irrigation, fertilization and pesticides is a necessity.

Seedling Yellows Experiment and Total Chlorophyll Content

Results showed that all the rootstock candidates have a SY reaction 8 months after inoculation of T36 isolate. The typical SY symptoms caused by CTV are a severe chlorosis, stunting and vein corking of sour orange, lemon or grapefruit. The SY symptoms are commonly observed in nurseries (Fraser, 1952) and greenhouses but they are not usually seen in the field (Roistacher, 1982). Shoot measurements in cm (Table 3-3 and Figure 3-10) and the total chlorophyll in mg/g tissue (Table 3-4) indicated that somatic hybrids A + 7-2-99-5 (35 cm) and A + SN7 (25 cm) were as bad as sour orange (30 cm) rootstock compared to their controls in terms of the stunting reaction. They also gave the same score of SY symptoms (3), the highest

score possible with low chlorophyll content (0.34 mg/g, 0.30 mg/g and 0.29 mg/g), respectively. Somatic hybrids A + HBJL-1, A+HBJL-3 and A+ HBJL-5 showed shoot length (76 cm, 80 cm, and 72 cm) with SY scores of 2.5, 2 and 3 respectively and the total chlorophyll content was 0.86 mg/g, 1.09 mg/g and 0.36 mg/g, respectively. Somatic hybrids; A+ 4-3-99-2, A+4-4-99-6 and A+ Chandler #A1 -11 produced shoot lengths of 99 cm, 63 cm and 59 cm, respectively with SY scores of 2.5, 2.5 and 3 respectively. Page + HBJL-3 had a score of 3 in terms of SY symptoms with shoot growth of 53cm. (Table 3-3 and Figures 3-8 and 3-9). Total chlorophyll data is presented in Table (3-9) and Figure (3-10). In general, there was a strong correlation between the losses of total chlorophyll content and the severity score of SY symptoms.

In conclusion, most of SY data was in contrast with data from the field top-working experiment. In the current SY study, the somatic hybrid A + 7-2-99-5 showed strong SY symptoms in the greenhouse study, and a high susceptibility to CTV in the top-working field study, and it was rated as a susceptible rootstock. However, several other tested somatic hybrid rootstocks (A + Chandler #A1-11, A+ HBJL-5,and A+ 4-4-99-6) showed a strong SY reaction in the greenhouse study, but none of these showed any SY reaction or any disease symptoms in the field and they were rated as tolerant or intermediate. Therefore, there is clearly no strong correlation between the SY and QD diseases, and the top-working approach provides a more reliable screen for CTV-QD resistance in the new rootstock candidates.

Starch content and biochemical aspects of CTV-QD problem

The results of the iodine staining showed that the starch content decreased in the roots in CTV-infected rootstock candidates as compared to the healthy controls (Figure 3-11). These visual results were supported by quantification of starch content (mg/g dry weight) in the leaves and the roots of the test rootstock candidates. Data is presented in Table (3-5) and Figures (3-12 and 3-13). The rootstock candidates; A+ Chandler #A1-11, A+ 7-2-99-5 and sour orange showed

increases in starch content in the leaves (125.51 mg/g ± 1.92 dry weight, 127.49 mg/g ± 2.83 dry weight and 135.52 mg/g ± 2.06 dry weight respectively) as compared to the healthy controls (Table 3-5). These rootstocks show severe SY symptoms in the greenhouse assay. These results were in contrast with the data from the field top-working experiment for the rootstock candidate A + Chandler #A1-11. The rootstock candidates A + 4-3-99-2 (63.82 mg/g ± 2.35), and A+HBJL-1 (84.58 mg/g ± 5.32) gave the lowest starch content in the leaves with no significant difference to the control (Table 3-5). The depletion of the starch content in the roots of CTV infected rootstocks was not severe. However, it was more pronounced in rootstocks, A+ 7-2-99-5 (79.56 mg/g ± 3.35), Page + HBJL-3 (84.58 mg/g dry weight ± 2.06) and the sour orange control (69.91 mg/g ± 3.92). In general, there was no significant difference in starch content (mg/g) in roots of healthy and CTV- infected rootstocks; A + HBJL-1 (122.57 mg/g ± 2.62), A+ HBJL-5 (123.35 mg\g ± 2.76) and A+ Chandler #A1-11(88.21 mg/g ± 2.59) compared to the healthy controls (129.95mg/g ± 2.29, 121.31mg/g ± 4.84 and 100.16 mg/g ± 5.59), respectively (Table 3-5). Interestingly, at the end of the experiment, plants were removed from the soil to examine the root systems. There were no observable differences between the root systems in the infected and healthy rootstocks. All showed healthy and strong root systems, including the sour orange rootstock, which supports the absence of QD phenomenon in the greenhouse after infection with the CTV- QD T36 isolate. The activities of sucrose synthase and sucrose phosphate synthase enzymes which in return affected the starch accumulation in the leaves were determined (data not shown). These two enzymes are among the enzymes that control the sucrose synthesis. In general these enzymes activities were 2-10 fold higher in the healthy tested leaves than in the CTV infected leaves of 'Hamlin' sweet orange grafted on sour orange rootstock.

In conclusion, the carbohydrate data suggests that CTV QD infection alters carbohydrate metabolism and this phenomenon should be further studied to understand the role of CTV-QD infection in the carbohydrate formation and translocation. This suggests that the CTV infection may alter some of the genes that control carbohydrate metabolism and targeting of starch translocation to the phloem, resulting in phloem necrosis. Further investigation to determine the relationship between carbohydrates and CTV-QD disease might provide an answer about the mechanism and affect of CTV QD infection on carbohydrate synthesis and transport, and help to explain why QD is difficult to read in the greenhouse. It could be because there is enough carbon available in greenhouse seedlings to temporarily carry out photosynthesis. If the QD is only a budunion necrosis problem, it should still be determined why there is budunion problem in the field with mature trees, but that is not obvious in the greenhouse. The role of carbohydrate metabolism and transport in the QD phenomenon requires further study.

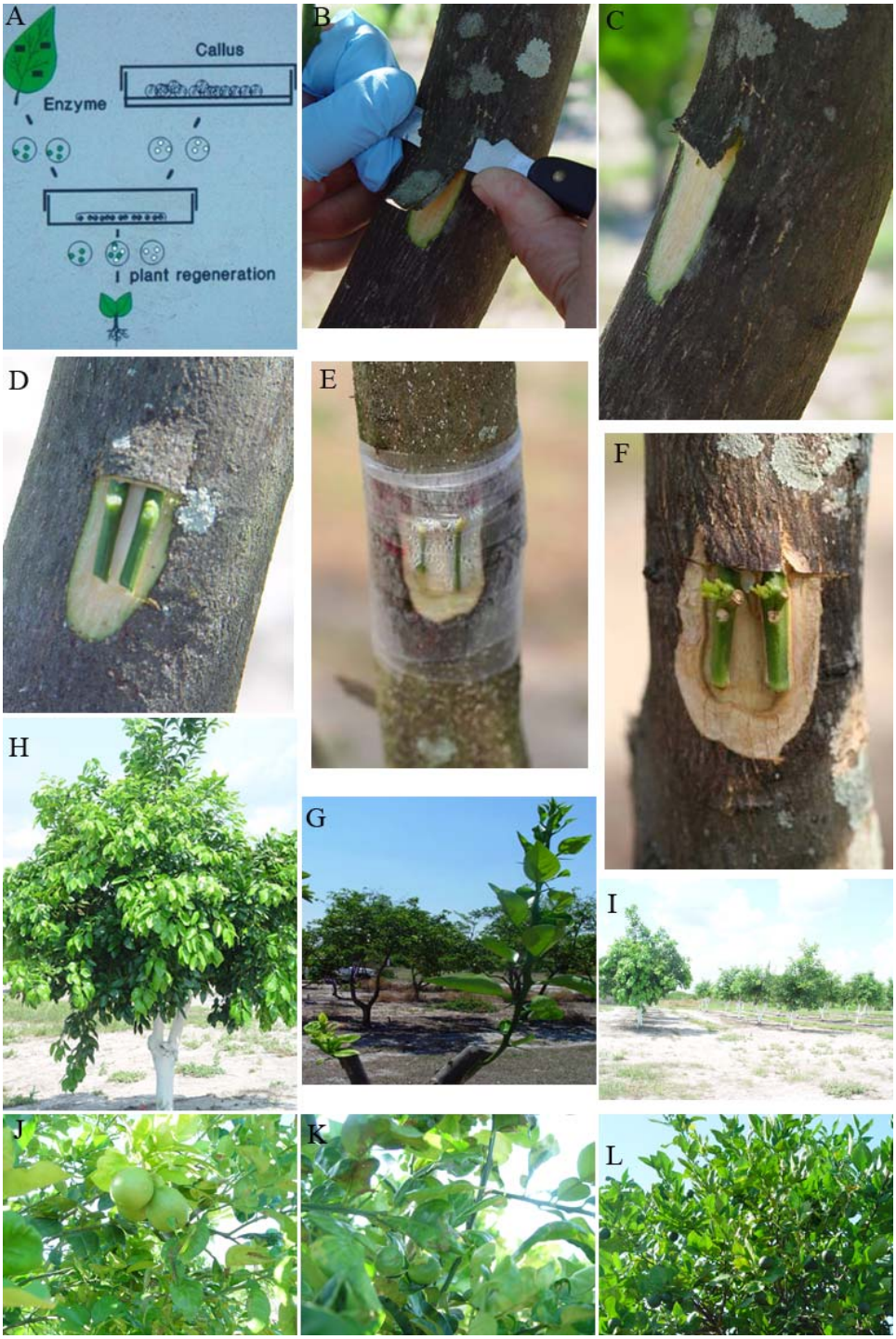

Figure 3-1. Summary of the top-working technique. A) Protoplast fusion protocol. B, C, D, E and F) Hanging bud steps. G) Sour orange to the left and 2247-OP-A2 to the right. H) Examples of the top-worked trees. I) Overview of the top-worked groove. J, K and L) Examples of the top-worked trees (4-3-99-2, 7-2-99-2 and (SRXSH) 99-5) respectively after fruiting.

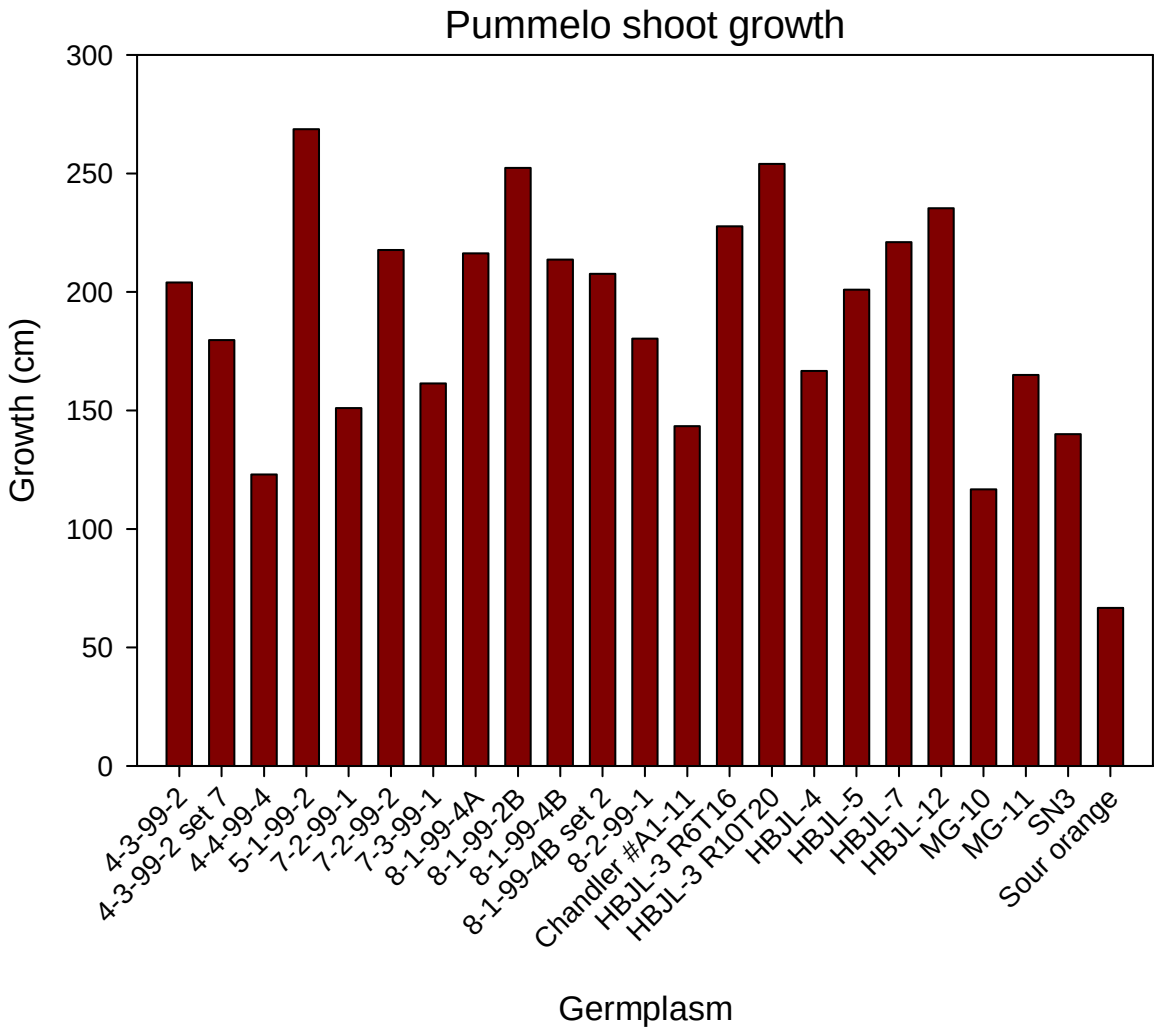

Figure 3-2. Shoot length (cm) of the pummel parents and the sour orange in average18 months after grafting.

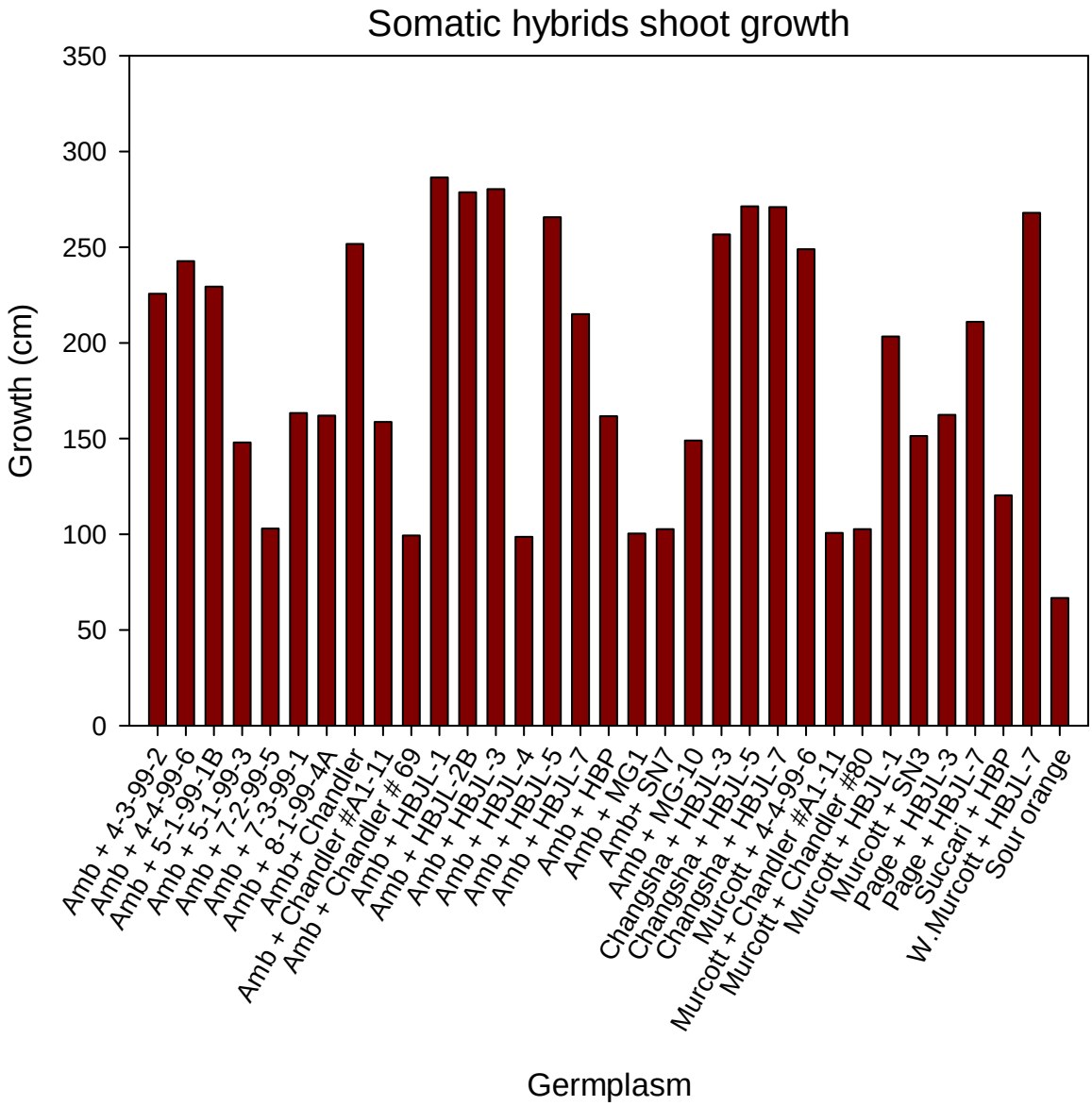

Figure 3-3. Shoot length (cm) of the somatic hybrids rootstock candidates and the sour orange in average18 months after grafting.

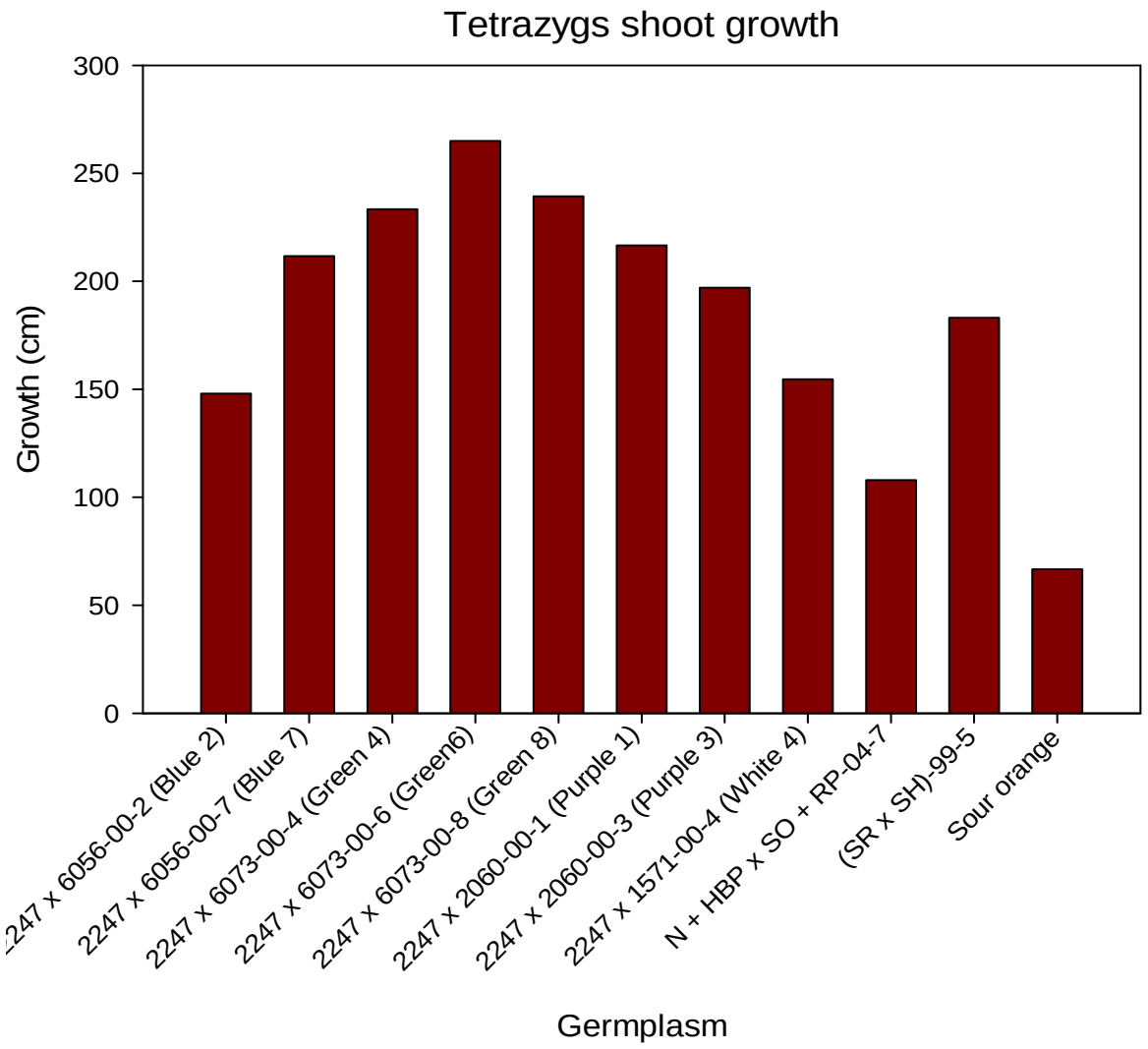

Figure 3-4. Shoot length (cm) of the tetrazygs rootstock candidates and the sour orange in average18 months after grafting.

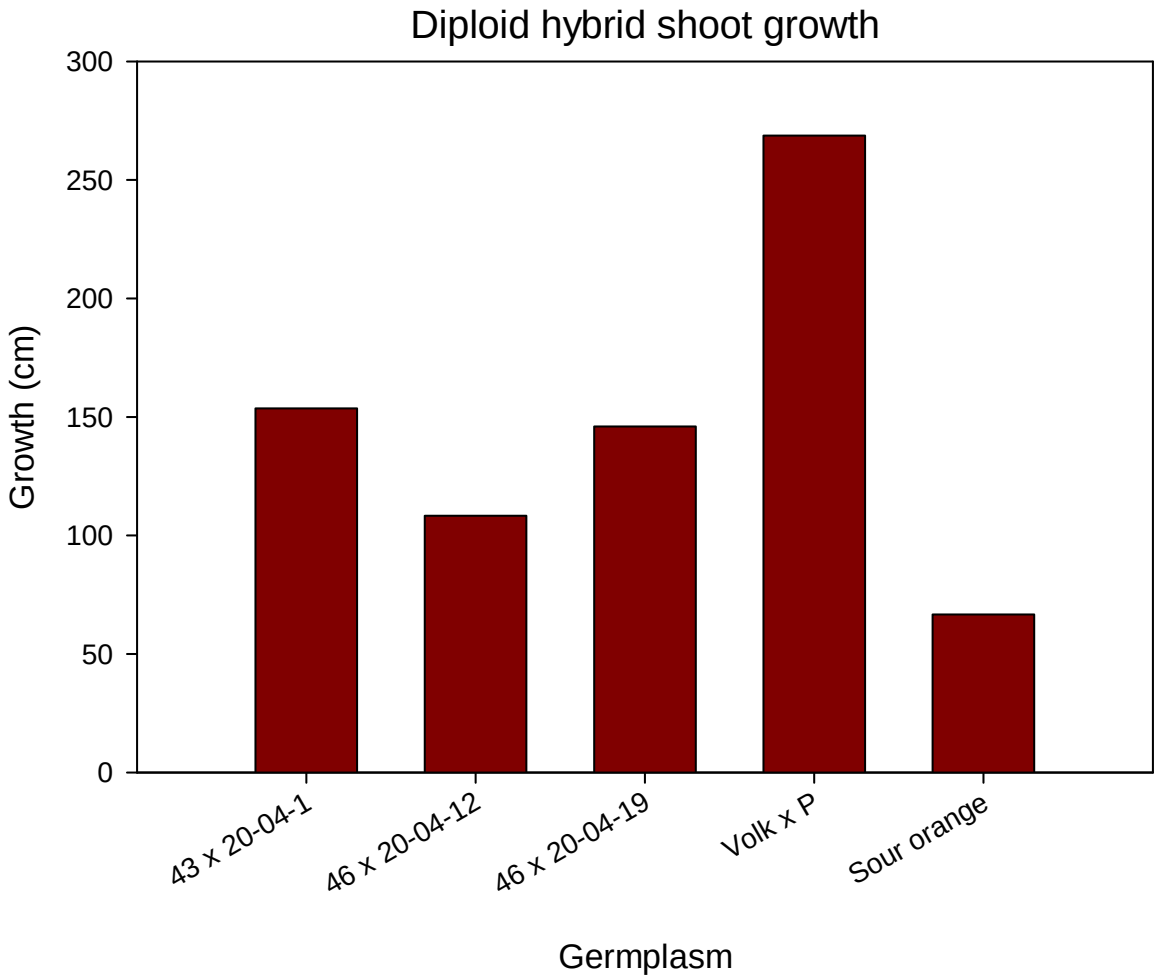

Figure 3-5. Shoot length (cm) of the diploid hybrids rootstock candidates and the sour orange in average18 months after grafting.

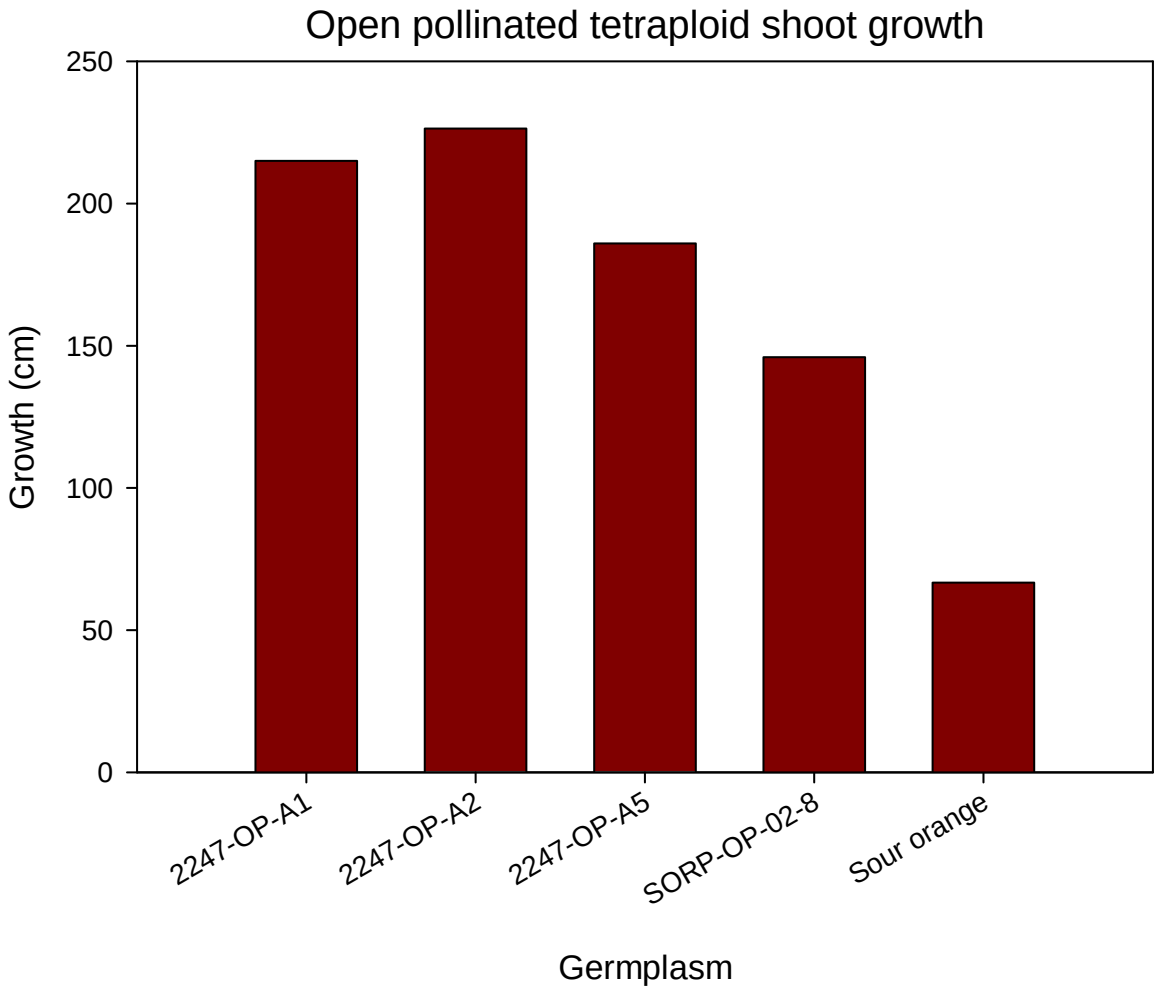

Figure 3-6. Shoot length (cm) of the open pollinated tetraploid rootstock candidates and the sour orange in average18 months after grafting.

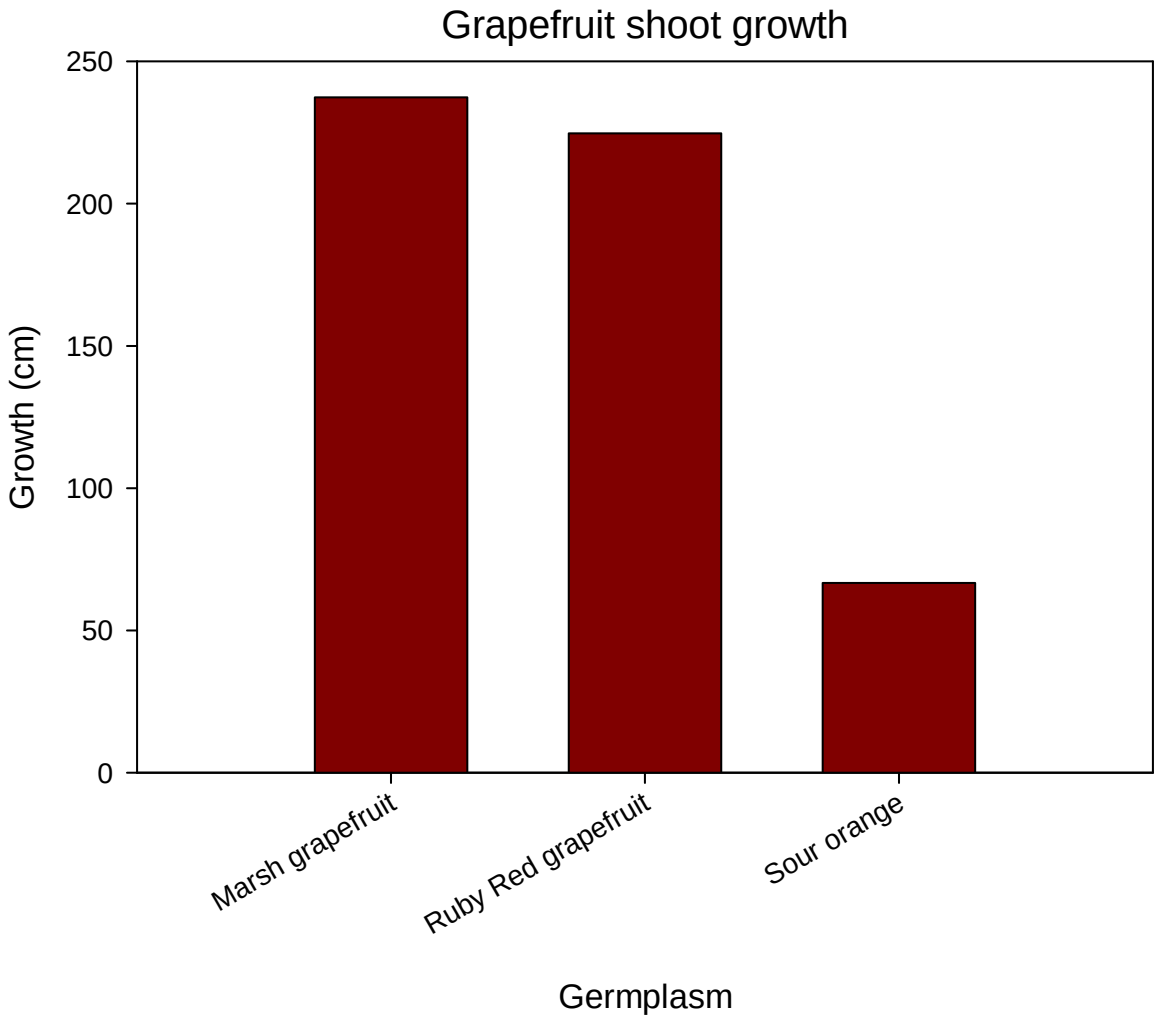

Figure 3-7. Shoot length (cm) of Marsh grapefruit, Ruby Red grapefruit and the sour orange in average18 months after grafting.

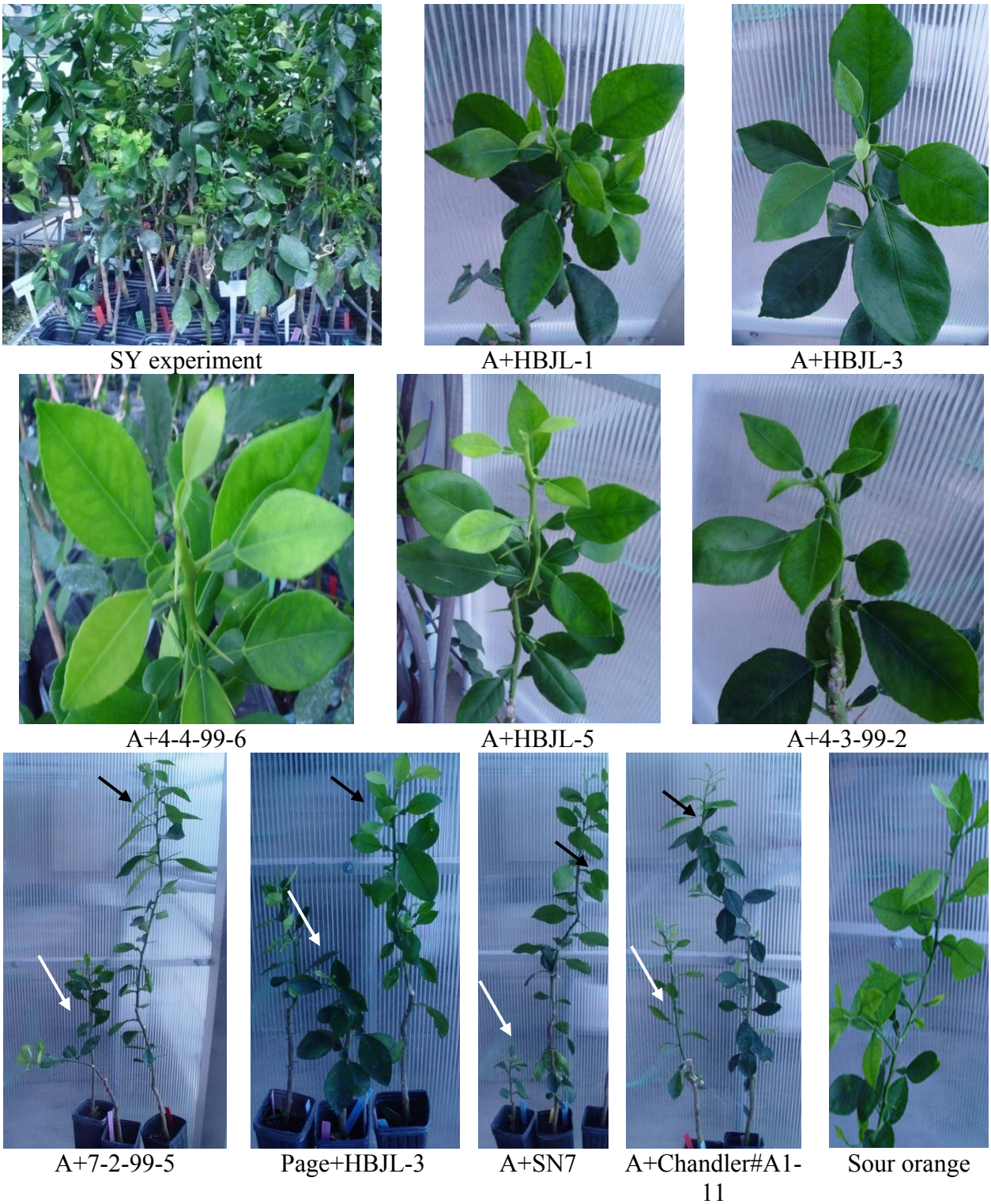

Figure 3-8. Seedling yellows symptoms of rootstock candidates 8 months after inoculation of T36 in the greenhouse. White arrows refer to rootstock candidates and black arrows refer to control plants.

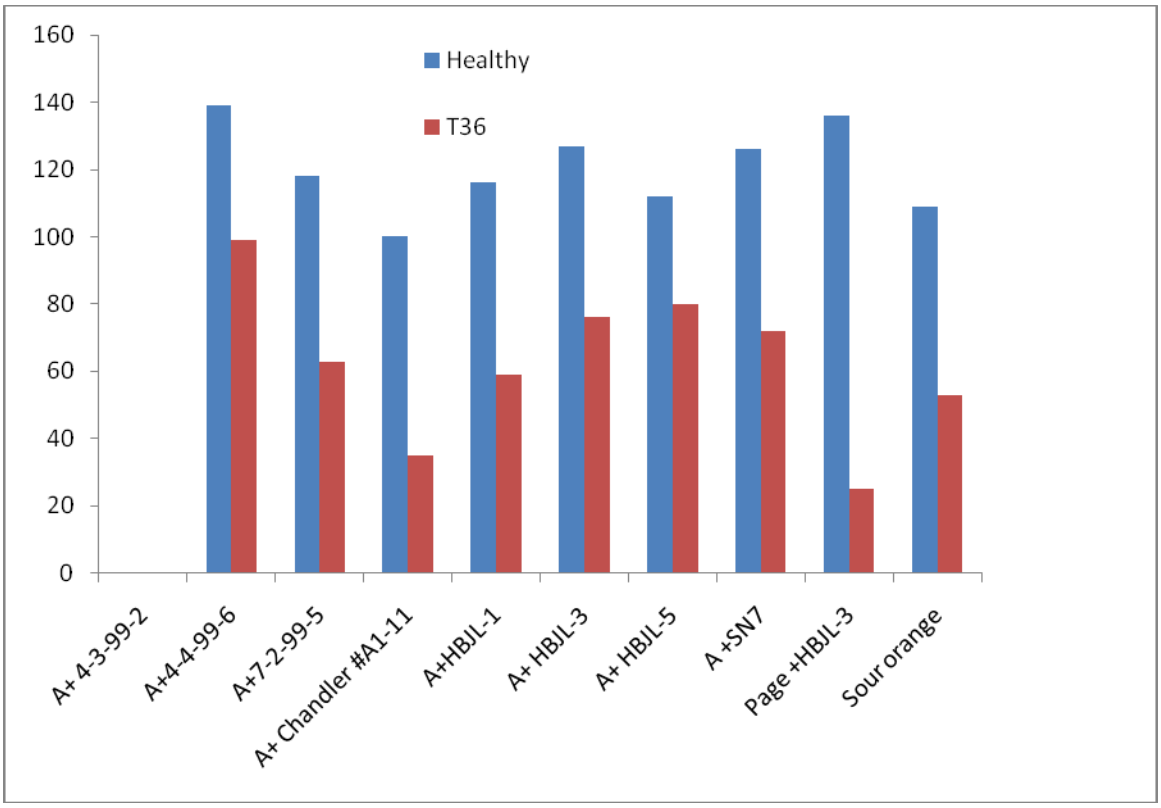

Figure 3-9. Shoot length (cm) and the seedling yellows symptoms of test rootstock candidates inoculated with T36 in the greenhouse 8 months after inoculation.

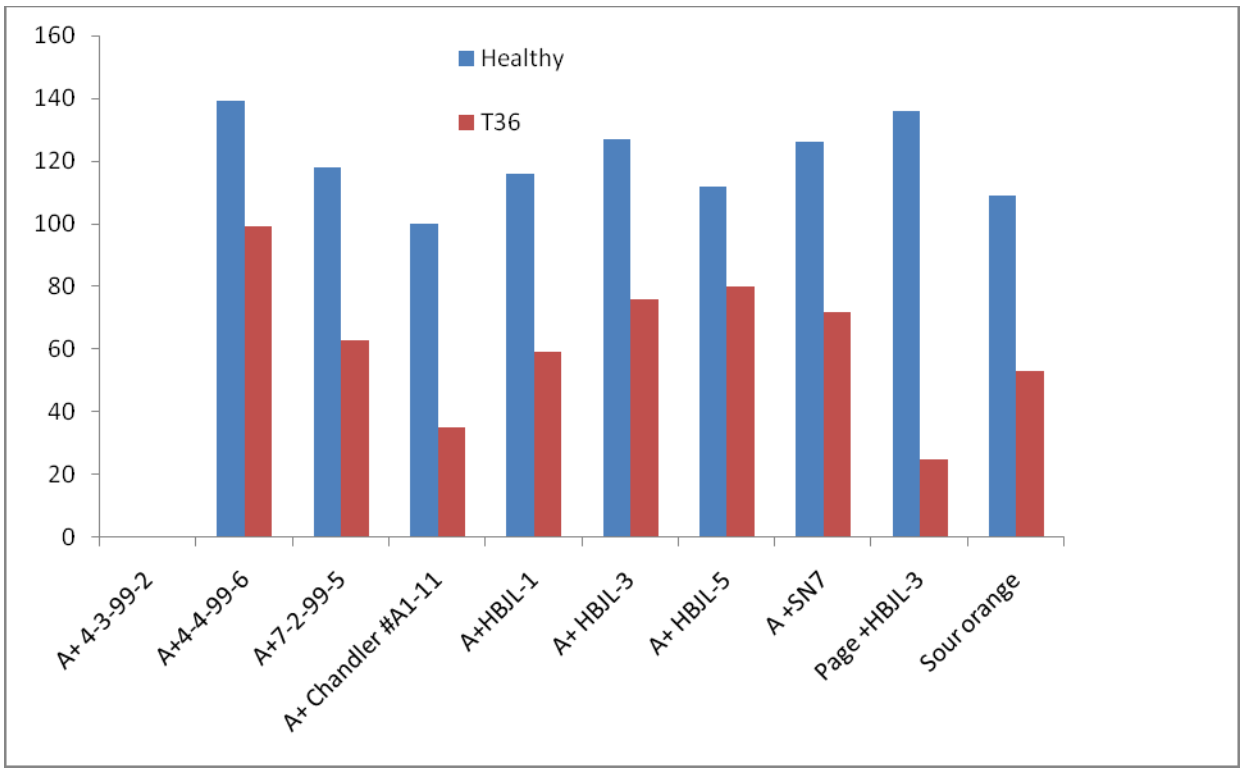

Figure 3-10. Total chlorophyll content (mg/g dry weight) in test rootstock candidates showing chlorosis symptoms 8 months after inoculation with T36 in the greenhouse.

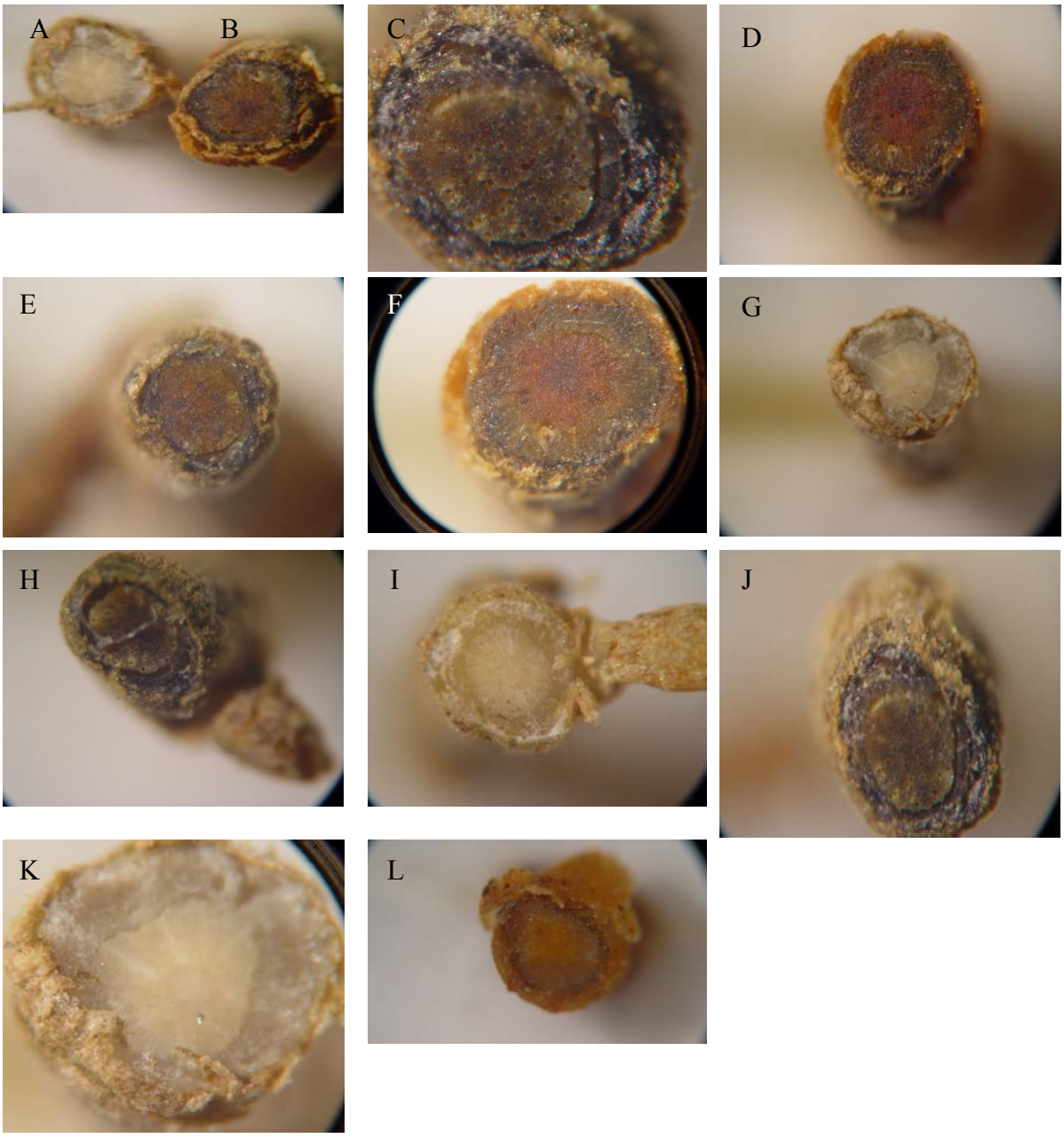

Figure 3-11. Iodine staining of the roots of the test rootstocks infected with CTV-T36. A) Root of sour orange CTV infected rootstock. B) Root of sour orange rootstock healthy control. C) Root of CTV infected A+4-3-99-2 rootstock. D) Root of CTV infected A+4-4-99-6 rootstock. E) Root of CTV- infected A+HBJL-1 rootstock. F) Root of CTV- infected A+HBJL-3 rootstock. G) Root of CTV- infected A+HBJL-5 rootstock. H) Root of CTV- infected A + Chandler #A1-11 rootstock. I) Root of CTV infected A+7-2-99-5 rootstock. J) Root of A+7-2-99-5 rootstock healthy. K) Root of CTV infected A+SN7 rootstock. L) Root of CTV infected Page +HBJL-3 rootstock.

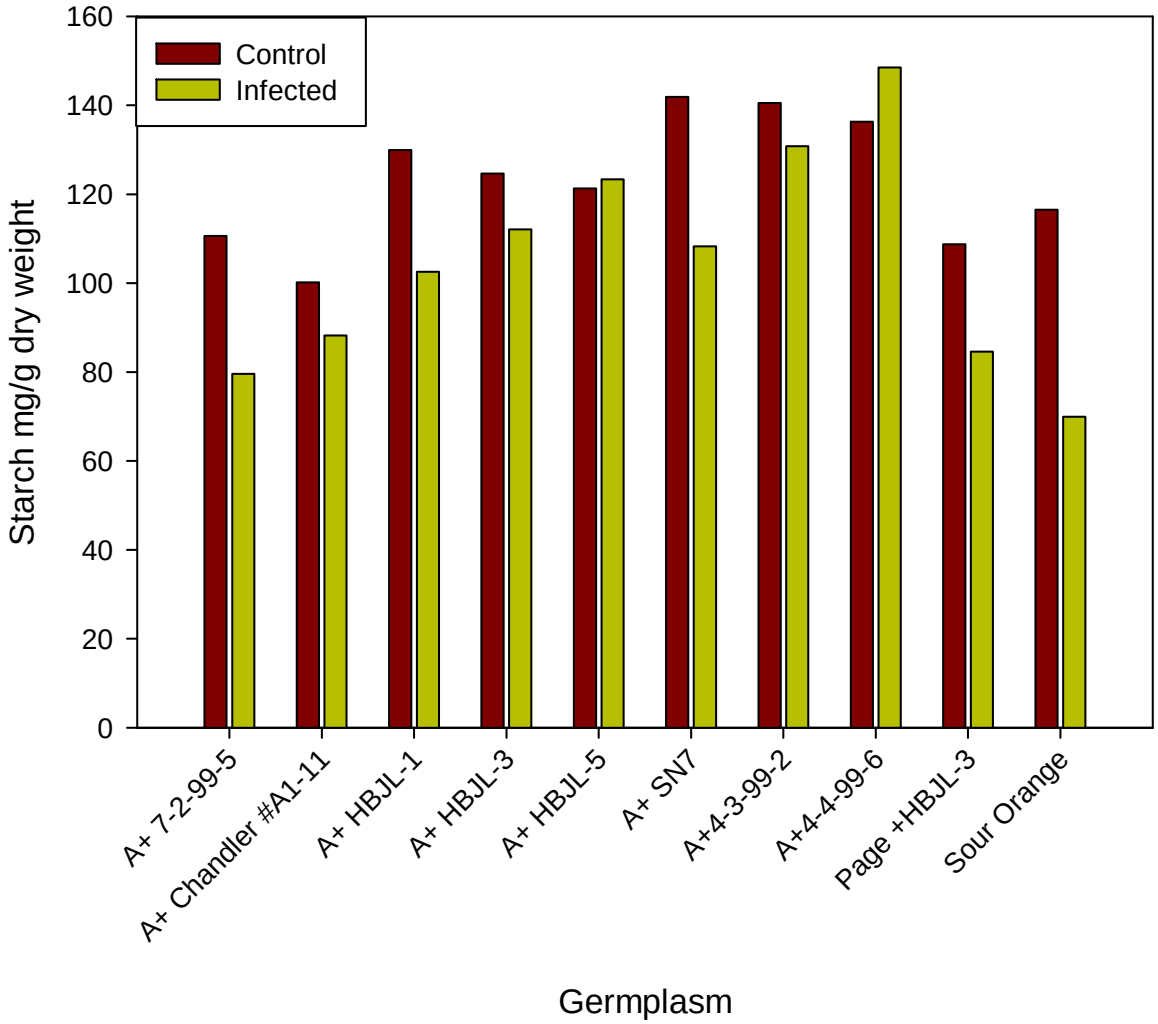

Figure 3-12. Starch content (mg/g dry weight) 12 months after inoculation of T36 CTV-QD isolate in the greenhouse.

Table 3-1. Identification and description of the germplasms included in the field top-working study.

Germplasm	Description
Pummelo parent (*Citrus. grandis* L. Osb.)	
4-3-99-2	Pummelo parent: selected seedling of Sha Tian You Pummelo
4-3-99-2 set 7	Pummelo parent: selected seedling of Sha Tian You Pummelo
4-4-99-4	Pummelo parent: selected seedling of Siamese Pummelo
5-1-99-2	Pummelo parent: selected seedling of Hirado Buntan Pummelo (HBP)
7-2-99-1	Pummelo parent: selected seedling of Large Pink Pummelo
7-2-99-2	Pummelo parent: selected seedling of Large Pink Pummelo
7-3-99-1	Pummelo parent: selected seedling of Siamese Sweet Pummelo
8-1-99-4A	Pummelo parent: selected seedling of Liang Ping Yau Pummelo
8-1-99-2B	Pummelo parent: selected seedling of Liang Ping Yau Pummelo
8-1-99-4B	Pummelo parent: selected seedling of Liang Ping Yau Pummelo
8-1-99-4B set2	Pummelo parent: selected seedling of Liang Ping Yau Pummelo
8-2-99-1	Pummelo parent: selected seedling of pummelo from the DPI
Chandler #A1-11	Pummelo parent: selected seedling of 'Chandler' pummelo
HBJL-3 R6T16	Pummelo parent: selected seedling of Hirado Buntan Pummelo
HBJL-3 R10T20	Pummelo parent: selected seedling of Hirado Buntan Pummelo
HBJL-4	Pummelo parent: selected seedling of Hirado Buntan Pummelo
HBJL-5	Pummelo parent: selected seedling of Hirado Buntan Pummelo
HBJL-7	Pummelo parent: selected seedling of Hirado Buntan Pummelo
HBJL-12	Pummelo parent: selected seedling of Hirado Buntan Pummelo
MG-10	Pummelo parent: selected seedling of Hirado Buntan Pummelo
MG-11	Pummelo parent: selected seedling of Hirado Buntan Pummelo
SN3	Pummelo parent: selected seedling of Hirado Buntan Pummelo
Somatic Hybrids	**Obtained from mandarin + pummelo protoplast fusion**
Amblycarpa (Amb) + 4-3-99-2	Somatic hybrid: Amblycarpa mandarin (*Citrus amblycarpa* Oche) + selected seedling of Sha Tian You Pummelo
Amb + 4-4-99-6	Somatic hybrid: Amblycarpa mandarin + selected seedling of Siamese Pummelo
Amb + 5-1-99-1B	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + 5-1-99-3	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + 7-2-99-5	Somatic hybrid: Amblycarpa mandarin + selected seedling of Large Pink Pummelo
Amb + 7-3-99-1	Somatic hybrid: Amblycarpa mandarin + selected seedling of Siamese sweet Pummelo
Amb + 8-1-99-4A	Somatic hybrid: Amblycarpa mandarin + selected seedling of Liang Ping Yau Pummelo
Amb + Chandler	Somatic hybrid: Amblycarpa mandarin + selected seedling of 'Chandler' pummelo
Amb + Chandler # 69	Somatic hybrid: Amblycarpa mandarin + selected seedling of 'Chandler' pummelo
Amb + Chandler #A1-11	Somatic hybrid: Amblycarpa mandarin + selected seedling of 'Chandler' pummelo

Table 3-1. Continued.

Germplasm	Description
Amb + HBJL-1	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBJL-2B	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBJL-3	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBJL-4	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBJL-5	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBJL-7	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + HBP	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + MG1	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + MG-10	Somatic hybrid: Amblycarpa mandarin + selected seedling of Hirado Buntan Pummelo
Amb + SN7	Somatic hybrid: Amblycarpa mandarin + selected seedling of Liang Ping Yau Pummelo
Changsha + HBJL-3	Somatic hybrid: 'Changsha' mandarin (*C. reticulata* Blanco) + selected seedling of Hirado Buntan Pummelo
Changsha + HBJL-5	Somatic hybrid: 'Changsha' mandarin + selected seedling of Hirado Buntan Pummelo
Changsha + HBJL-7	Somatic hybrid: 'Changsha' mandarin + selected seedling of Hirado Buntan Pummelo
Murcott + 4-4-99-6	Somatic hybrid: 'Murcott' tangor (*C. reticulata* Blanco x *C. sinensis* Osbeck) + selected seedling of Siamese Pummelo
Murcott + Chandler #80	Somatic hybrid: 'Murcott' + selected seedling of 'Chandler' pummelo # 80
Murcott + Chandler #A1-11	Somatic hybrid: 'Murcott' + selected seedling of 'Chandler' pummelo # A1-11
Murcott + HBJL-1	Somatic hybrid: 'Murcott' + selected seedling of Hirado Buntan Pummelo
Murcott + SN3	Somatic hybrid: 'Murcott' + selected seedling of Hirado Buntan Pummelo
Page + HBJL-3	Somatic hybrid: 'Page' tangelo [(Minneola(*C. reticulata* Blanco X *C. paradisi* Macf) x Clementine mandarin (*C. reticulata* Blanco)] + selected seedling of Hirado Buntan Pummelo
Page + HBJL-7	Somatic hybrid: 'Page' tangelo' + selected seedling of Hirado Buntan Pummelo
Succari + HBP	Somatic hybrid: 'Succari' Sweet orange + Hirado Buntan Pummelo
W.Murcott + HBJL-7	Somatic hybrid: 'W. Murcott' tangor (*C. reticulata* Blanco x *C. sinensis* Osbeck) + selected seedling of Hirado Buntan Pummelo

Table 3-1. Continued.

Germplasm	Description
Tetrazygs	**Origin: from crosses of allotetraploid somatic hybrids**
2247 x 6056-00-2 (Blue 2)	Tetrazygy: 'Nova'* mandarin hybrid + HBP somatic hybrid/ Sour Orange (S.O)**+ Palestine sweet lime (PSL)
2247 x 6056-00-7 (Blue 7)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ S.O + PSL somatic hybrid
2247 x 6073-00-4 (Green 4)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ S.O + Carrizo citrange somatic hybrid
2247 x 6073-00-6 (Green6)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ S.O + Carrizo citrange somatic hybrid
2247 x 6073-00-8 (Green 8)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ S.O + Carrizo citrange somatic hybrid
2247 x 2060-00-1 (Purple 1)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ Cleopatra mandarin (Cleo) + S.O somatic hybrid
2247 x 2060-00-3 (Purple 3)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ Cleo + S.O somatic hybrid
2247 x 1571-00-4 (White 4)	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid / Succari sweet orange + Argentine trifoliate orange (*Poncirus trifoliata)* somatic hybrid
N + HBP x SO + RP-04-7	Tetrazygy: 'Nova' mandarin + HBP somatic hybrid/ S.O + rangpur (RP) somatic hybrid
(SR x SH) 99-5	Tetrazygy: S.O + RP somatic hybrid / Cleo + Sour orange somatic hybrid
Diploid Hybrids	**Obtained from conventional crosses**
43 x 20-04-1	Diploid Hybrid: Ling Ping Yau sdlg. Pummelo x Cleopatra mandarin
46 x 20-04-12	Diploid Hybrid: Hirado Buntan Pummelo x Cleopatra
46 x 20-04-19	Diploid Hybrid: HBP x Cleopatra
Volk x P	Diploid Hybrid : Volkamerian lemon (*C. Volkameriana*) / unknown pummelo
OP tetraploids	**Source: open pollination of allotetraploid somatic hybrid**
2247-OP-A1	Tetraploid : selected Mandarin/ pummelo seedling from open pollination of (Nova + HBP zyg somatic hybrid)
2247-OP-A2	Tetraploid : Mandarin/ pummelo seedling from open pollination of (Nova + HBP zyg somatic hybrid)
2247-OP-A5	Tetraploid : Mandarin/ pummelo seedling from open pollination of (Nova + HBP zyg somatic hybrid)
SORP-OP-02-8	Tetraploid : Mandarin/ pummelo seedling from open pollination of (S.O + rangpur somatic hybrid)
Grapefruit *Citrus Paradisi* Macfad	**Commercial cultivars**
Marsh grapefruit	'Marsh' Grapefruit, buds from DPI***
Ruby Red grapefruit	'Ruby Red' Grapefruit, buds from DPI

*Nova mandarin: Fina 'Clementine' and Orlando 'tangelo' (Duncan grapefruit X Dancy tangerine) made by F.G. Gardner and J. Bellows in 1942 and released in 1964 (Saunt, 1990).
**Sour orange: (*Cirus aurantium* L.).
***DPI:- Division of Plant Industry in Winter Haven Florida.

Table 3-2. Shoot growth of the rootstock candidates and the sour orange in average18 months after grafting (means were separated using the LSD separation of means at *p=0.05*).

Germplasm	Shoot growth (cm) ± StDev	Gemplasm	Shoot growth (cm) ± StDev	germplasm	Shoot growth (cm) ± StDev
Pummelo		**Somatic Hybrids**		**Tetrazygs**	
4-3-99-2	204.00 ± 36.10	Amb + 7-3-99-1	163.33 ± 10.02	2247 x 6056-00-2 (Blue 2)	148.00 ± 62.23
4-3-99-2 set 7	179.67 ± 21.20	Amb + 8-1-99-4A	162.00 ± 36.59	2247 x 6056-00-7 (Blue 7)	211.67 ± 29.19
4-4-99-4	123.00 ± 21.63	Amb+ Chandler	251.67 ± 31.01	2247 x 6073-00-4 (Green 4)	233.33 ± 50.29
5-1-99-2	268.67 ± 56.50	Amb + Chandler #A1-11	158.67 ± 14.36	2247 x 6073-00-6 (Green6)	265.00 ± 34.07
7-2-99-1	151.00 ± 48.77	Amb + Chandler # 69	99.33 ± 8.02	2247 x 6073-00-8 (Green 8)	239.33 ± 22.81
7-2-99-2	217.67 ± 38.44	Amb + HBJL-1	286.33 ± 13.32	2247 x 2060-00-1 (Purple 1)	216.67 ± 48.21
7-3-99-1	161.33 ± 09.29	Amb + HBJL-2B	278.67 ± 29.54	2247 x 2060-00-3 (Purple 3)	197.00 ± 11.14
8-1-99-4A	216.33 ± 16.29	Amb + HBJL-3	280.33 ± 23.54	2247 x 1571-00-4 (White 4)	154.67 ± 14.01
8-1-99-2B	252.33 ± 34.00	Amb + HBJL-4	98.67 ± 18.01	N + HBP x SO + RP-04-7	108.00 ± 38.74
8-1-99-4B	213.67 ± 41.68	Amb + HBJL-5	265.67 ± 12.50	(SR x SH)-99-5	183.17 ±15.97
8-1-99-4B set 2	207.67 ± 15.63	Amb + HBJL-7	215.00 ± 29.51	**Diploid Hybrid**	
8-2-99-1	180.33 ± 27.61	Amb + HBP	161.67 ± 37.10	43 x 20-04-1	153.67 ± 12.22
Chandler #A1-11	143.33 ± 24.01	Amb + MG1	100.33 ± 19.86	46 x 20-04-12	108.33 ± 19.86
HBJL-3 R6T16	227.67 ± 25.42	Amb+ SN7	102.67 ± 07.37	46 x 20-04-19	146.00 ± 08.00
HBJL-3 R10T20	254.00 ± 25.24	Amb + MG-10	149.00 ± 18.25	Volk x P	268.67 ± 20.74
HBJL-4	166.67 ± 16.26	Changsha + HBJL-3	256.67 ± 15.63	**Open pollinated tetraploid**	
HBJL-5	201.00 ± 14.11	Changsha + HBJL-5	271.33 ± 15.04	2247-OP-A1	215.00 ± 22.61
HBJL-7	221.00 ± 24.76	Changsha + HBJL-7	271.00 ± 15.62	2247-OP-A2	226.33 ± 05.69
HBJL-12	235.33 ± 13.58	Murcott + 4-4-99-6	249.00 ± 25.51	2247-OP-A5	186.00 ± 24.43
MG-10	116.67 ± 16.50	Murcott + Chandler #A1-11	100.67 ± 09.29	SORP-OP-02-8	146.00 ± 06.56
MG-11	165.00 ± 6.00	Murcott + Chandler #80	102.67 ± 06.66	**Grapefruit**	
SN3	140.00 ± 11.14	Murcott + HBJL-1	203.33 ± 06.51	Marsh grapefruit	237.33 ± 14.84
Somatic Hybrids		Murcott + SN3	151.33 ± 09.07	Ruby Red grapefruit	224.67 ± 48.01
Amb + 4-3-99-2	225.67 ± 22.37	Page + HBJL-3	162.33 ± 10.69		
Amb + 4-4-99-6	242.67 ± 24.21	Page + HBJL-7	211.00 ± 17.58		
Amb + 5-1-99-1B	229.33 ± 12.01	Succari + HBP	120.33 ± 22.37		
Amb + 5-1-99-3	148.00 ± 35.79	W.Murcott + HBJL-7	268.00 ± 07.94		
Amb + 7-2-99-5	103.00 ± 34.60				
Sour orange			66.67 ± 2.52		
LSD			41.197		
P value			0.0001		

Table 3-3. Shoot length (cm) and the seedling yellows symptoms of test rootstock candidates inoculated with T36 in the greenhouse 8 months after inoculation.

Germplasm	Shoot length (cm)		Symptoms	Resistance level based
Rootstock	Healthy	T36	SY score	on performance in the field
A+ 4-3-99-2	139	99	2.5	Resistant
A+ 4-4-99-6	118	63	2.5	Intermediate
A+ 7-2-99-5	100	35	3.0	Susceptible
A+ Chandler #A1-11	116	59	3.0	Tolerant
A+ HBJL-1	127	76	2.5	Resistant
A+ HBJL-3	112	80	2.0	Intermediate
A+ HBJL-5	126	72	3.0	Intermediate
A + SN7	136	25	2.5	Susceptible
Page + HBJL-3	109	53	3.0	Slightly tolerant
Sour orange (S.O)	124	30	3.0	Susceptible

Table 3-4. Total chlorophyll content (mg/g) in test rootstock candidates showing chlorosis symptoms 8 months after inoculation with T36 in the greenhouse.

Germplasm	Total chlorophyll (mg/g)	
Rootstock	Healthy	T36
A+ 4-3-99-2	1.21	0.52
A+ 4-4-99-6	1.4	0.75
A+ 7-2-99-5	1.73	0.34
A+ Chandler #A1-11	0.99	0.42
A+ HBJL-1	1.32	0.86
A+ HBJL-3	1.52	1.09
A+ HBJL-5	0.81	0.36
A + SN7	0.67	0.30
Page + HBJL-3	1.83	0.52
S.O	1.09	0.29

Table 3-5. Summary of the starch content (mg/g dry weight) in 'Hamlin' sweet orange leaf and the rootstocks roots (means were separated using the LSD separation of means at *p=0.05*).

	Starch content in 'Hamlin' sweet orange leaf (mg/g dry weight ± StDev)		Starch content in the rootstocks roots (mg/g dry weight ± StDev)	
Rootstock	Healthy control	CTV-infected	Healthy control	CTV-infected
A+4-3-99-2	54.12 ± 4.69	63.82 ± 2.35	140.50 ± 5.29	130.82 ± 2.02
A+4-4-99-6	70.76 ± 10.13	86.66 ± 3.23	136.29 ± 7.50	148.52 ± 3.72
A+HBJL-1	76.33 ± 7.64	84.58 ± 5.32	129.95 ± 2.29	122.57 ± 2.62
A+HBJL-3	80.46 ± 12.76	101.69 ± 3.86	124.64 ± 4.75	112.07 ± 2.61
A+HBJL-5	90.87 ± 10.59	112.40 ± 4.94	121.31 ± 4.84	123.35 ± 2.76
A+Chandler #A1-11	89.67 ± 6.04	125.51 ± 1.92	100.16 ± 5.59	88.21 ± 2.59
A+7-2-99-5	97.01 ± 9.62	127.49 ± 2.83	110.59 ± 4.74	79.56 ± 3.35
A+SN7	88.07 ± 2.16	113.02 ± 2.07	141.89 ± 4.68	108.27 ± 7.61
Page+HBJL-3	71.19 ± 2.46	121.81 ± 3.09	108.72 ± 7.41	84.58 ± 2.06
Sour orange	64.54 ± 6.58	135.52 ± 2.06	116.49 ± 9.09	69.91 ± 3.92
LSD	10.13		8.11	
P value	0.0001			

CHAPTER 4
USE OF SEROLOGICAL METHODS TO DETERMINE CITRUS TRISTEZA VIRUS (CTV) STATUS AND RESISTANCE IN TOP-WORKED ROOTSTOCK CANDIDATES TO REPLACE SOUR ORANGE

Introduction

Citrus tristeza virus is often a concern wherever citrus is produced commercially. CTV isolates differ in the symptoms they cause depending on the isolate, the host and or the scion-rootstock combination. From the disease management point of view, the stem pitting and the quick decline (QD) diseases are the two major disease syndromes produced by CTV infection. Certain isolates cause the stem pitting of scions regardless of the rootstock, which reduces vigor, fruit yield and quality on the infected trees. Variously sized pits or grooves in the wood often contain a yellow gum and irregular growth of the phloem occurs in the area of these xylem pits (Brlansky et al., 2002). Other isolates cause decline and death of citrus trees grafted on sour orange (*Citrus aurantium* L.), the most desirable horticultural rootstock. The QD is caused by a virus-induced phloem necrosis in the bark of the rootstock just below the bud union that prevents the movement of carbohydrates from the canopy to the roots. Lack of carbohydrates supply in the root system causes the roots to degenerate and inhibits formation of new fibrous roots that result in the decline of the infected trees (Garnsey et al., 1987a). The QD problem is more severe and can occur on sweet orange, mandarin and grapefruit scions grafted on sour orange rootstock. Millions of citrus trees on sour orange rootstock were lost due to the quick decline disease caused by CTV. Therefore the use of sour orange rootstock is no longer feasible and less desirable rootstocks are being utilized. The primary rootstocks used currently in Florida are trifoliate hybrids, and in general they are not adapted to high pH, calcareous soils (Grosser et al., 2000; Grosser et al., 2004a; Bauer et al., 2005). The CREC citrus improvement program is focusing mainly on developing new rootstocks for CTV-induced QD resistance with the effort to

replace sour orange rootstock. Screening the rootstock candidates for CTV-QD resistance is required as a part of the rootstock improvement program.

Several techniques have been developed for CTV detection and differentiation of CTV isolates. Biological indexing was applied by inoculating a selected group of citrus genotypes (Garnsey et al., 1987b). The major disadvantage of this biological indexing is the time required to complete the indexing (Lee et al., 1994). Also the electron microscopy (EM) of negatively stained extracts was another method used for detection of CTV infected trees (Bar-Joseph et al., 1989). This technique received limited application because of the high cost, it was time consuming and required specific skills (Rocha-Peña and Lee, 1991). The development of quick, accurate serological tests for CTV was not possible until purification methods for CTV were developed. Antisera was then prepared against purified virus and used in diverse serological techniques (Rocha-Peña and Lee, 1991). Serological tests introduced a fast and reliable a technique to screen for CTV infection on a large scale and have been used for long time to detect CTV (Gonsalves et al., 1978; Bar-Joseph et al., 1979b; Garnsey et al., 1979; Brlansky et al., 1984; Rocha Peña et al., 1991). Polyclonal antibodies have been made in several animal species against different CTV isolates (Rocha Peña et al., 1991). Monoclonal antibody MCA13 was raised against a decline- inducing CTV isolate (T36) collected from a sweet orange grafted onto sour orange rootstock in Florida (Permar et al., 1990). This antibody differentiates between mild and severe CTV isolates. It reacts with decline isolates from Florida and a majority of decline and stem pitting isolates from various citrus growing regions (Permar et al., 1990). The MCA13 reactive site was mapped to a single amino acid in the coat protein (CP) by in vitro studies using *Echerichia coli* system (Pappu et al., 1993). Mutation of a single nucleotide resulting in the change of the amino acid phenylalanine to tyrosine at the position 124 of the CP prevented the

MCA13 reactivity of a severe isolate, whereas the CP of a mild isolate with a position 124 change from tyrosine to phenylalanine reacted positively with MCA13, monoclonal antibody (Pappu et al., 1993). Several studies on serological detection of different CTV isolates with a number of polyclonal and monoclonal antibodies suggested that multiple epitopes exist in the CTV coat protein (Brlansky et al., 1984; Vela et al., 1988). Enzyme-linked immunosorbent assay (ELISA) is the most convenient, reliable, and relatively inexpensive procedure. Therefore, ELISA is widely used to measure the concentration of soluble proteins including viral protein such as CTV (Rocha-Peña and Lee, 1991). In general, the protein is attached to the antibody coated on an assay plate and detected using a three-step process. Color development after adding the substrate is quantified and is proportional to the viral protein bounds to the plate (Garnsey and Cambra, 1991). The serological techniques such as, ELISA and direct tissue blots immunoassay (DTBI) allow screening for large number of samples. DTBI is rapid, required little sample preparation and could be stored at room temperature for 30 days at least prior to assay (Garnsey et al., 1993). In addition, western blot analysis can be used to detect a specific protein in a tissue extract using specific antibodies to the target protein (Gutiérrez et al., 1997).

Materials and Methods

ELISA

Plant materials

Samples were collected from 15-year- old field trees (source) of ‘Hamlin’ sweet orange on Carrizo citrange prior and after the top-working, as well as from the newly top- grafted scions (that included preselected candidate rootstock hybrids, some of their pummelo parents and two grapefruit varieties). The total number of the tested citrus genotypes was 74 (72 test selections and Ruby Red and Marsh grapefruit), and their identity is described in Table (3-1). Marsh and Ruby red grapefruit were used here to compare the CTV titer in grapefruit with sweet orange and

the tested materials. All 74 test genotypes were top-worked as described in Chapter (3) along with the sour orange. Samples from the tested materials were collected and assayed 18 months after the successful top-working graft. One important point to consider when performing serological tests for CTV detection is the selection of the tissue that contains the virus particles. CTV is a phloem-limited virus and therefore, it is present at highest concentrations in phloem-rich tissues (Bar-Joseph et al., 1979b). The best tissues for CTV detection have proven to be bark, petioles, and midribs of recent flushes (Garnsey et al., 1979). Time of sample collection also is a critical factor and needs to be considered. Prolonged hot weather can result in the uneven distribution of CTV in grapefruit and sometimes in sweet orange. Therefore, for routine serological tests, field samples are collected preferably in the spring or autumn (Lee et al., 1988). Ambient temperatures above 30°C are known to suppress the field symptoms and detection of CTV through serological tests (Roistacher et al., 1974; Mathews et al., 1997).

Visual assessments of symptoms were made periodically over almost a two- year period according to methods detailed by Garnsey et al., (1987b) and a severity score (0-3) was assigned to each symptom in each graft (rootstock candidates and sour orange) for the individual top-worked trees, where 0 = no symptoms and 3 = severe CTV symptoms (data not presented). The shoot growth of all the top-worked citrus test genotypes was also measured (Table3-2). Tissue samples of healthy and positive controls were also included in the serological tests. In this study, the serological tests were used to determine the CTV titer mainly in the rootstock candidates and the corresponding sour orange graft using polyclonal and monoclonal (MCA13) antibodies.

ELISA method

Enzyme-linked immunosorbent assay (ELISA) was used to estimate the virus concentration in the CTV- infected plants using polyclonal and monoclonal CTV specific antibodies (Rocha-Peña and Lee, 1991). The ELISA test was performed 3 times at 6 months

intervals but the data presented here is from the samples collected 18 months after grafting since it is believed that CTV symptoms would appear on infected trees 10-12 months after inoculation (McGovern et al., 1994; Al-Senan et al., 1997).

The broad spectrum or general ELISA was conducted using two CTV polyclonal antisera (anti-CTV 1052 and 1052 IgG alkalin phosphatase labeled), kindly provided by Dr. R. H. Brlansky (Citrus Research and Education Center, University of Florida, Lake Alfred, FL). For the general polyclonal (broad spectrum) ELISA, the double antibody sandwich direct (DAS) ELISA method was carried out according to Garnsey and Cambra (1991). The MCA13 ELISA test was conducted using monoclonal MCA13 antiserum (purchased from Tom Permar, at Nokomis Corporation) and the procedure was done according to Permar et al., (1990). For both ELISA assays, wells of costar high binding (Corning, Acton, MA) or Immulon 2-HB microtiter 96 well plates were rinsed with deionized water to remove polystyrene fragments (McLaughlin et al., 1981). After that, they were coated with 200 µL of the rabbit polyclonal coating antiserum IgG 1052 developed against T36 CTV isolate. The coating antibody was diluted to 1:10,000 in sodium carbonate coating buffer pH 9.6 (see appendix A) for both general and monoclonal MCA13 assays. Then the plates were incubated for overnight at 4°C. After incubation, the antibody was discarded and plates were washed three times with phosphate-buffered saline with Tween- 20 (PBST; 0.02 M phosphate, 0.14 M sodium chloride at pH 7.4, 0.1 % [v/v] Tween-20). The bark (0.5 g) from each sample was pulverized in 10 ml extraction buffer (PBST) using a KLECO tissue pulverizer. The homogenized sap (200 µL) for each sample was added to duplicate test wells on the antibody-coated plates and incubated at 4°C overnight. The plates for DAS-ELISA were then rinsed with PBST for 3 times 10 min each and 200 µL per well of the 1052 antibody conjugated with- Alkaline phosphatase (AP) at a dilution of 1:10,000 in conjugate

buffer (PBST and 0.2% [w/v] bovine serum albumin, BSA). For the monoclonal test, Double Antibody Sandwich Indirect (DAS-I) ELISA was performed using MCA13 specific monoclonal antibody. The MCA13 plates were washed as previous and 200 µL per well of the MCA13, monoclonal antibody was added at a dilution of 1:30,000 in the antibody buffer (PBST and 0.2% [w/v] BSA). The MCA13 plates were then incubated at 37°C for 4 h. After washing the plates three times, 10 min each with PBST, a 200 µL aliquots of goat Anti-Mouse IgG antibody (Whole Molecule) – (AP) , Sigma A-3562 (GAM) for MCA13 ELISA DAS-I at 1:30,000 dilutions in conjugate buffer (PBST and 0.2% [w/v] BSA) were added and incubated under the same conditions. Polyclonal and MCA13 plates were again washed three times, 10 min each with PBST and phosphatase substrate (1µg/ml; ρ-nitrophenyl phosphate Sigma S-0942 in 10% [v/v] triethanolamine, pH 9.8), was added. The plates were kept in the dark at room temperature until color development was complete. The resulting yellow color was measured at 405 nm (OD_{405}) during the reaction (1-3h.) using a microplate reader (Bio-Rad 550, BioRad, Hercules, CA). The data represented the average OD_{405} of duplicated samples of the tested materials, healthy control, CTV- infected (positive) samples and extraction buffer controls in each test. The buffer value was Subtracted from all the values and samples were considered positive when their average OD_{405} value was more than twice that of the healthy control (Clark et al., 1988; Lee et al., 2005).

Direct Tissue Blots Immunoassay (DTBI)

Samples of the top-worked rootstock candidates were collected a year after grafting. Due to the large number of samples and the limited amount of the antibody, only the most important samples were selected from the 74 test genotypes. The selection was based on ELISA results using the specific monoclonal antibody, MCA13 (Table 4-4). Seventeen MCA13 negative samples and 21 of the MCA13 positive samples were selected for this assay (Table 4-1). Reaction of the grafted materials to the MCA13 monoclonal antibody was tested by DTBI in

order to confirm the MCA13 reaction results especially for the negative results. Tissue blots were prepared as described by Garnsey et al., (1993). Three young stems were taken from each tree, then cut and the fresh- cut stem was pressed onto a nitrocellulose membrane. Stems of healthy and positive greenhouse controls were included. The membrane was air dried and then blocked in PBS + 1% BSA for 1 h. Blocking solution was removed and the primary antibody, MCA13 at a 1:20,000 dilution in antibody buffer (PBS + 1% BSA) was added. The membrane was incubated for three h at room temperature with shaking at 25 RPM. After this, membranes were washed three times in PBST with gentle agitation for 5 min each. After the final wash, secondary antibody GAM-AP was added at 1:15,000 dilutions and the membrane was incubated for either 2 h at 37°C or for overnight at room temperature. Then the membrane was washed as previously. The blots were treated with a mixture of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (BCIP) and ρ-nitro blue tetrazolium (NBT) (Sigma B-1911) till the development of the purple color (Garnsey et al., 1993).

Western Blot Analysis

To carry out western blot analysis, total protein was extracted from the samples previously tested by direct tissue blot immunoassay **(**Table 4-1). About 0.2 g tissue from the tested materials was collected 18 months after top-working. Tissue was ground in liquid nitrogen. To isolate the soluble fractions, the ground tissue was thawed in an equal amount of phosphate buffered saline, PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4) and 200 µl extraction buffer [50 mM Tris-HCl, pH 6.5; 1 mM EDTA; 150 mM NaCl; 0.1% Triton X-100]. The following protease inhibitors: 2 µg/ml Antipain; 2 µg/ml Leupeptin; 2 µg/ml Aprotinin; 1 mM of 4-[2-aminoethyl]-benzenesulfonyl fluoride (AEBSF) Sigma; and 5% of 2- mercapto-ethanol were added immediately prior to using the extraction buffer. Samples were incubated

with shaking for 30 min at 4ºC and then centrifuged at 10,000 RPM for 10 min at 4ºC. The supernatant was re-centrifuged at 13,000 RPM for another 10 min at 4ºC. Total protein concentration was determined in the supernatant by the Bradford assay using BSA as a standard (Bradford, 1979), then samples were either immediately subjected to western blot analyses or stored at -80ºC till needed. For western analysis, aliquots of the soluble plant extracts containing 100 µg total proteins were mixed with an equal volume of dissociation buffer (140 mM SDS, 160 mM Tris-HCl pH 7.8, 1% (v/v) glycerol, 142 mM β-mercapto-ethanol) then boiled for 5 min. Samples were separated on a precast 12% polyacrylamide Tris-HCl gel (BioRad) in a Mini-Protean III cell (Bio-Rad) according to Laemmli (1970) using Tris-glycine as the SDS-PAGE electrophoresis buffer (Appendix B). Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon™-P; Millipore Corporation, Bedford, MA, USA, Cat. No. IPVH 000 10) using Trans-Blot Cell (BioRad) using the transfer buffer (Appendix B). The non-specific binding sites on the membrane were then blocked with Blotto [5% non-fat dried milk in TTBS (100 mM Tris-HCl, pH 7.9; 150 mM NaCl; 0.1% Tween 20) with shaking for one h at room temperature. The membrane was incubated with the primary monoclonal antibody (MCA13) at 1:30,000 dilutions (in TBS with 2% BSA) at room temperature with shaking overnight. After that, the membrane was washed for 10 min each in TTBS. The blot then was incubated with the secondary antibody (GAM-AP) in TBS + 2% BSA at 1:20,000 for 3 h at room temperature with shaking, followed by three 10-min. washes in TTBS. The membrane blots were developed using BCIP/NBT liquid substrate system.

Results and Discussion

ELISA

ELISA data for the source samples before and after top-working, as well as the newly top-worked test genotypes, using CTV polyclonal and the monoclonal antibody MCA13 are presented in Tables (4-2), (4-3) and (4-4), respectively. The ELISA data presented as OD values at 405 nm is the average of two replications per sample after a 2-h reaction. Positive (+) values are higher and minus (-) values are lower than the value of twice the value of the healthy control (Clark et al., 1988).

The entire source 'Hamlin' sweet orange trees were CTV positive using both the polyclonal and MCA13 monoclonal ELISA. Therefore, all of the source trees were characterized as MCA13 positive (Table 4-2). Of the 72 test genotypes plus the Marsh and Ruby Red grapefruit (Table 3-1), only eight genotypes were negative (pummelo seedlings 7-2-99-1, 8-1-99-4B set 2, HBJL-3 R10T20, HBJL-5; somatic hybrids Amb +4-3-99-2, Amb +5-1-99-3, Murcott + HBJL -1; and open-pollinated tetraploid 2247-OP-A2), and the remainder were positive for CTV in the polyclonal ELISA test . When the same samples were tested with the monoclonal antibody, ten more candidates were found to be MCA13 negative (Table 4-4). The test hybrids that showed negative results by MCA13 monoclonal antibody were pummelo seedlings 5-1-99-2, 7-2-99-1, 8-1-99-2B, 8-1-99-4B set 2, Chandler #A1-11, HBJL3 R10T20, HBJL-5; somatic hybrids Amb +4-3-99-2, Amb +5-1-99-3, Amb +Chandler, Amb + HBJL -1, Amb + HBJL -2B, Murcott + HBJL -1, W. Murcott + HBJL -7; tetrazyg 2247 x 6073-00-6 (GREEN 6); diploid hybrid Volk x P; and tetrazyg 2247-OP-A2. All of these showed a high shoot growth. The positive values of the polyclonal ELISA ranged from OD_{405} 0.08 for pummelo 4-3-99-2 set 7 to 0.74 for diploid hybrid Volk x P, whereas the OD_{405} values for the corresponding sour orange control were 1.416 and 2.850 respectively. The value for the healthy control was OD_{405} 0.035. In

general the OD_{405} for sour orange ranged from 1.09-3.185 (Table 4-3). For MCA13 ELISA, positive values ranged from OD_{405} 0.060 for 8-1-99-4B to 0.374 for 46x 20-04-12 whereas the OD_{405} values for the corresponding sour orange control were 1.348 and 1.345 respectively. For MCA13 ELISA, data are shown for each category in Figures (4-1 to 4-6). In the Pummelo seedling group, data ranged from OD_{405} 0.078 for 4-3-99-2set7 to 0.261 for 8-1-99-4A (Figure 4-1). Group 2 (somatic hybrids) showed values between 0.065 for Changsha + HBJL-7 and 0.521 for Amb + MG-1 (Figure 4-2). The tetrazygs group showed OD_{405} values between 0.064 to 0.308 for Green 4 and (SRXSH) 99-5 respectively (Figure 4-3). For the diploid hybrids, the lowest CTV titer was shown in Volk x P (0.029) and the highest OD_{405} value was 0.374 for 46x20-04-12 (Figure 4-4). In the open pollinated tetraploid group the OD_{405} value varied from 0.017 for 2247-OP-A5 to 0.219 for Sorp-OP-02-8 (Figure 4-5). Marsh and Ruby Red grapefruit were used here to test the difference in the severe CTV accumulation in the field compared to the 'Hamlin' sweet orange (Figure 4-6). Ruby Red showed a lower value (0.116) than Marsh grapefruit (0.153), and 'Hamlin' sweet orange showed a very high value (OD_{405} +1.105) compared to both grapefruit varieties. This data was in agreement with (Bar-Joseph and Lee, 1989), who stated that sweet orange is more sensitive to infection than grapefruit. In general the OD_{405} for sour orange ranged from 0.981-2.861(Table 4-4) and the value for the healthy control was OD_{405} 0.026.

Direct Tissue Blot Immunoassay (DTBI)

Table (4-1) represents a list of the samples selected for direct tissue blot immunoassay using the MCA13, monoclonal antibody based on the ELISA, MCA13 data. Tissue prints were quickly performed and were as sensitive as ELISA in detecting CTV. The results are shown in Table (4-6) and the prints of representative samples are shown in Figure (4-7). The imprint of the CTV-infected stems was clearly visible with deep purple- stained area indicating the presence of the CTV virion in the phloem of the stems (Figure 4-7). The healthy tissue imprint showed no color

that was easily distinguished from the intense purple color in the stained phloem of the CTV-infected samples. The results were in agreement with the ELISA data. DTBI is a reliable and sensitive procedure for CTV detection and provides a fast tool to screen a large number of samples (Garnsey et al., 1993).

Western Blot Analysis

Samples listed in Table (4-1) were further analyzed by western blotting for the CTV coat protein (CP) using the MCA13 monoclonal antibody. The specific bands were developed on the membrane in purple color. Strong purple bands corresponding to the coat protein, 25- kDa in size were detected in the infected samples indicating the presence of CTV quick decline isolate from Florida (Figure 2-8 A-E). The sour orange corresponding to the listed MCA13 negative samples (1-7) were tested and the results are shown in Figure (4-8 E).

Conclusions

Rootstock candidates developed in efforts to replace sour orange rootstock were screened using a top-working technique by grafting each of 72 selections, mostly mandarin + pummelo somatic hybrids, but also including selected parental pummelo seedlings, along with sour orange. Test genotypes were top-worked onto established CTV- infected 'Hamlin' field trees. The virus infection was then detected by serological techniques including tissue blot immunoassay (TBIA), double antibody sandwich enzyme –linked immunosorbent assay (DAS-ELISA) and western analysis. DAS-ELISA using polyclonal antibodies has previously been used to evaluate virus titer in citrus plants (Garnsey, et al 1985 and Lee et al, 1991). Positive reaction for some samples was not achieved unless reaction with the substrate was continued for 2 h. This may reflect a low titer of the virus in those plants. A higher titer in the MCA13-ELISA may be relative estimate of the severe CTV infection, since the MCA13 monoclonal antibody reacts especially with severe CTV isolate (Permar et al., 1990). Its use provides a tool to screen for severe CTV infection,

especially in the Florida budwood registration program to prevent propagation of budwood containing potentially damaging isolates, while allowing propagation of budwood carrying mild isolates already endemic in the state (Sieburth, 2000). The relatively quick tissue print method using MCA13 was determined to be a good method for high throughput and to validate traditional ELISA. Seventeen of the test genotypes were MCA13 negative in this study, and the data revealed various degrees of CTV resistance/tolerance in the remaining test genotypes. The rootstock candidates were divided into 5 categories based on the performance in the field (shoot growth and CTV symptoms) in relation to MCA13 (DAS-I) ELISA the MCA13 –ELISA results combined with the shoot length data: resistant; tolerant, intermediate, slightly tolerant and susceptible (Table 4-5). Hybrid rootstock candidates from the resistant and highly tolerant groups should definitely be included in further studies to determine their rootstock potential.

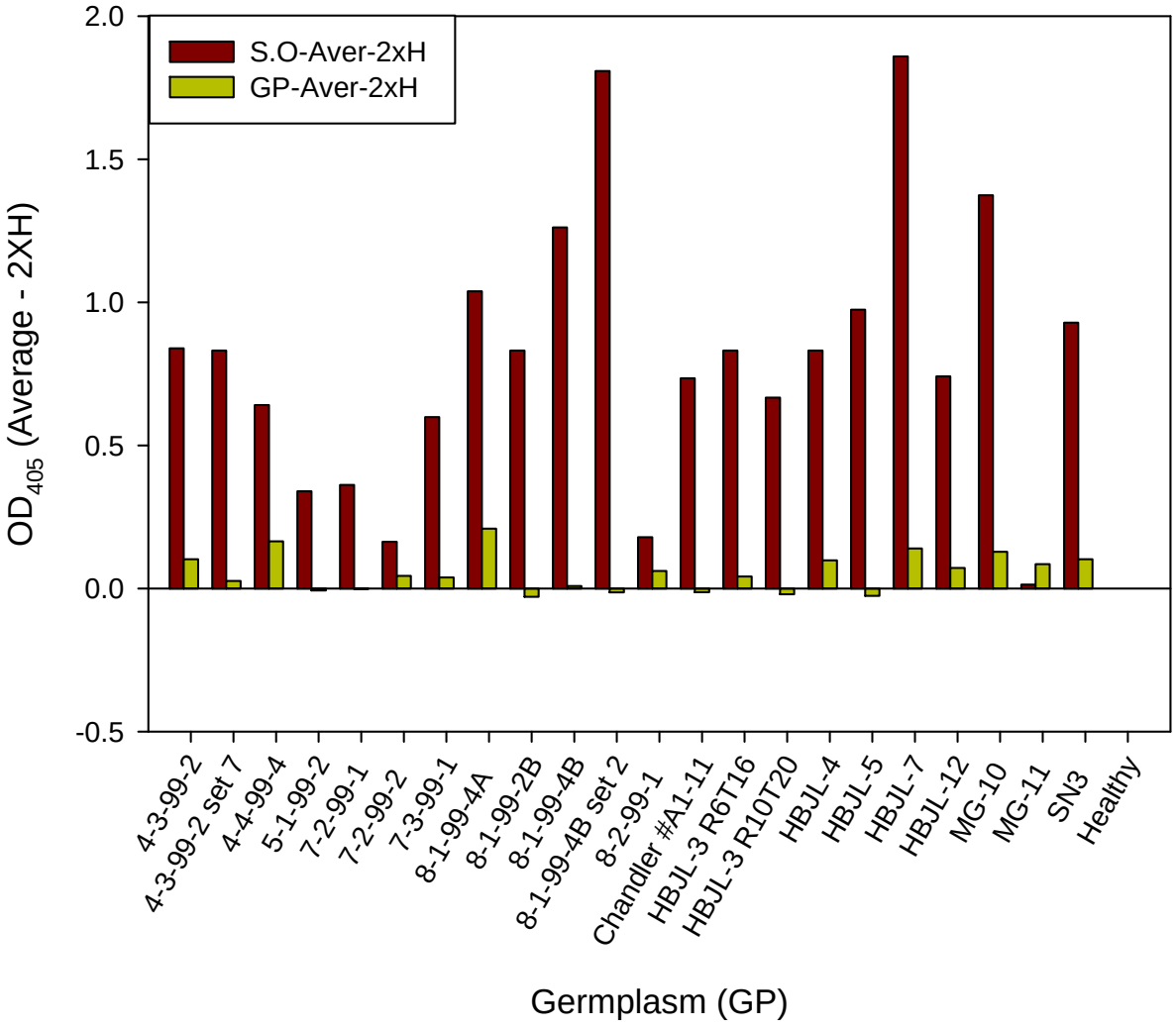

Figure 4-1. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for top-worked test genotypes (pummelo seedling parent group) and sour orange control, collected 18 months after top-work grafting.

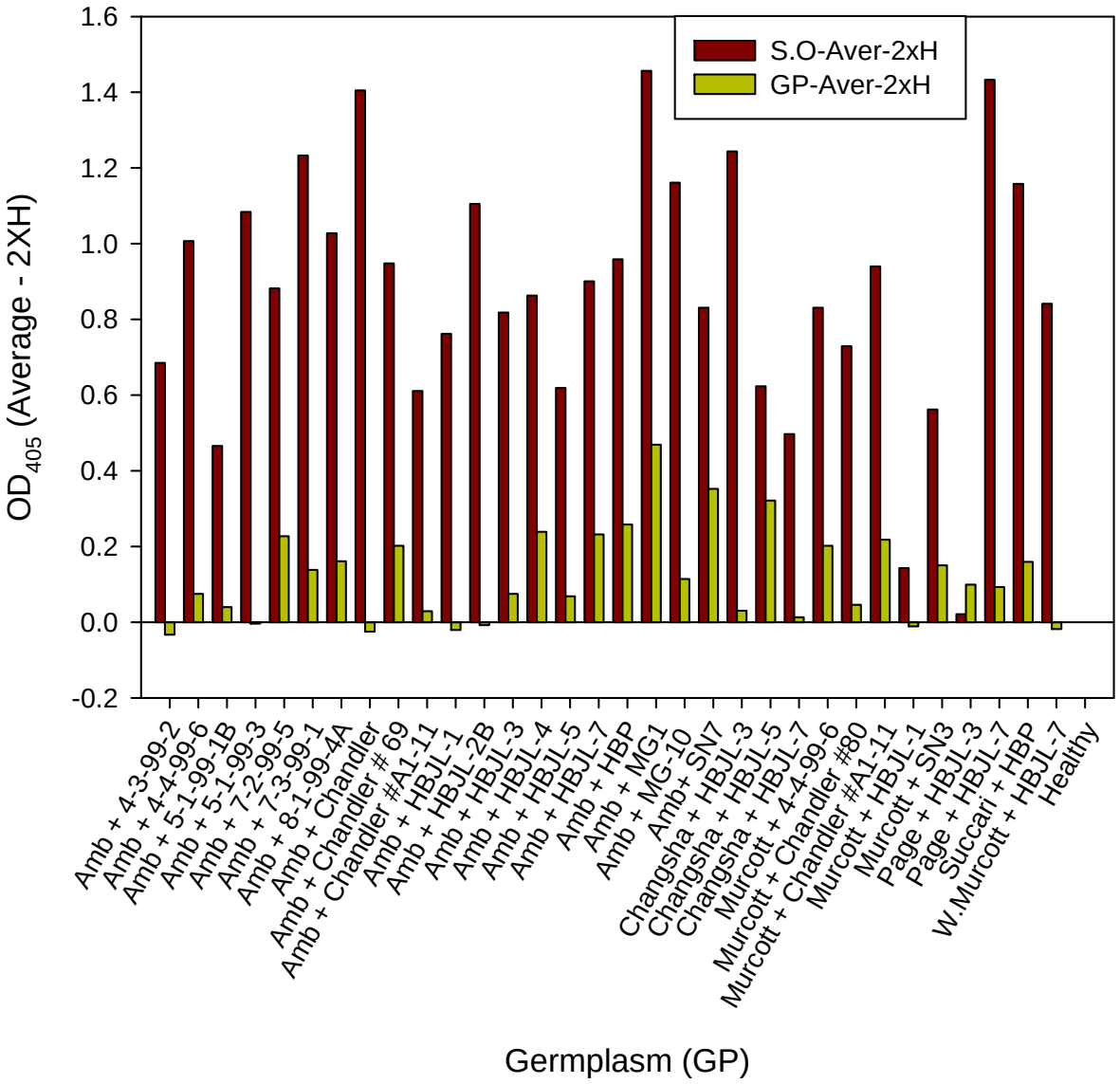

Figure 4-2. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for top-worked test genotypes (somatic hybrid group) and sour orange control collected 18 months after grafting.

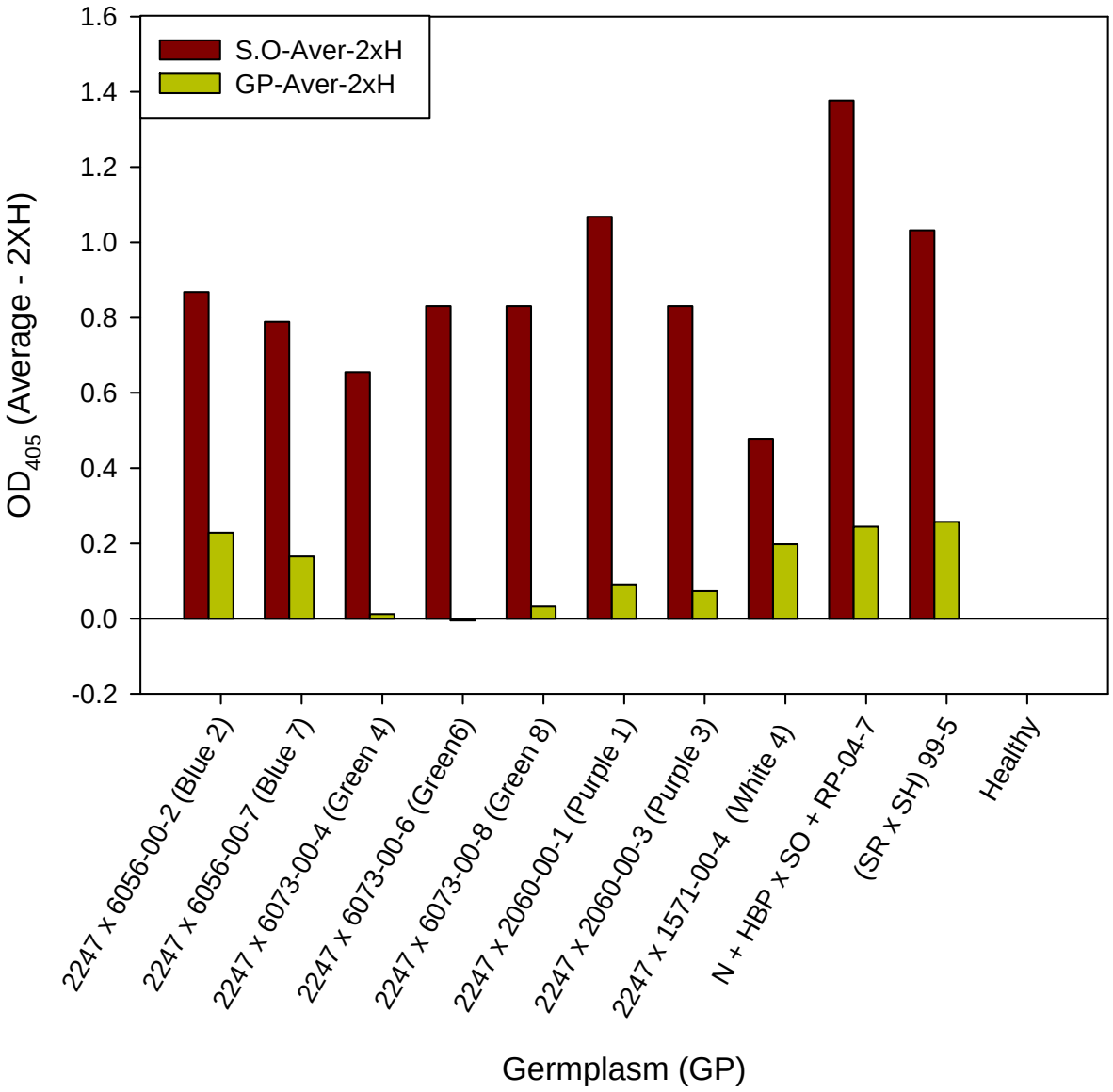

Figure 4-3. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for top-worked test genotypes (tetrazyg group) and sour orange control collected 18 months after grafting.

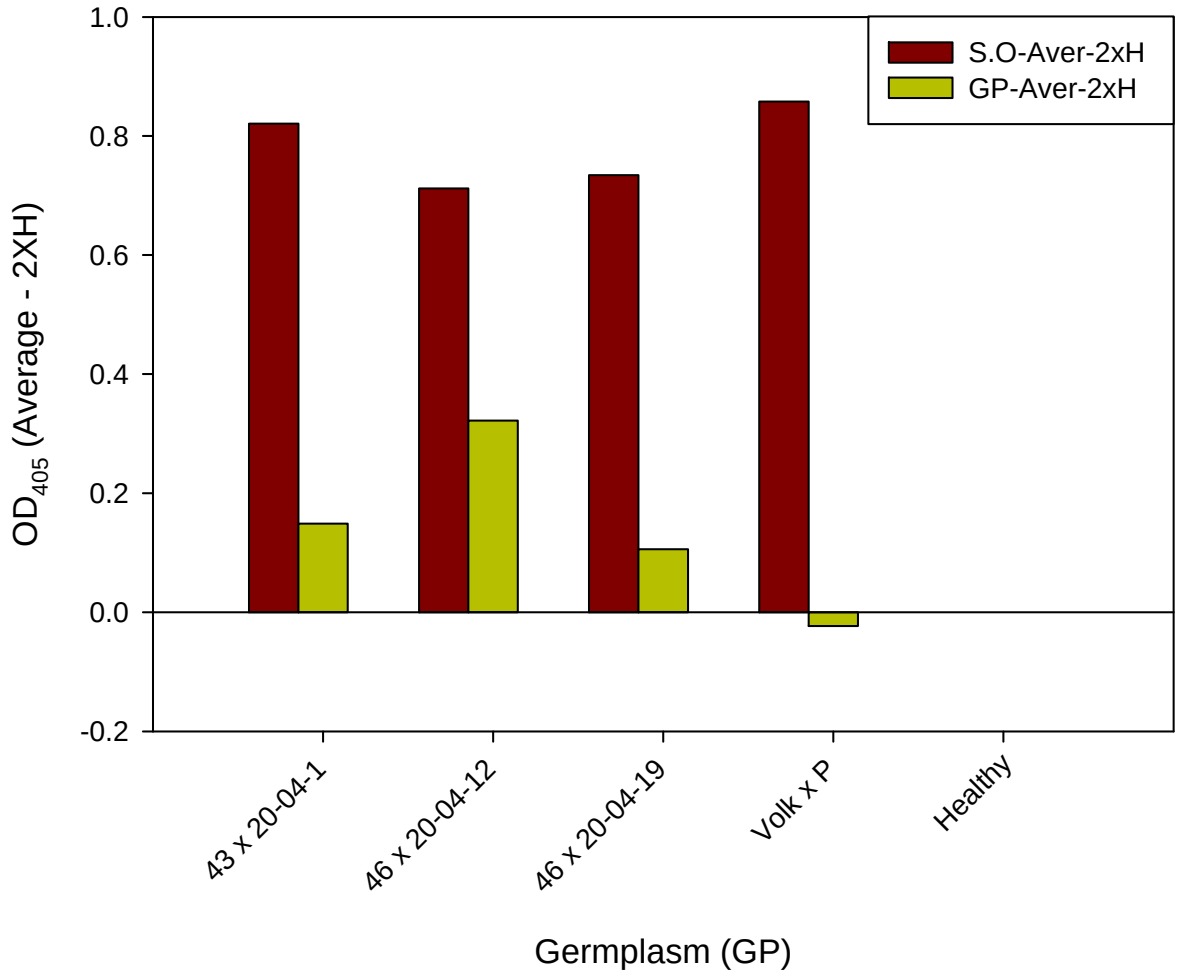

Figure 4-4. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for top-worked test genotypes the grafted rootstock candidates (Diploid hybrid group) and sour orange control collected 18 months after grafting.

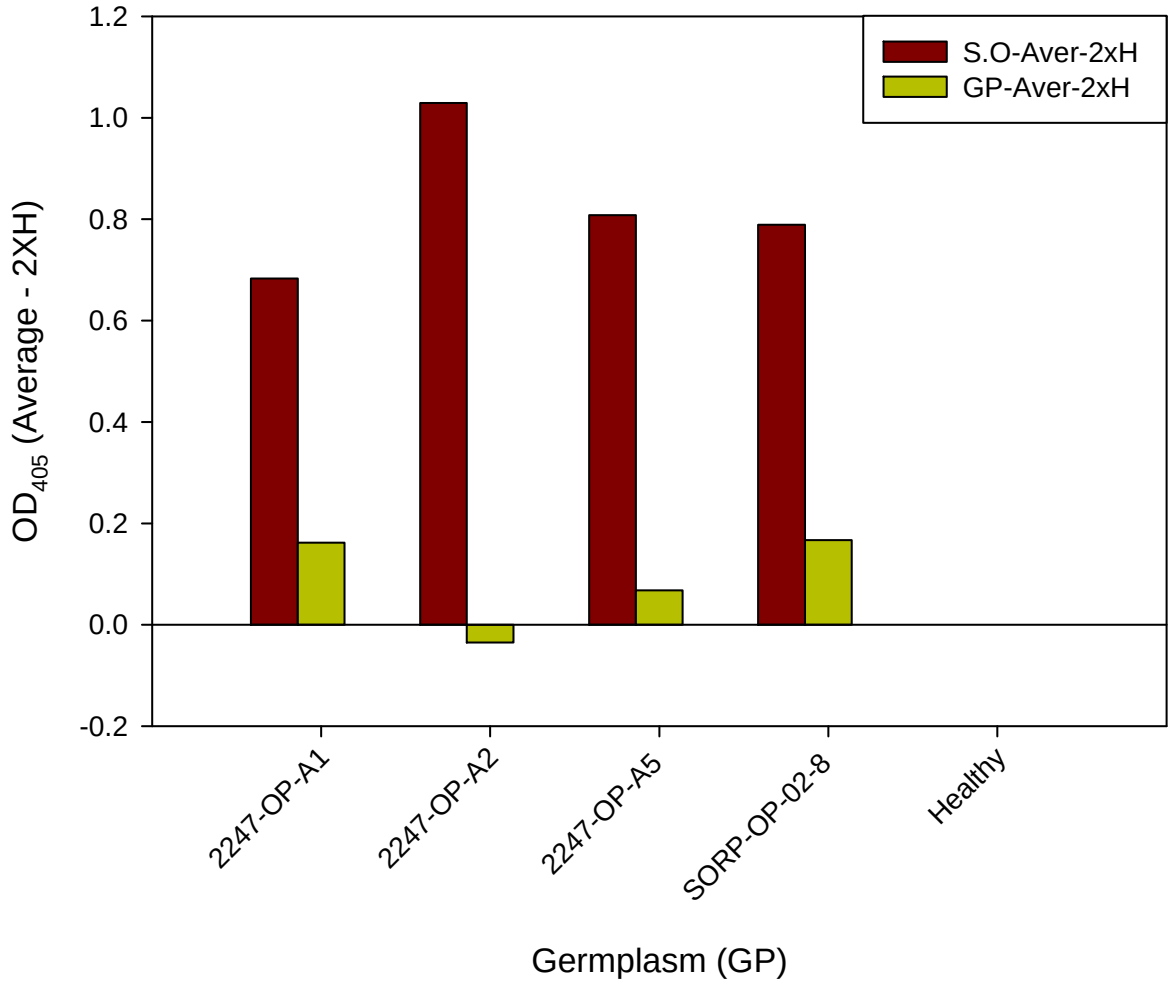

Figure 4-5. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for the grafted rootstock candidates (OP) tetraploids group and sour orange control collected 18 months after grafting.

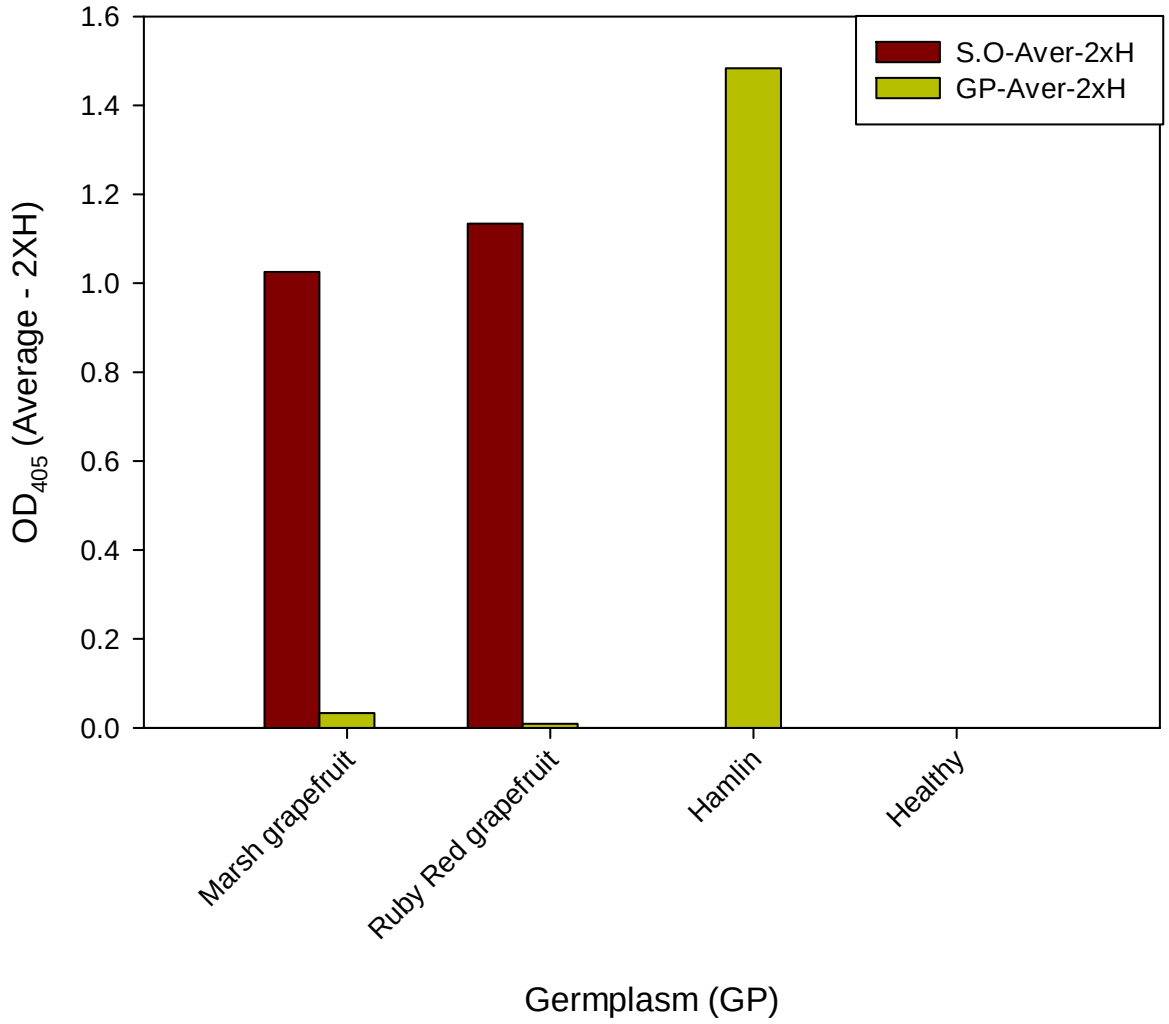

Figure 4-6. CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for top-worked commercial scions and sour orange control collected 18 months after grafting.

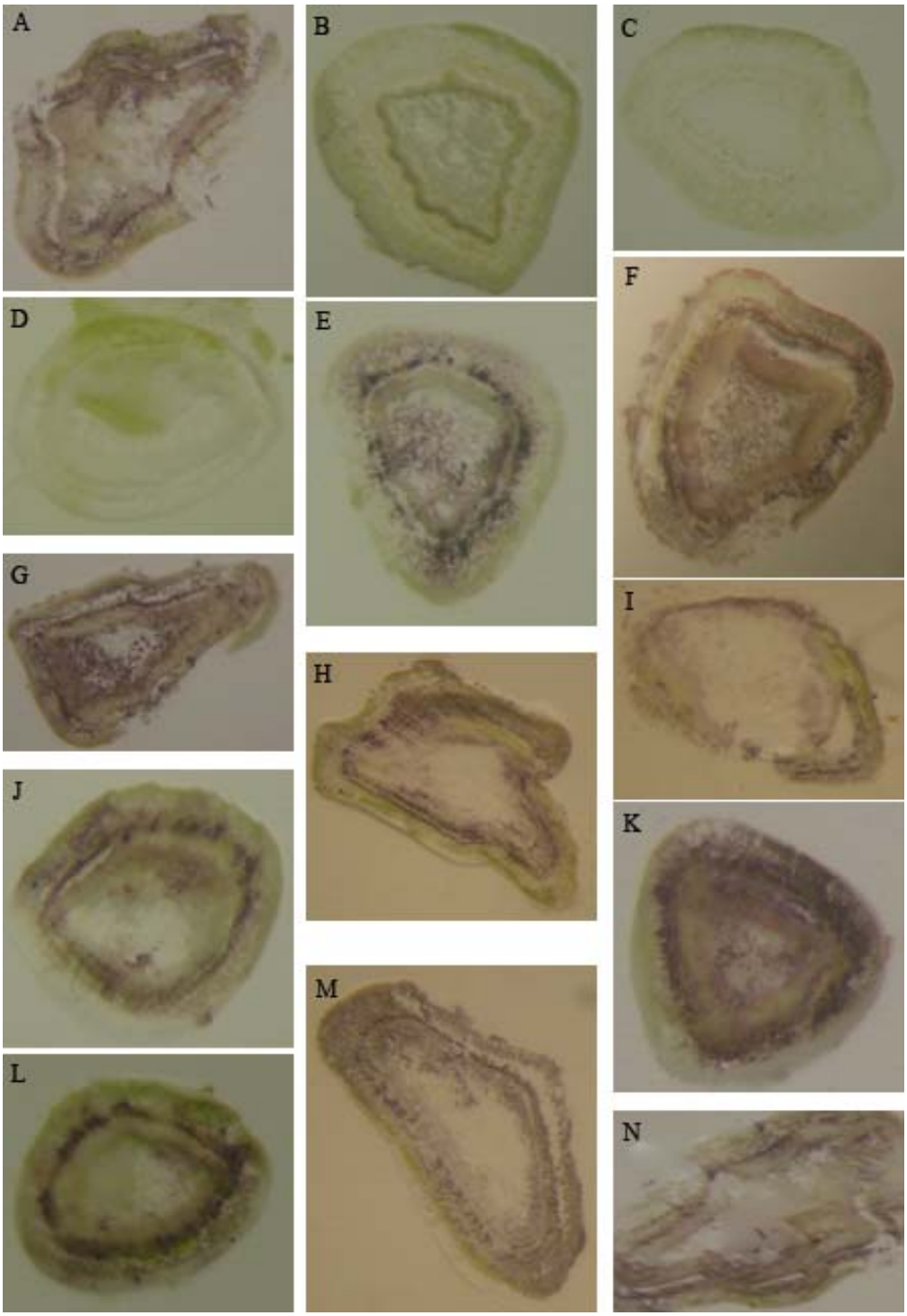

Figure 4-7. Tissue prints of representative healthy and CTV positive and top-worked rootstock candidates after incubation with the MCA13 DTBI. A) Positive control. B) Healthy control. C and D) Examples of the CTV- MCA13 samples (Table 4-1).E-N) Examples of the CTV- infected samples (Table 4-1).

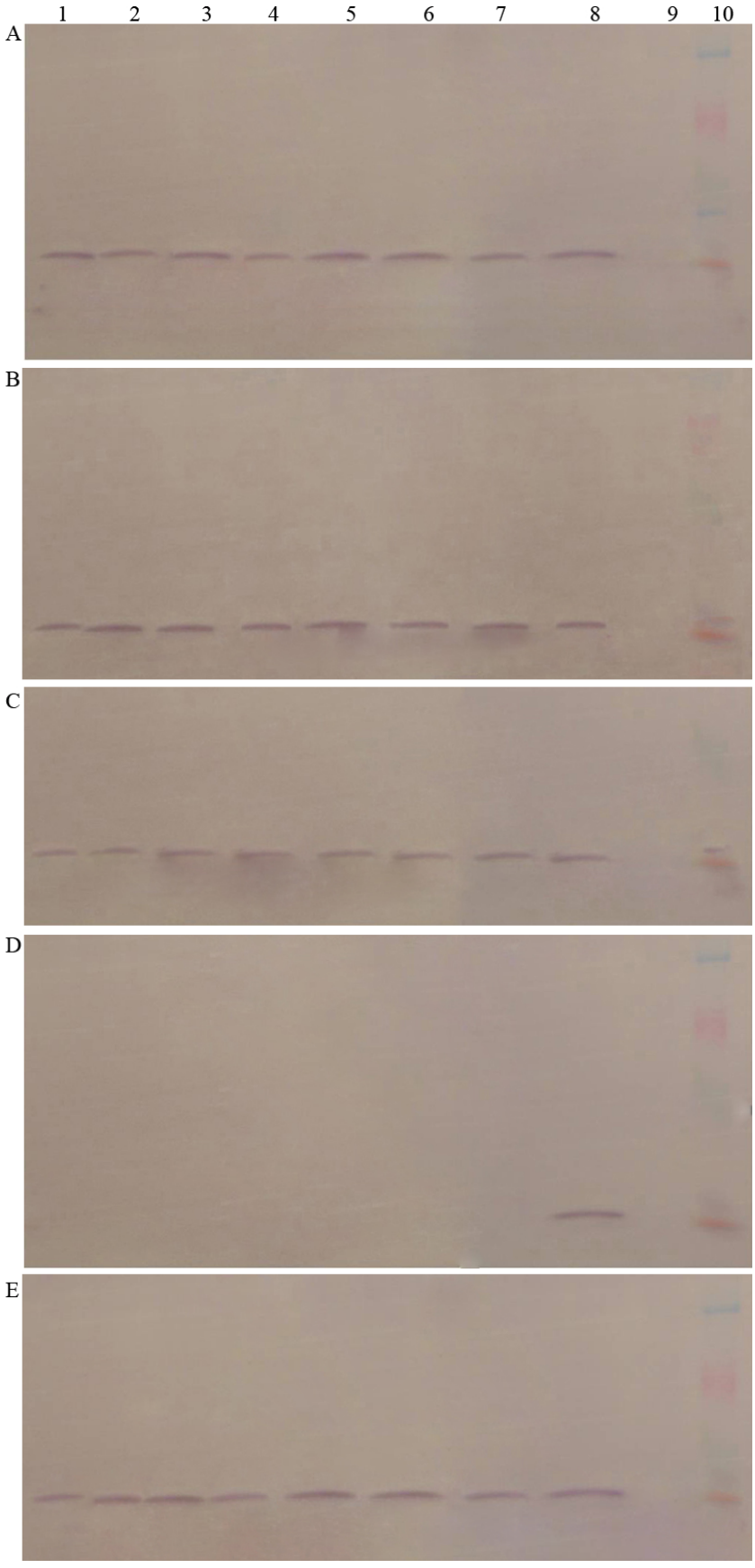
1
2
3
4
5
6
7
8
9
10
A
B
C
D
E

Figure 4-8. Western blot analysis of total soluble protein of healthy and infected samples using the MCA13 monoclonal antibody. A) Lanes 1-7, CTV- MCA13 positive samples 1-7 of selected rootstock candidates (Table 4-1); lane 8, greenhouse CTV positive control; lane 9, greenhouse CTV negative control; and lane10, Kaleidoscope pre-stained protein standard. B) Lane 1-7, CTV- MCA13 positive samples 7-14 of selected rootstock candidates (Table 4-1); lane 8, greenhouse CTV positive control; lane 9, greenhouse CTV negative control; and lane10, Kaleidoscope pre-stained protein standard. C) Lane1-7, more CTV- MCA13 positive samples 14-21 of selected rootstock candidates (Table 4-1); lane 8, greenhouse CTV positive control; lane 9, greenhouse CTV negative control; and lane10, Kaleidoscope pre-stained protein standard. D) Lane 1-7, representative of CTV- MCA13 negative samples 1-7 of selected rootstock candidates (Table 4-1); lane 8, greenhouse CTV positive control; lane 9, greenhouse CTV negative control; lane10, Kaleidoscope pre-stained protein standard. E) Lane 1-7, representative of sour orange graft corresponding to the negative samples 1-7 of selected rootstock candidates (Table 4-1); lane 8, greenhouse CTV positive control; lane 9, greenhouse CTV negative control; and lane10, Kaleidoscope pre-stained protein standard.

Table 4-1. Samples selected from the top- worked rootstock candidates to be further tested by direct tissue blots immunoassay and western analysis based on ELISA MCA13 results.

	ELISA MCA13 negative samples		Selected ELISA MCA13 positive samples
1	7-2-99-1	1	4-4-99-4
2	8-1-99-2B	2	Amb +8-1-99-4A
3	8-1-99-4B set 2	3	2247x 6056-00-7 (Blue7)
4	HBJL3 R10T20	4	2247-1571-00-4 (White4)
5	Amb +5-1-99-3	5	2247-OP-A1
6	Amb + HBJL -2B	6	Sorp-OP-02-8
7	VolkX P	7	8-199-4A
8	HBJL-5	8	Amb + 7-2-99-5
9	Chandler #A1-11	9	Amb +Chandler #69
10	5-1-99-2	10	Amb+HBJL-4
11	Amb +Chandler	11	Amb+HBJL-7
12	Amb + HBJL -1	12	Amb +HBP
13	Amb +4-3-99-2	13	Amb +MG1
14	Murcott + HBJL -1	14	Amb +SN7
15	W. Murcott + HBJL -7	15	Changsha+HBJL-5
16	2247 x 6073-00-6 (GREEN 6)	16	Murcott+ 4-4-99-6
17	2247-OP-A2	17	Murcott+ Chandler #A1-11
		18	2247x 6056-00-2 (Blue2)
		19	N+HBP x SO+RP-04-7
		20	(SRxSH)99-5
		21	46x20-04-12

Table 4-2. Summary of polyclonal and the MCA13, monoclonal Enzyme-linked Immunosorbent Assays (ELISA) results for the source trees prior to the top-working.

Source(S)	OD_{405} Value[a]		Source	OD_{405} Value[a]		Source	OD_{405} Value[a]	
	Poly	MCA13		Poly	MCA13		Poly	MCA13
S1	2.011	1.728	**S26**	2.613	1.216	**S51**	2.038	1.166
S2	1.558	1.195	**S27**	1.725	1.236	**S52**	1.709	1.070
S3	3.132	2.480	**S28**	1.150	0.812	**S53**	2.160	1.657
S4	1.940	1.062	**S29**	1.371	1.270	**S54**	1.135	1.001
S5	1.514	1.171	**S30**	1.502	1.201	**S55**	2.223	1.194
S6	1.970	1.713	**S31**	2.294	1.385	**S56**	2.023	1.970
S7	2.658	1.351	**S32**	2.127	1.817	**S57**	1.816	1.602
S8	2.404	1.844	**S33**	1.768	1.052	**S58**	1.919	1.796
S9	2.247	1.686	**S34**	2.613	1.645	**S59**	1.601	1.386
S10	2.167	1.157	**S35**	3.490	2.040	**S60**	1.830	1.193
S11	2.260	1.129	**S36**	2.026	0.947	**S61**	2.018	1.736
S12	2.513	1.220	**S37**	1.884	0.783	**S62**	1.340	1.031
S13	1.342	1.154	**S38**	3.185	1.501	**S63**	1.529	2.430
S14	1.941	1.195	**S39**	1.746	1.193	**S64**	1.391	2.107
S15	2.065	1.276	**S40**	1.331	1.001	**S65**	2.163	2.064
S16	1.099	0.987	**S41**	2.019	1.708	**S66**	2.240	2.101
S17	1.830	1.358	**S42**	1.013	0.746	**S67**	1.020	0.790
S18	1.409	1.193	**S43**	2.113	2.055	**S68**	1.806	1.653
S19	2.435	1.077	**S44**	2.144	2.362	**S69**	2.409	2.027
S20	2.452	1.170	**S45**	2.107	1.189	**S70**	2.063	1.518
S21	1.719	1.291	**S46**	1.135	0.906	**S71**	1.817	1.768
S22	1.491	1.213	**S47**	1.612	1.018	**S72**	1.109	0.643
S23	2.393	1.170	**S48**	2.108	2.000	**S73**	1.241	1.066
S24	1.857	1.355	**S49**	2.189	1.880	**S74**	1.019	1.000
S25	2.120	1.423	**S50**	2.210	1.285	**S75**	1.743	1.317
Healthy	0.04^{x}	0.032^{y}						

[a] Average (Avr) of two replications per samples after a 2h reaction.
[x,y] Healthy control for polyclonal and,MCA13 monoclonal, ELISA respectively.

Table 4-3. Summary of the CTV polyclonal antibody Enzyme-linked Immunosorbent Assays (ELISA) results for the source trees, grafted rootstock candidates and sour orange control collected 18 months after top-working.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
Pummelo							
1	2.400	2.330	0.975	0.905	4-3-99-2	0.186	0.116
2	1.416	1.346	1.052	0.982	4-3-99-2 set 7	0.080	0.010
3	3.185	3.115	0.850	0.780	4-4-99-4	0.242	0.172
4	1.910	1.840	0.551	0.481	5-1-99-2	0.153	0.083
5	1.452	1.382	0.568	0.498	7-2-99-1	0.067	-0.003
6	2.283	2.213	0.377	0.307	7-2-99-2	0.190	0.120
7	2.585	2.515	0.954	0.884	7-3-99-1	0.118	0.048
8	2.538	2.468	1.461	1.391	8-1-99-4A	0.289	0.219
9	2.147	2.077	1.052	0.982	8-1-99-2B	0.122	0.052
10	2.014	1.944	1.560	1.490	8-1-99-4B	0.093	0.023
11	2.190	2.120	1.074	1.004	8-1-99-4B set 2	0.051	-0.019
12	2.470	2.400	0.275	0.205	8-2-99-1	0.164	0.094
13	1.487	1.417	0.858	0.788	Chandler #A1-11	0.145	0.075
14	1.763	1.693	1.052	0.982	HBJL-3 R6T16	0.160	0.090
15	2.220	2.150	0.886	0.816	HBJL-3 R10T20	0.041	-0.029
16	1.381	1.311	1.052	0.982	HBJL-4	0.276	0.206
17	1.240	1.170	1.179	1.109	HBJL-5	0.043	-0.027
18	1.556	1.486	2.028	1.958	HBJL-7	0.210	0.140
19	2.169	2.099	0.997	0.927	HBJL-12	0.154	0.084
20	2.638	2.568	1.500	1.430	MG-10	0.270	0.200
21	1.090	1.020	0.761	0.691	MG-11	0.186	0.116
22	1.451	1.381	1.140	1.070	SN3	0.194	0.124
Somatic Hybrid							
23	2.944	2.874	0.827	0.757	Amb + 4-3-99-2	0.030	-0.040
24	1.685	1.615	1.192	1.122	Amb + 4-4-99-6	0.171	0.101
25	2.436	2.366	0.693	0.623	Amb + 5-1-99-1B	0.139	0.069
26	2.423	2.353	1.216	1.146	Amb + 5-1-99-3	0.052	-0.018
27	1.640	1.570	1.019	0.949	Amb + 7-2-99-5	0.315	0.245
28	1.497	1.427	1.586	1.516	Amb + 7-3-99-1	0.224	0.154
29	1.138	1.068	1.195	1.125	Amb + 8-1-99-4A	0.263	0.193
30	1.290	1.220	1.640	1.570	Amb + Chandler	0.186	0.116
31	2.461	2.3910	0.834	0.764	Amb + Chandler #A1-11	0.134	0.064
32	2.343	2.273	1.071	1.001	Amb + Chandler # 69	0.326	0.256
33	1.664	1.594	0.965	0.895	Amb + HBJL-1	0.158	0.088
34	2.731	2.661	1.390	1.320	Amb + HBJL-2B	0.237	0.167
35	3.116	3.046	0.953	0.883	Amb + HBJL-3	0.139	0.069

Table 4-3. Continued.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
36	1.900	1.830	1.045	0.975	Amb + HBJL-4	0.340	0.270
37	1.537	1.467	0.797	0.727	Amb + HBJL-5	0.142	0.072
38	2.769	2.699	1.140	1.070	Amb + HBJL-7	0.354	0.284
39	1.475	1.405	0.981	0.911	Amb + HBP	0.373	0.303
40	1.538	1.468	1.720	1.650	Amb + MG1	0.676	0.606
41	1.843	1.773	1.052	0.982	Amb+ SN7	0.421	0.351
42	1.564	1.494	1.300	1.230	Amb + MG-10	0.185	0.115
43	1.781	1.711	1.416	1.346	Changsha + HBJL-3	0.094	0.024
44	1.930	1.860	1.094	1.024	Changsha + HBJL-5	0.467	0.397
45	1.857	1.787	0.834	0.764	Changsha + HBJL-7	0.086	0.016
46	1.249	1.179	1.052	0.982	Murcott + 4-4-99-6	0.279	0.209
47	1.380	1.310	1.038	0.968	Murcott + Chandler #A1-11	0.310	0.240
48	1.786	1.716	0.849	0.779	Murcott + Chandler #80	0.144	0.074
49	1.735	1.665	0.242	0.172	Murcott + HBJL-1	0.050	-0.020
50	1.884	1.814	0.770	0.700	Murcott + SN3	0.240	0.170
51	2.472	2.402	0.093	0.023	Page + HBJL-3	0.165	0.095
52	1.610	1.540	1.696	1.626	Page + HBJL-7	0.173	0.103
53	2.436	2.366	1.370	1.300	Succari + HBP	0.226	0.156
54	1.553	1.483	1.010	0.940	W.Murcott + HBJL-7	0.197	0.127
Tetrazygs							
55	1.795	1.725	1.260	1.190	2247 x 6056-00-2 (Blue 2)	0.340	0.270
56	2.361	2.291	0.937	0.867	2247 x 6056-00-7 (Blue 7)	0.242	0.172
57	1.590	1.520	0.850	0.780	2247 x 6073-00-4 (Green 4)	0.094	0.024
58	1.454	1.384	1.052	0.982	2247 x 6073-00-6 (Green6)	0.133	0.063
59	1.416	1.346	1.052	0.982	2247 x 6073-00-8 (Green 8)	0.150	0.080
60	1.430	1.360	1.319	1.249	2247 x 2060-00-1 (Purple 1)	0.198	0.128
61	2.557	2.487	1.052	0.982	2247 x 2060-00-3 (Purple 3)	0.167	0.097
62	1.783	1.713	0.723	0.653	2247 x 1571-004 (White 4)	0.277	0.207
63	1.840	1.770	1.515	1.445	N + HBP x SO + RP-04-7	0.346	0.276
64	1.214	1.144	1.244	1.174	(SR x SH) 99-5	0.435	0.365
Diploid Hybrids							
65	1.912	1.842	1.027	0.957	43 x 20-04-1	0.232	0.162
66	1.450	1.380	0.831	0.761	46 x 20-04-12	0.415	0.345
67	1.616	1.546	0.974	0.904	46 x 20-04-19	0.176	0.106
68	2.850	2.780	1.030	0.960	Volk x P	0.740	0.670
Open pollinated (OP) tetraploids							
69	1.853	1.783	0.848	0.778	2247-OP-A1	0.241	0.171
70	1.334	1.264	1.146	1.076	2247-OP-A2	0.047	-0.023
71	1.287	1.217	1.059	0.989	2247-OP-A5	0.149	0.079

Table 4-3. Continued.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
72	1.480	1.410	0.987	0.917	SORP-OP-02-8	0.250	0.180
Grapefruit							
73	1.425	1.355	1.224	1.154	Marsh grapefruit	0.171	0.101
74	1.582	1.512	1.363	1.293	Ruby Red grapefruit	0.142	0.072

Healthy = 0.035 and 2X healthy= 0.07.
'Hamlin' sweet orange = 1.205.

Table 4-4. Summary of the CTV monoclonal, MCA13 antibody Enzyme-linked Immunosorbent Assays-Indirect (ELISA-I) results for the source trees, grafted rootstock candidates and sour orange control collected 18 months after grafting.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
Pummelo							
1	2.031	1.979	0.891	0.839	4-3-99-2	0.154	0.102
2	1.504	1.452	0.883	0.831	4-3-99-2 set 7	0.078	0.026
3	2.861	2.809	0.693	0.641	4-4-99-4	0.217	0.165
4	2.093	2.041	0.392	0.340	5-1-99-2	0.045	-0.007
5	1.317	1.265	0.414	0.362	7-2-99-1	0.050	-0.002
6	1.822	1.770	0.215	0. 3	7-2-99-2	0.096	0.044
7	2.370	2.318	0.651	0.599	7-3-99-1	0.090	0.038
8	2.105	2.053	1.090	1.038	8-1-99-4A	0.261	0.209
9	2.214	2.162	0.883	0.831	8-1-99-2B	0.023	-0.029
10	1.737	1.685	1.314	1.262	8-1-99-4B	0.060	0.008
11	2.146	2.094	1.860	1.808	8-1-99-4B set 2	0.038	-0.014
12	1.950	1.898	0.231	0.179	8-2-99-1	0.113	0.061
13	1.523	1.471	0.787	0.735	Chandler #A1-11	0.039	-0.013
14	1.464	1.412	0.883	0.831	HBJL-3 R6T16	0.094	0.042
15	2.101	2.049	0.719	0.667	HBJL-3 R10T20	0.032	-0.020
16	1.146	1.094	0.883	0.831	HBJL-4	0.150	0.098
17	0.981	0.929	1.026	0.974	HBJL-5	0.026	-0.026
18	1.357	1.305	1.911	1.859	HBJL-7	0.192	0.140
19	1.960	1.908	0.793	0.741	HBJL-12	0.124	0.072
20	2.153	2.101	1.426	1.374	MG-10	0.180	0.128
21	1.981	1.929	0.065	0.013	MG-11	0.137	0.085
22	1.219	1.167	0.981	0.929	SN3	0.154	0.102
Somatic Hybrid							
23	2.577	2.525	0.737	0.685	Amb + 4-3-99-2	0.019	-0.033
24	1.452	1.400	1.059	1.007	Amb + 4-4-99-6	0.127	0.075
25	2.320	2.268	0.518	0.466	Amb + 5-1-99-1B	0.092	0.040
26	2.293	2.241	1.136	1.084	Amb + 5-1-99-3	0.048	-0.004
27	1.490	1.438	0.934	0.882	Amb + 7-2-99-5	0.279	0.227
28	1.371	1.319	1.285	1.233	Amb + 7-3-99-1	0.190	0.138
29	1.005	0.953	1.080	1.028	Amb + 8-1-99-4A	0.213	0.161
30	1.926	1.874	1.457	1.405	Amb + Chandler	0.027	-0.025
31	1.770	1.718	0.663	0.611	Amb + Chandler #A1-11	0.081	0.029
32	1.415	1.363	1.000	0.948	Amb + Chandler # 69	0.254	0.202
33	1.310	1.258	0.814	0.762	Amb + HBJL-1	0.031	-0.021
34	2.4920	2.440	1.157	1.105	Amb + HBJL-2B	0.044	-0.008
35	2.704	2.652	0.870	0.818	Amb + HBJL-3	0.127	0.075

Table 4-4. Continued.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
36	1.583	1.531	0.915	0.863	Amb + HBJL-4	0.291	0.239
37	1.442	1.390	0.671	0.619	Amb + HBJL-5	0.120	0.068
38	1.717	1.665	0.953	0.901	Amb + HBJL-7	0.284	0.232
39	1.307	1.255	1.011	0.959	Amb + HBP	0.310	0.258
40	1.459	1.407	1.509	1.457	Amb + MG1	0.521	0.469
41	1.621	1.569	0.883	0.831	Amb+ SN7	0.404	0.352
42	1.493	1.441	1.2130	1.1610	Amb + MG-10	0.166	0.114
43	1.595	1.543	1.296	1.244	Changsha + HBJL-3	0.082	0.030
44	1.318	1.266	0.675	0.623	Changsha + HBJL-5	0.373	0.321
45	1.408	1.356	0.549	0.497	Changsha + HBJL-7	0.065	0.013
46	1.065	1.013	0.883	0.831	Murcott + 4-4-99-6	0.254	0.202
47	1.213	1.1610	0.992	0.940	Murcott + Chandler #A1-11	0.270	0.218
48	1.661	1.609	0.781	0.729	Murcott + Chandler #80	0.098	0.046
49	1.584	1.532	0.1950	0.1430	Murcott + HBJL-1	0.041	-0.011
50	1.753	1.701	0.614	0.562	Murcott + SN3	0.202	0.150
51	1.904	1.852	0.073	0.021	Page + HBJL-3	0.151	0.099
52	1.574	1.522	1.485	1.433	Page + HBJL-7	0.145	0.093
53	1.600	1.548	1.210	1.158	Succari + HBP	0.211	0.159
54	1.413	1.361	0.893	0.841	W.Murcott + HBJL-7	0.034	-0.018
Tetrazygs							
55	1.621	1.569	0.920	0.868	2247 x 6056-00-2 (Blue 2)	0.280	0.228
56	2.201	2.149	0.841	0.789	2247 x 6056-00-7 (Blue 7)	0.217	0.165
57	1.348	1.296	0.707	0.655	2247 x 6073-00-4 (Green 4)	0.0640	0.012
58	1.293	1.241	0.883	0.831	2247 x 6073-00-6 (Green6)	0.046	-0.006
59	1.375	1.323	0.883	0.831	2247 x 6073-00-8 (Green 8)	0.084	0.032
60	1.341	1.289	1.120	1.068	2247 x 2060-00-1 (Purple 1)	0.143	0.091
61	2.184	2.132	0.883	0.831	2247 x 2060-00-3 (Purple 3)	0.125	0.073
62	1.477	1.425	0.530	0.478	2247 x 1571-00-4 (White 4)	0.250	0.198
63	1.610	1.558	1.429	1.377	N + HBP x SO + RP-04-7	0.296	0.244
64	1.173	1.121	1.084	1.032	(SR x SH) 99-5	0.308	0.256
Diploid Hybrids							
65	1.867	1.815	0.873	0.821	43 x 20-04-1	0.201	0.149
66	1.345	1.293	0.764	0.712	46 x 20-04-12	0.374	0.322
67	1.426	1.374	0.786	0.734	46 x 20-04-19	0.158	0.106
68	2.540	2.488	0.910	0.858	Volk x P	0.029	-0.023
Open pollinated (OP) tetraploids							
69	1.706	1.654	0.735	0.683	2247-OP-A1	0.214	0.162
70	1.192	1.140	1.081	1.029	2247-OP-A2	0.017	-0.035
71	1.126	1.074	0.860	0.808	2247-OP-A5	0.120	0.068

Table 4-4. Continued.

	Sour Orange		source		Germplasm		
	Average	Avr-2xH	Average	Avr-2xH		Average	Avr-2xH
72	1.163	1.111	0.841	0.789	SORP-OP-02-8	0.219	0.167
Grapefruit							
73	1.310	1.258	1.077	1.025	Marsh grapefruit	0.153	0.101
74	1.207	1.155	1.186	1.134	Ruby Red grapefruit	0.116	0.064

Healthy = 0.026 and 2X healthy= (OD_{405}) 0.052.
'Hamlin' sweet orange = (OD_{405}) 1.105.

Table 4-5. Summary of rootstock candidates categories based on the performance in the field (shoot growth and CTV symptoms) in relation to MCA13 (DAS-I) ELISA.

Resistant	Tolerant	Slightly tolerant	Susceptible
5-1-99-2	4-3-99-2 set 7	4-3-99-2	8-1-99-4A
7-2-99-1	7-2-99-2	4-4-99-4	Amb + 7-2-99-5
8-1-99-2B	7-3-99-1	HBJL-7	Amb + Chandler # 69
8-1-99-4B set 2	8-1-99-4B	MG-10	Amb + HBJL-4
Chandler #A1-11	HBJL-3 R6T16	SN3	Amb + HBJL-7
HBJL-3 R10T20	Amb + 5-1-99-1B	Amb + 7-3-99-1	Amb + HBP
HBJL-5	Amb + Chandler #A1-11	Amb + 8-1-99-4A	Amb + MG1
Amb +4-3-99-2	Changsha + HBJL-3	Amb + MG-10	Amb+ SN7
Amb +5-1-99-3	Changsha + HBJL-7	Murcott + SN3	Changsha + HBJL-5
Amb +Chandler	Murcott + Chandler #80	Page + HBJL-3	Murcott + 4-4-99-6
Amb + HBJL -1	2247 x 6073-00-4 (Green 4)	Succari + HBP	Murcott + Chandler #A1-11
Amb + HBJL -2B	2247 x 6073-00-8 (Green 8)	2247 x 6056-00-7 (Blue 7)	2247 x 6056-00-2 (Blue2) 2)
Murcott + HBJL -1		2247 x 1571-00-4 (White 4)	N + HBP x SO + RP-04-7
	Intermediate		
WMurcott + HBJL -7	8-2-99-1	43 x 20-04-1	(SRXSH) 99-5
2247 x 6073-00-6 (GREEN 6)	HBJL-4	46 x 20-04-19	46 x 20-04-12
VolkX P	HBJL-12	2247-OP-A1	
2247-OP-A2	MG-11	SORP-OP-02-8	
	Amb + 4-4-99-6	Marsh grapefruit	
	Amb + HBJL-3		
	Amb + HBJL-5		
	Page + HBJL-7		
	2247 x 2060-00-1 (Purple 1)		
	2247 x 2060-00-3 (Purple 3)		
	2247-OP-A5		
	Ruby Red grapefruit		

Table 4-6. Summary of the serological tests results on the rootstock candidates + Marsh and Ruby Red grapefruit.

	Germplasm	Polyclonal Average	MCA13 Average	(DTBI)	Western blot
Pummelo					
1	4-3-99-2	0.186/+ [a]	0.154	NA	NA
2	4-3-99-2 set 7	0.08	0.078	NA	NA
3	4-4-99-4	0.242	0.217	+	+
4	5-1-99-2	0.153	0.045/-	-	-
5	7-2-99-1	0.067/-	0.050/-	-	-
6	7-2-99-2	0.19	0.096	NA	NA
7	7-3-99-1	0.118	0.09	NA	NA
8	8-1-99-4A	0.289	0.261	+	+
9	8-1-99-2B	0.122	0.023/-	-	-
10	8-1-99-4B	0.093	0.060/-	-	-
11	8-1-99-4B set 2	0.051/-	0.038/-	-	-
12	8-2-99-1	0.164	0.113	NA	NA
13	Chandler #A1-11	0.145	0.039/-	-	-
14	HBJL-3 R6T16	0.16	0.094	NA	NA
15	HBJL-3 R10T20	0.041/-	0.032/-	-	-
16	HBJL-4	0.276	0.15	NA	NA
17	HBJL-5	0.043/-	0.026/-	NA	NA
18	HBJL-7	0.21	0.192	NA	NA
19	HBJL-12	0.154	0.124	NA	NA
20	MG-10	0.27	0.18	NA	NA
21	MG-11	0.186	0.137	NA	NA
22	SN3	0.194	0.154	NA	NA
Somatic Hybrid					
23	Amb + 4-3-99-2	0.030/-	0.019/-	-	-
24	Amb + 4-4-99-6	0.171	0.127	NA	NA
25	Amb + 5-1-99-1B	0.139	0.092	NA	NA
26	Amb + 5-1-99-3	0.052/-	0.048/-	-	-
27	Amb + 7-2-99-5	0.315	0.279	+	+
28	Amb + 7-3-99-1	0.224	0.19	NA	NA
29	Amb + 8-1-99-4A	0.263	0.213	+	+
30	Amb + Chandler	0.186	0.027/-	-	-
31	Amb + Chandler #A1-11	0.134	0.081	NA	NA
32	Amb + Chandler # 69	0.326	0.254	+	+
33	Amb + HBJL-1	0.158	0.031/-	-	-
34	Amb + HBJL-2B	0.237	0.044/-	-	-
35	Amb + HBJL-3	0.139	0.127	NA	NA
36	Amb + HBJL-4	0.34	0.291	+	+
37	Amb + HBJL-5	0.142	0.12	NA	NA
38	Amb + HBJL-7	0.354	0.284	+	+
39	Amb + HBP	0.373	0.31	+	+
40	Amb + MG1	0.676	0.521	+	+

Table 4-6. Continued.

Germplasm		Polyclonal Average	MCA13 Average	(DTBI)	Western blot
41	Amb+ SN7	0.421[a]	0.404	+	+
42	Amb + MG-10	0.185	0.166	NA	NA
43	Changsha + HBJL-3	0.094	0.082	NA	NA
44	Changsha + HBJL-5	0.467	0.373	+	+
45	Changsha + HBJL-7	0.086	0.065	NA	NA
46	Murcott + 4-4-99-6	0.279	0.254	+	+
47	Murcott + Chandler #A1-11	0.31	0.27	+ NA	+ NA
48	Murcott + Chandler #80	0.144	0.098	NA	NA
49	Murcott + HBJL-1/-	0.05	0.041/-	-	-
50	Murcott + SN3	0.24	0.202	NA	NA
51	Page + HBJL-3	0.165	0.151	NA	NA
52	Page + HBJL-7	0.173	0.145	NA	NA
53	Succari + HBP	0.226	0.211	NA	NA
54	W.Murcott + HBJL-7	0.197	0.034/-	-	-
Tetrazygs					
55	2247 x 6056-00-2 (Blue 2)	0.34	0.28	+	+
56	2247 x 6056-00-7 (Blue 7)	0.242	0.217	+	+
57	2247 x 6073-00-4 (Green 4)	0.094	0.064	-	-
58	2247 x 6073-00-6 (Green6)	0.133	0.046/-	NA	NA
59	2247 x 6073-00-8 (Green 8)	0.15	0.084	NA	NA
60	2247 x 2060-00-1 (Purple 1)	0.198	0.143	NA	NA
61	2247 x 2060-00-3 (Purple 3)	0.167	0.125	NA	NA
62	2247 x 1571-00-4 (White 4)	0.277	0.25	+	+
63	N + HBP x SO + RP-04-7	0.346	0.296	+	+
64	(SR x SH) 99-5	0.308	0.256	+	+
Diploid Hybrids					
65	43 x 20-04-1	0.232	0.201	NA	NA
66	46 x 20-04-12	0.415	0.374	+	+
67	46 x 20-04-19	0.176	0.158	NA	NA
Open pollinated (OP) tetraploids					
68	Volk x P	0.74	0.029/-	-	-
69	2247-OP-A1	0.241	0.214	+	+
70	2247-OP-A2	0.047/-[b]	0.017/-	-	-
71	2247-OP-A5	0.149	0.12	NA	NA
72	SORP-OP-02-8	0.25	0.219	+	+
Grapefruit					
73	Marsh grapefruit	0.171	0.153	NA	NA
74	Ruby Red grapefruit	0.142	0.116	NA	NA

Healthy for polyclonal ELISA = 0.035 and 2X healthy= 0.070.
Healthy for MCA13 ELISA = 0.026 and 2X healthy= 0.052.
[a, b]OD Values higher than 2x healthy value are positive (+) and values lower than 2x healthy are negative respectively. NA = not applicable.

CHAPTER 5
MOLECULAR CHARECTERIZATION OF CITRUS TRISTEZA VIRUS (CTV) IN SELECTED HYBRID ROOTSTOCK CANDIDATES TO POTENTIALLY REPLACE SOUR ORANGE

Introduction

Citrus tristeza virus (CTV), genus *Closterovirus,* family *Closteroviridae* is the causal agent of devastating epidemics that changed the course of the citrus industry worldwide, killing millions of citrus trees on sour orange rootstock (Moreno et al., 2008). CTV has a narrow host range that is limited mostly to the genus Citrus in the family *Rutaceae*. Most of the species, cultivars and hybrids of citrus are infected by CTV (Muller and Garnsey, 1984). CTV causes different symptoms on different hosts. The most important diseases caused by CTV are quick-decline (QD), on sour orange rootstock and stem-pitting on grapefruit (SPG) (Garnsey et al., 1987a; Rocha-Pena et al., 1995). The virus is phloem-limited and transmitted by aphids in a semi-persistent manner and by infected buds. *Toxoptera citricida* (Kirkaldy), commonly known as the brown citrus aphid (BCA), is the most efficient vector of CTV (Hermosa de Mendoza et al., 1984; Yokomi et al., 1994). The breakdown of cross protection against CTV- decline inducing isolates of CTV in grapefruit trees has been reported following the introduction of the BCA into Florida (Powell et al., 2003). The incidence of all strains of CTV has increased in south Florida, following the introduction of BCA in Florida. However the increase of severe strains has been greater than that of the mild strains (Halbert et al., 2004).

CTV virions are composed of two capsid proteins and a single-stranded, positive-sense genomic RNA (gRNA) of ~20 kb, containing 12 open reading frames (ORFs) and two un-translated regions (UTRs). The 3' UTR is highly conserved among different CTV isolates with nucleotide identities as high as 97%, whereas the 5' UTR region is highly variable with nucleotide identities as low as 44% (Karasev et al., 1995). Two conserved blocks of genes, ORF

1a & 1b and ORFs 3 to ORFs 7 have been identified in CTV that also are conserved in other Closteroviruses (Karasev, 2000).

Field isolates of CTV exist as complex populations consisting of a number of different CTV genotypes, with large sequence variation among the genotypes. Thus, CTV isolates are populations of CTV genotypes, in which one genotype may predominate (Ayllon et al., 1999a; Hilf et al., 1999). Characterization of the population structure is crucial to understanding the biology and evolution of CTV isolates, and may have important implications in the selection of pre-immunizing isolates (Iglesias et al., 2005), and the breeding of resistant scions and rootstocks. CTV isolates differ in type and severity of symptoms induced in different citrus species and cultivars, and in their aphid transmissibility have been reported worldwide (Roistacher and Moreno, 1991). These factors complicate the screening for resistance to CTV-induced diseases in citrus breeding programs. A more thorough understanding of CTV field biology should facilitate the improvement of screening methods and subsequently the development of resistant cultivars.

Several methods have been described for the characterization of CTV field isolates. The standard method is a biological characterization using a panel of indicator plants developed by Garnsey et al., (1987b). The serological differentiation of CTV isolates has been reported using the monoclonal antibody MCA 13 (Permar et al., 1990). Monoclonal antibody, MCA13 discriminates between severe and mild CTV isolates by reacting only to the severe isolates. The major disadvantage of MCA13 is that it is not able to differentiate between the QD isolates and the SP isolates. Therefore, this antibody is not always useful, especially in mixed infection of CTV. Molecular characterization of CTV isolates by PCR-based and molecular hybridization techniques has been developed for CTV detection (Mathews et al., 1997; Cambra et al., 2000;

Roy et al., 2005) and strain differentiation (Cevik et al., 1996b; Hilf and Garnsey, 2000; Niblett et al., 2000; Sieburth et al., 2005), allowing for more thorough characterization of field isolates.

Characterization of CTV isolates on the basis of the full genetic sequence provides the best comparison, but it is a difficult and time consuming process. The present molecular techniques were used to better understand the population diversity of CTV in 'Hamlin' sweet orange field trees used in the previously described top-working study. The molecular techniques including multiple molecular markers (MMM) and heteroduplex mobility assay (HMA), followed by the DNA sequencing of the amplified region, were applied to detect the different CTV genotypes residing in the 'Hamlin' interstock, and subsequently the differential movement of CTV genotypes from this interstock into the top-worked test hybrid rootstock candidates. CTV titer in top-worked trees was estimated using quantitative real time PCR (qRT-PCR). The working hypothesis was that there may be differential movement of the CTV genotypes contained in the original 'Hamlin' interstock isolate into the newly top-worked test material, thus the possibility of differential resistances/susceptibilities among the test hybrid rootstock candidates maybe revealed.

Multiple Molecular Markers (MMM)

MMM is a method used for molecular characterization of CTV isolates and identification of specific CTV genotypes. The MMM method is based on the amplification of selected regions of the CTV genome using CTV genotype specific primers, designed from non-conserved regions of VT, T3, T30 and T36 CTV isolates. The method provides a rapid technique for the detection of CTV genotypes (Hilf and Garnsey, 2000). MMM method can be used to characterize unknown CTV isolates based on the sequence specific amplification of RT-PCR products, producing a profile designated as the "Isolate Genotype" (Hilf and Garnsey, 2000). The MMM method provides a rapid technique for the detection of CTV genotypes and also provides an

initial assessment of the molecular variability within the CTV population from different citrus growing regions of the world (Hilf and Garnsey, 2000). Based on the MMM analysis of over 400 accessions from Florida, T36 and/or T30 genotypes were the primary CTV genotypes detected in commercial citrus trees in Florida, followed by the VT genotype, detected in some Meyer lemon trees, while the T3 genotype was never detected in commercial citrus (Hilf and Garnsey, 2002). It is very important that the complete MMM profile is considered, not only the reaction to one or two primer markers (Brlansky et al., 2003). For example, an isolate was designated as a T36 genotype if it reacted with at least the T36 Pol; however, this isolate may not react with all T36 markers (T36 5' and T36 K-17). VT genotype and T30 genotype also were designated if reactions occurred with the VT-Pol and T30 Pol, respectively. Moreover, T3 genotype was designated only when there is a reaction with both T3-K17 and VT-Pol, and/or VT-5' (Brlansky et al., 2003).

Heteroduplex Mobility Assay (HMA)

Heteroduplex mobility assay is a simple method for the detection and estimation of the genotypic variations between viral strains. The DNA heteroduplexes are formed as a result of nucleotide differences between closely related sequences, upon denaturation and re-annealing of the sequences (Delwart et al., 1993). The DNA heteroduplexes, thus formed, have a reduced mobility on polyacrylamide gel electrophoresis (Delwart et al., 1993). HMA analysis has been used for the characterization of several RNA viruses in human and in plant RNA and DNA viruses (Cai et al., 1991; Delwart et al., 1993; Lin et al., 2000; Berry and C., 2001). HMA was developed for the detection of unknown CTV genotypes present in the mixed infections of CTV, which cannot be detected by other PCR- based detection methods (Biswas et al., 2004). The sensitivity of HMA has been reported to be about 5 %, however, sequence differences as low as 2.3 % have been reported (Berry and C., 2001).

Quantitative Real-Time PCR (qRT-PCR) Method to Determine and Quantify CTV Accumulation

None of the serological or molecular methods used provides a reliable estimation of virus accumulation. In contrast to conventional PCR where only the amount of end product is determined (Freeman et al., 1999), real-time PCR allows tracking of the changes of PCR product during the reaction. QRT-PCR has been reported for detection of viruses from different woody plants. qRT-PCR has been reported for the detection for viruses in different insect vectors (Boonham et al., 2002; Fabre et al., 2003; Olmos et al., 2005) as well as from different woody plants (Marbot et al., 2003; Schneider et al., 2004; Varga and James, 2005; Osman and Rowhani, 2006; Varga and James, 2006; Osman et al., 2007). There are some recent reports about using qRT-PCR to detect and quantify CTV from citrus and aphids (Ruiz-Ruiz et al., 2007; Saponari et al., 2008) Quantification of CTV titer by using reverse transcriptase quantitative real time PCR (qRT-PCR) is very useful in evaluating the tested hybrid rootstock candidates for CTV resistance.

Use of the qRT - PCR analysis will add more sensitivity and accuracy without the need for post-PCR analysis. This will minimize the labor and the biohazard of using the Ethidium Bromide (a carcinogenic agent). QRT - PCR is very sensitive and it can detect as little as a 2 fold change. The Real Time PCR technique is based on monitoring the fluorescence emitted from double -stranded DNA binding dye (SYPR® Green I) or Flourophore- labeled specific probes that hybridize with target sequences during the exponential phase of the PCR reaction (In TaqMan assay). This fluorescent signal is proportional to the accumulation of PCR product generated which is proportional to the quantity of initial DNA template in the sample (Livak et al., 1995). Fluorescence levels are detected during each cycle of amplification by specialized instrumentation. During the early cycles of amplification, the fluorescence level is low, but at a

critical point, fluorescence accumulates to a level detectable by the instrument. This point is called the threshold cycle (Ct) and depends primarily on the starting amount of nucleic acid (Heid et al., 1996). The higher the initial amount of nucleic acid in the reaction, the smaller the Ct values. In practice, there is a linear relationship between the log of the starting quantity of the template and its Ct value during the real-time PCR reaction. Accordingly, the Ct is defined as the cycle at which the fluorescence reaction reaches the threshold line. This technique is currently widely used in the medical field to estimate the viral load (Hubert and Niesters, 2001). Real Time PCR can be used to analyze and quantify the virus titer in a large number of known samples in less than 3h. With the RNA viruses like CTV, it is not easy to get a high quality cDNA to be used in the time-consuming conventional PCR, but here the cDNA is made in the same tube and at the same time with a very high efficiency. QRT-PCR is a rapid, quantitative, reliable and a very sensitive method. Using the qRT-PCR required less RNA as compared to the current methods that require the extraction of larger quantities of RNA from the infected materials.

Materials and Methods

Multiple Molecular Markers (MMM)

Plant materials and virus isolates

The CTV isolate pre-existing in the 'Hamlin' interstock of all top-worked trees (designated TW) was obtained from North-40 field trees at the Citrus Research and Education Center (CREC) Lake Alfred, Florida, USA. This isolate is MCA-13 positive by the ELISA test using the monoclonal antibody MCA 13, which has been reported to discriminate between mild and severe isolates in Florida (Permar et al., 1990). Samples of eleven selected top-worked representative rootstock candidates (Pummelo seedlings HBJL-3R10T20, HBJL-5, and 8-1-99-2B; and somatic hybrids A+7-2-99-5, A+5-1-99-3, A+ HBJL-5, Page + HBJL-3 , Page + HBJL-7, A + Chandler

#A1-11, A+4-3-99-2; and open-pollinated tetraploid 2247-OP-A2), along with the corresponding sour orange as a control, and the corresponding source isolate from the interstock were collected 16 months after successful top-work grafting. These rootstock representatives were chosen based on the MCA13, ELISA results to represent various levels of CTV resistance in the test candidate rootstock population as described in Chapter 4 (Table 4-5). Category I, the highly CTV tolerant selections, was represented by the somatic hybrid A + Chandler #A1-11. Category II, the selections showing an intermediate level of CTV resistance was represented by somatic hybrids A+ HBJL-5 and Page + HBJL-7. Category III, the slightly resistant selections was represented by the somatic hybrid Page + HBJL3. Category IV, the highly susceptible selections, was represented by the somatic hybrid A+ 7-2-99-5. Finally, Category V, the resistant selections, was represented by somatic hybrids A+ 4-3-3-99-2 and A+5-1-99-3; open-pollinated tetraploid 2247OP-A2; and pummelo seedlings HBJL-3R10T20, HBJL-5, and 8-1-99-2B (Table5-5).

Multiple molecular markers primers

Nine pairs of specific primers developed by Hilf and Garnsey (2000) and one CTV universal primer, T36CP (Table 5-1) were used for genotyping of CTV in the source ('Hamlin' interstock) and the top-worked materials (test rootstock candidates and the corresponding control sour orange). The MMM primers are designed from four different regions of the CTV genome (CP, POL, 5' and K17; Figure 5-1) of T36,T30, T3 isolates from Florida and VT isolate from Israel. Ten pairs of genotype specific primer pairs designated as T36POL, T36 5', T36K17, T30POL, T30 5', T30K17, VT POL, VT5', VTK17,T3 K17 and the universal primer T36CP were synthesized (Integrated DNA technologies Inc., Coralville, IA), (Hilf and Garnsey, 2000). The universal T36 CP primer pair was obtained from the T36 sequence and is considered to be the general marker for CTV, and it is not useful for isolate discrimination since all isolates of

CTV are expected to amplify with primers designed to the CP gene region of the viral genome due to the high similarity between different CTV isolates in this region.

Total RNA isolation and complementary DNA (cDNA) synthesis

An analysis of variable sequences in the first three open reading frames (Figure 5.1) showed that the first ORF 1a is probably the most reliable for such analysis (Manjunath, K. L., unpublished data). A 403- base region (nucleotide 1081-1484) was selected for analysis of population mixtures by HMA. Bark of CTV-infected tissue (100 mg) was ground in liquid nitrogen and the total RNA was extracted by using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The extraction was re-suspended in 30-40 µl of RNase-free water depending on the pellet size, and either used immediately or stored at -80º C for later use. Ten µl the RNA extract was used to synthesize the first strand complimentary DNA (cDNA) using a mixture of antisense primers as shown in Table (5-1). Reverse transcription was carried out using a final volume of 25 µl using Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR master mix was prepared as in appendix C. For better results, cDNAs were purified using a QIA quick PCR purification kit (QIAGEN, Valencia, CA), according to manufacturer's protocol and the final elution was made in 40 µl of elution buffer (EB). The CDNA purification step was very important in order to obtain good PCR amplification with the different MMM primers.

Polymerase chain reaction (PCR)

PCR reactions were carried out from each sample in 25 µl PCR reaction volume. Twelve PCR amplifications were carried out using the ten primer pairs (Table 5-1). For each primer pair, 2.5 µl of the purified cDNAs was amplified in a 25 µl reaction volume in1X PCR reaction mixture using GoTaq® Green Master Mix 2X (see appendix c) was used. PCR was performed using a programmable thermo-cycler. The PCR profile was summarized in appendix c. The RT-

PCR products were analyzed on 1% agarose gels in 1X TAE buffer (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0) containing 200 ng of ethidium bromide per ml. Ten µl of PCR product and 5 µl of a 100bp DNA ladder were loaded. DNA bands were visualized using a standard UV-imaging system.

Heteroduplex Mobility Assay (HMA)

Plant materials and virus isolates

Based on the MMM analysis, five somatic hybrid rootstock candidates (A+ Chandler #A1-11, Page+HBJL-3, A+7-2-99-5, Page + HBJL-7, A+HBJL-5 and) were selected as representative hybrid rootstock candidates from each of the 5 resistance categories (based on MCA13-ELISA results), along with the corresponding sour orange control and the source isolate from the interstock. The somatic hybrid A + Chandler #A1-11 was chosen as a representative of the highly tolerant rootstock candidates. The somatic hybrid Page + HBJL-3 represented the slightly tolerant group. The somatic hybrid A+ 7-2-99-5 was chosen as a representative of the susceptible rootstock candidates. The somatic hybrids A+ HBJL-5 and Page + HBJL-7 were chosen as representatives of the intermediate resistant rootstocks. The resistant group was not represented here since the MCA13-ELISA results showed no CTV replication and because there was no PCR amplification in the MMM analysis. Samples were collected approximately 16 months after top-work grafting. The virus infection was detected by serological techniques such as double antibody sandwich enzyme –linked immunosorbent assay indirect (DASI-ELISA) as in Chapter (4).

Total RNA isolation and complementary DNA (cDNA) synthesis

CTV-infected tissue (100 mg) from bark was pulverized in liquid nitrogen and the total RNA extracted using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The final total RNA extraction was re-suspended in 40 µl of RNase-

free water. The RNA extract was either used immediately or stored at -80º C. For the first strand complimentary DNA (cDNA) synthesis, 10 µl of total RNA was mixed separately with the antisense primer, CN 491 (5'GTGTARGTCCCRCGCATMGGAACC 3') (200 nM). The preparation was gently mixed then centrifuged at 10,000 rpm for 10 s, then incubated at in water bath at 70º C for 10 min and transferred to ice for 5 min. A reaction mixture was prepared by adding 5X first strand buffer (Invitrogen), 0.1 M dithiothreiotol (DTT), 200 µM of dNTPs mixture (Promega, Madison, WI) and nucleotide free water. This reaction mixture was incubated at 42 C for 2 min in a water bath. and then kept at room temperature for 10 min. Twenty U of Superscript II RNase H-Reverse transcriptase (Invitrogen, Carlsbad, CA) and 40 U of RNasin (Promega, Madison, WI) was added to the reaction mixture and centrifuged at 10, 000 rpm for 10 s. Nine µl of this mixture was added to each tube containing the RNA preparations. Twenty µl of the total content was incubated at 50º C for 1h, 72ºC for 15 min. and then transferred to ice for slow cooling. The 50 µl reaction volume containing 5 µl of the cDNA was used in the PCR reaction carried by 5 U of Taq DNA polymerase (Promega, Madison, WI) in 1X PCR reaction buffer, 200 nM of each of CN 488 (5'TGTTCCGTCCTGSGCGGAAYAATT 3') and CN 491 (5'GTGTARGTCCCRCGCATMGGAACC3') primer pair, 1.5 mM MgCl2 and 200 µM of dNTPs mixture. The reaction was carried out in a programmable thermo cycler. A-30 cycle PCR was performed according to the following steps: denaturation at 94º C for 2 min and 94º C for 30 s, annealing at 62º C for 45 s, and primer extension at 72º C for 45 s, (10 min at 72º C for the last extension step). The PCR products (5 µl) were analyzed by electrophoresis on 1% agarose gel in 1X TAE buffer (40 mM Tris-Acetate and 1 mM EDTA, pH 8.0). DNA bands were visualized under a UV image system.

DNA purification, cloning and transformation

The 403 bp region (1081-10484 nt) was amplified by RT- PCR from total RNA. Twenty µl of the PCR product was loaded on 0.8% agarose gel and the DNA bands were separate by electrophoresis at low voltage for better separation. The amplified band was excised using a sterilized razor blade, and purified by using QIAGEN Gel Purification kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Final elution was made in 40 µl of the elution buffer. Two µl of the purified DNA was run on 1% agarose gel to confirm the purification step. The purified PCR products were then ligated into pGEM-T Easy plasmid vector according to the manufacturer (Promega, A1360). Three µl of the gel purified PCR product was mixed with 5 µl 2X rapid ligation buffer, 1 µl of the T4 DNA ligase, 1 µl of pGEM-T Easy vector (50ng) and 2 µl of Promega nuclease-free water. For better ligation product, the ratio should be 1 vector to 3 DNA. The ligation reaction was performed at 4 º C for overnight. Three µl of the ligation reaction mixture was then added to the 50 µl of the DH-5α *E. coli* chemical competent cells, which were then incubated on ice for 30 min. The cells were transferred by heat-shock method at 42º C for 45s-1min, transferred to ice for 10-15 min and 800 µl of Luria-Bertani (LB) medium pH7 (10 g trypton, 5g yeast extract, 5g NaCl, and 15g agar) was added to the mixture. The cells were grown at 37 ºC and 210 rpm for 45min-1 h and three volumes 50, 100, and 150 µl cells were plated on LB agar plates containing 50 µg/ml of kanamycin and 80 ng/ml of X-gal. The plates were left open to dry in the hood then incubated at 37 ºC overnight. Master plates with 50 colonies each were prepared with the white colonies by subculture on a fresh LB agar plate with kanamycin. The master plates were kept at 4º C until the colony PCR reaction was performed on them.

Colony PCR and heteroduplex mobility assay (HMA)

The transformed colonies were screened for the target insert using colony PCR by extraction of individual colonies in an extraction buffer (1 % Triton X100, 20mM Tris HCl, pH 8.0 and 2mM EDTA, pH 8.0). The colony extracts were then heated at 95 ºC for 10 min. Five µl of the colony extract was used in a final volume of 50 µl for the PCR reaction using CN 488 and CN 491 primer pair. PCR amplification conditions were 94º C for 2 min; 30 cycles of 94º C for 30 s, 62º C for 45 s, 72º C for 45 s; followed by incubation at 72º C for 10 min. The PCR products were analyzed using 1% agarose gels and visualized on a UV image system. About 25-30 clones from each test sample were used for the formation of heteroduplexes following a protocol slightly modified from (Delwart et al., 1993). The modification of this protocol was done by K.L. Manjunath. Colony PCR product (4.5 µl) from the reference clone was mixed with the equal volume of the test clone and 1 µl of 10X annealing buffer (100 mM tris-HCl, pH7.8, 1M NaCl and 20 mM EDTA). The reaction was done in a thermocycler block where the DNA mixture(reference DNA+ the tested colony DNA) was denatured at 95º C for 10 min, then slowly annealed at 68º C for 1 h and then kept at 4º C for 10 min. The mixture was then electrophoresed on 10 % Criterion™ precast polyacrylamide gel (Biorad) in chilled Tris-borate EDTA (TBE) buffer (0.088 M Tris-borate, 0.08 M boric acid and 0.02M EDTA) at 120 volts for 3.5 h at 4° C in a Criterion™ cell (Biorad). The Biorad unit was disassembled and the gel was cut from the upper edge for labeling the lanes and carefully stained in 1X TBE buffer containing 200 ng/ml of ethidium bromide for 20 min. A UV imaging system was used for visualization of DNA heteroduplex pattern. Tested clones that showed heteroduplex formation during the first screening were selected for the second HMA screening by using one of these clones as a new reference clone. Therefore, the total number of clones from each sample were reduced to 2-3

different groups (genotypes), based on the sequence differences after 2-3 HMA screenings indicated by the different HtD patterns.

DNA miniprep, sequencing and sequence analysis

Two to three clones from each group were cultured from the master plate in 5ml LB medium without antibiotics and incubated over night at 37°C with shaking at 210rpm. The DNA miniprep was done using Miniprep Quiaprep spin miniprep from QUIAGEN. The DNA concentration was measured before sending for sequencing using the nanodrop at OD260. These clones were sequenced at the DNA Sequencing Core Laboratory at University of Florida, Gainesville, FL. These sequences were aligned with other CTV-full length sequences available in the database using CLUSTAL X (Thompson et al., 1997). The phylogenetic relationship among the sequences of the amplified regions from the tested CTV isolates, using the universal primers (CN 488 and CN 491), was determined using program CLUSTAL X. The dendograms were generated using the TreeView program (version 1.6.6.), and then the Genedoc version 2.6.002 program (Nicholas and Nicholas, 1997).

Quantitative Teal-Time PCR (qRT-PCR) Method to Determine and Quantify CTV Accumulation

Plant materials and virus isolates

Ten selected representative rootstock candidates were chosen based on the ELISA, MCA13 results and according to the seedlings available. Somatic hybrid rootstock candidates Page + HBJL-3, A + SN7, A+4-3-99-2, A+HBJL-1, A+ HBJL-5, A+HBJL-3, A+7-2-99-5, A + Chandler #A1-11, Page + HBJL-7, A+4-4-99-6, and control sour orange were inoculated in the greenhouse with the CTV, T36- QD isolate kindly provided by the Dr. W.O. Dawson laboratory. Samples were collected approximately 12 months after inoculation. The virus infection was detected by double antibody sandwich enzyme –linked immunosorbent assay indirect (DASI-

ELISA). Samples of pummelo seedlings HBJL-3, HBJL-5, and 8-1-99-2B, the somatic hybrid A+5-1-99-3, and open-pollinated tetraploid 2247-OP-A2 were collected 16 months after the successful top-working. The test rootstocks designation by ELISA-MCA13 is presented in Table (5-5).

RT-PCR primers

The conserved region of the T36-CTV coat protein gene was used to design the primers. Forward primer, start position (69):- TGCCGAGTCTTCTTTCAGTTCCGT and reverse primer, start position (172):- TGTTCAAAGCAGCGTTCTGTTGGG. Primers were designed with the Primer Express 2.0 software (Applied Biosystems-Perkin-Elmer, Foster City, CA, USA). Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). This primer pair can be used to detect and quantify the CTV in all infected sample regardless of the isolate.

RNA extraction

Total RNA from the test samples and the controls was extracted by RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions and as previously described in the MMM and HMA section. Purified RNA was measured by UV absorption at 260 nm, whereas RNA purity was evaluated based on the UV absorption ratio at 260/280 nm. The standard curve was generated from purified T36 (5 folds). This standard curve was used for relative quantification of the CTV titer in the unknown samples. A no-template control (NTC) was also prepared as a negative control for the analysis.

PCR conditions

Quantitative multiplex real-time PCR (qRT-PCR) assay was done in a fluorometric thermal cycler (ABI PRISMTM 7000 Sequence Detection System, Applied Biosystems-Perkin-Elmer, Foster City, CA) in a final volume of 25 µl. The reaction mixture contained 1x SYBR Green Mix (2X) (Applied Biosystems), the RNA sample and an optimal concentration specific

primer. The amplification conditions were one cycle of one 30 min cycle at 48ºC to synthesize the cDNA, and then one cycle of one 2 min cycle at 50ºC and a 10 min cycle at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Fluorescence was monitored during the 60ºC annealing step. The data was analyzed with ABI PRISMTM 7000 SDS software ver.1.1 provided by P. E. Applied Biosystems. A standard curve was generated using purified RNA from the T36 isolate kindly provided by Dr. William O. Dawson's laboratory (CREC) and five-fold serial dilutions were prepared and used to obtain the standard curve. PCR amplification efficiency of the reaction is an important factor when using a relative quantification method. The common logarithm of dilution series of RNA was plotted against the Ct values of those dilutions. The PCR efficiency was calculated from the equation E = 10-(1/slope)- 1 as described by Ginzinger (2002). The ideal slope should be -3.32 for 100% PCR efficiency, which means that the PCR product concentration doubles during every cycle within the exponential phase of the reaction (Gibson et al., 1996).

Results and Discussion

Multiple Molecular Markers (MMM)

Based on the amplification with the different MMM, a specific genotype profile (Isolate Genotypes) was assigned to each isolate according to Hilf and Garnsey (2000, 2002). The results of MMM analysis for the tested samples are presented in Tables (5-2) and (5-3), and in Figure (5-2). All the interstock source isolates were designated as group I and contained a mixture of T36, T30 and VT genotypes, as amplifications were obtained with the entire three markers specific to the T36, T30 and VT isolates (Figure 5-3 A) and (Tables 5-2 and 5-3). As expected, PCR products were obtained with the universal primer used as a positive control: T36 CP. There were no products obtained with the markers specific to the T3 isolate. The somatic hybrids A+ Chandler #A1-11, A + HBJL-5 and Page + HBJL-7 representative isolate of group II

(intermediate to high CTV tolerance), amplified with the T30 and T36 specific primers, but didn't amplify with either T3 or VT-pol. Therefore, the isolate in these hybrids contained T30 and T36 only, but not VT (Figure 5-3 B, E and F and Tables 5-2 and 5-3). In group III (CTV susceptible), the representative rootstock candidate somatic hybrid A+ 7-2-99-5 showed amplification with primers specific to T30, T36 and VT, showing similar genotypes as the source isolate except, this isolate didn't amplify with T36 5', T36 K17, T30 5 or T30 K17. This isolate also didn't amplify with primers specific to T3. As expected, this isolate amplified with the universal primer, T36CP (Figure 5-3 C). The profile is described in Tables (5-2) and (5-3). Group IV (slightly CTV tolerant), represented by the rootstock candidate Page + HBJL-3 contained two different genotypes T30 and VT, as amplifications were obtained with the Primers specific to these isolates; T30 pol and VTpol respectively (Figure 5-3 D). The complete profile is presented in Tables (5-2) and (5-3). All sour orange samples corresponding to the test rootstocks gave the same profile and it was similar to the source isolate. The isolate found in the control sour orange contained T30, T36 and VT. This isolate reacted with T36 pol, T365', T30pol, T305', T30K17, VTpol, VT5', and VTK17, in addition to the amplification with T36 CP primer. There was no amplification with T36 K17 or the primers specific to T3. Thus, the sour orange isolate (same as group III) had a profile very similar to the source isolate except that the sour orange isolate lacked the amplification with T36 K17 primer (Figure 5-3 G) and Tables (5-2) and (5-3). Finally, a few of the test rootstocks namely somatic hybrids A+4-3-99-2and A+5-1-99-3, open-pollinated tetraploid 2247-OP-A2, and pummelo seedlings; HBJL-3R10T20, HBJL-5, and 8-1-99-2B didn't amplify for any of the tested CTV genotypes, indicating a broad-based resistance to CTV replication (designated Group V).

The Heteroduplex Mobility Assay (HMA)

HMA results showed a range of differential virus movement as demonstrated by the different genotypes found in each rootstock candidate. HMA results supported the MMM results used to classify rootstock candidates into different groups (I-IV, not including the CTV resistant group V) based on the number and combination of detected genotypes that successfully migrated from the 'Hamlin' interstock into the test hybrids. Figures (5-4) and (5-5) showed the different patterns of HMA, indicating different CTV genotypes. HMA of the source isolate, A+7-2-99-5 rootstock and sour orange control showed three different HtD patterns (Figures 5-4 A and C and 5-5 A), respectively. Rootstocks, A+ Chandler # A1-11, Page+ HBJL-3 and A +HBJL-5 show only two different patterns of HtD based on the amplification with the universal primer pair 488, 491 (Figures 5-4 B and D; and 5-5 B and C), respectively. Colonies with different HMA patterns were sequenced. The dendogram in Figure (5-6) was generated in TreeView to determine the relationships among the tested hybrids according to CTV genotypes and also between CTV genotypes in these test rootstocks and the most commonly known CTV isolates from the GenBank database [Accession number, AF260651 (T30), Y1842 (T385), AB 046398 (NUAGA), EU937519 (VT), AF001623 (SY568), AY340974 (QAHA).and U16304 (T36)]. The comparison of nucleotide sequence identities of the different genotypes from the rootstock candidate representatives (A+7-2-99-5, A+Chandler#A1-11, Page+HBJL-3, 4Page+HBJL-7, A+HBJL-5), sour orange, and the source isolate, obtained after heteroduplex mobility assay (HMA) of the 403 bp amplicon from CTV genome (ORF1a) with sequenced CTV isolates from the GenBank database is shown in Table (5-4) and the phylogenetic tree showing genetic relationships of the different CTV genotypes is presented in Figure (5-6). The number before each rootstock indicates the colony number used for DNA sequencing. Rootstock candidate, A+7-2-99-5 acquired a nucleotide sequence closely related to both the T30 and T385 isolate with 98 %

sequence homology. It was also clustered with T36 (96 % sequence homology). The nucleotide sequence of CTV in this rootstock was distantly related to the NUAGA CTV isolate (only 89% similar). The SY568, VT and QAHA CTV isolate from Egypt shared nucleotide similarities of 94%, 92% and 92% respectively. The mild isolate (T30), QD isolate (T36) and the VT SP isolates from Israel were the most important CTV isolate to determine the sequence homology between them and the test rootstocks in this study. The isolate in sour orange was closely related to that of A+7-2-99-5 and the source, and all clustered with T30 and T36 CTV isolates with nucleotide identity of 99% and 96%, respectively. The nucleotide sequence from this isolate shared only 85% homology with the VT isolate. The source isolate was closely related to T30, T36 with 99 % and 96% similarity, respectively, than to the VT (91% nucleotide homology). Isolates found in the Page + HBJL-7 rootstock was more similar to both T30 and T385 isolate (99% and 98%) than to VT and T36 (90% and 91%), respectively. In the phylogenetic tree, the isolate from rootstock candidate A+ HBJL-5 is grouped with T30 and T36 isolates with sequence homology 99% and 92%, respectively. This isolate shared sequence identity with VT (89%) and in the tree it was not included with the same group with VT. The isolate from A+ Chandler A1 which has a nucleotide sequence highly similar to the T30 sequence (98%). Therefore; it was grouped with the T30 isolate. It was also grouped with T36 isolated with sequence homology (91%). This isolate is more distantly related to VT isolate (89%) and it was not clustered with theVT group in the phylogenetic tree. Page + HBJL-3 was closely related to T30 (98%) and shared 92% nucleotide sequence identity with VT isolate. This isolate was more distantly related to T36 (80% sequence homology) and it did not group with T36. In general there was a strong correlation between the identity of the sequence homology and the generated phylogenetic tree. The highest nucleotide sequence homology with any tested isolate and the VT isolate from the

GenBank was 92%, whereas the nucleotide sequence homology with the other isolates from Florida, T30 and T36, was 99%. These results proved that the VT isolate from Israel is more distantly related to the isolates found in the current work and shows the need for the complete sequence of the VT isolate currently found to compare with the VT isolate from Israel. Since VT is found as a mixture, aphid transmission could be a useful tool to separate the CTV genotypes in this isolate as needed to sequence the pure VT isolate.

Quantitative Real-Time PCR (qRT-PCR) Method to Determine and Quantify CTV Accumulation

Analysis of qRT-PCR data on the rootstock candidates inoculated with T36 in a small greenhouse companion study revealed that some of the tested rootstocks including the somatic hybrids A+7-2-99-5 and A+SN7 showed high CTV titers (4.996 ng/µL and 4.400 ng/µL, respectively) by qRT-PCR, with very low Ct values (13.14±0.04 and 13.31±0.098, respectively), indicating that these rootstock candidates are susceptible to CTV infection and replication. On the other hand, rootstocks such as somatic hybrids A+HBJL-1, A+4-3-99-2, and A + Chandler #A1 showed very low CTV titer, with higher Ct values (25.33±0.3,; 23.55 ±0.0 and 21.93±0.569, respectively), and the virus titer was 0.001 ng/µL, 0.002 ng/µL and 0.008 ng/µL respectively, suggesting some level of tolerance to CTV replication. The somatic hybrids A+ HBJL-5, A+ HBJL-3, and A+4-4-99-6 showed intermediate CTV titers (0.415ng/µL, 0.235ng/µL and 1.139ng/µL, respectively). Above all the test rootstocks, sour orange showed the highest CTV titer, 16.07 ng/µL with Ct =11.55±0.05, as expected for a susceptible control. In addition, five rootstock candidates from the top-working grafts (somatic hybrid A+5-1-99-3, open-pollinated tetraploid 2247-OP-A2, and pummelo seedlings; HBJL-3 R10T20, HBJL-5, and 8-1-99-2B) were tested by qRT-PCR since they were negative by MMM and MCA13- ELISA. Rootstock candidate A+5-1-99-3 showed a CTV titer = 0.019 ng/ µL with a high Ct value (20.71±0.216),

and 2247-OP-A2 rootstock candidate showed a CTV titer=0.01 ng/ µL with high Ct (21.67±1.318). The CTV titer in rootstocks; HBJL-3 R10T20, HBJL-5, and 8-1-99-2B was, 0.033 ng/µL, 0.029 ng/µL, and 0.089 ng/µL respectively.

Summary and Conclusions

Field isolates of CTV often are present mixtures of different CTV genotypes (Mawassi et al., 1995a; Mawassi et al., 1995b). The differential selection of host to different genes has been reported (Ayllon et al., 1999b; Ayllon et al., 1999a). The population diversity of CTV may change due to several effects, such as grafting with a different citrus genotype. In some cases, this can lead to the formation of new CTV genotypes and therefore be partially responsible for the broad biological, serological and also molecular variability among CTV isolates (Ayllon et al., 1999b; Ayllon et al., 1999a) . The molecular characterization of the CTV field isolates in the top-worked hybrid rootstock candidates using MMM and HMA conducted in this study also showed significant changes in the population structure of CTV isolates moving from the 'Hamlin' sweet orange interstock into the newly grafted top-worked hybrid material. The changes in the CTV genotype composition also suggest differential selection properties of different citrus hosts (test rootstock candidates).

Using MMM, an isolate was designated as T36 genotype if it reacted with at least the PCR marker for the T36 Pol, however it may not react with all the T36 markers. The T30 genotype and the VT genotype also were designated if a reaction occurred with T30 pol and VT pol markers, respectively. The T3 genotype should react with not only T3 K17, but also with the VT pol and/or with the VT 5' markers (Brlansky et al., 2003). The isolate, T36 CP, and the universal; primer pair CN 488, and 491 are used as a control. The strength of the amplified band can be used as an indicator of which genotype is dominant in each sub-isolate. Using the MMM and the sequence analysis in the HMA, the hybrid rootstock candidates that allowed CTV

replication were divided into 4 groups (I-IV) in Table (5-3) based on the different combination of CTV genotypes that were observed. Group V was composed of hybrid rootstock candidates that did not show any amplification with the MMM, indicating no CTV replication from any of the viral genotypes, indicating broad resistance to CTV.

The present study demonstrated both qualitative and quantitative changes in the original CTV genotypes found in the original 'Hamlin' interstock isolate upon top-working with genetically different hybrid rootstock candidates. The changes in the CTV genotypes suggest specific selection pressures by the host scion on the viral sequences. Interesting enough is that all the top-worked sour orange samples gave the exact MMM profile that was very similar to the one found in the 'Hamlin' interstock source isolate. Most of the known molecular methods for CTV detection are limited by the lack of information available for CTV sequences. New and better molecular tools are required for the fast and efficient detection of new CTV genotypes. The HMA and the sequence information generated on this study provide valuable information about the population diversity of CTV. Moreover, this study suggests a distant relationship of the VT isolate found in the Florida field under this study and the VT isolate known as stem pitting (SP) isolate from Israel. Determining the complete sequence of the Florida field VT isolate will be very helpful for comparison with the complete genome sequence of the VT isolate from Israel, as needed to ultimately prove that this Florida VT isolate may be different from the Israeli VT isolate that causes economically damaging SP. This suggestion is supported by the fact that neither the source 'Hamlin' interstock nor the hybrid rootstock candidates containing the Florida VT isolate developed any stem pitting symptoms over a 2-year period of observation.

Quantitative real time PCR is very useful in different purposes including potential association of the symptoms severity with accumulation of specific variants, evaluation of

resistance of citrus varieties to different viruses (Ruiz-Ruiz et al., 2007). Based on the analysis of the qRT-PCR results, some of the tested rootstocks, such as the somatic hybrid A+7-2-99-5, showed a very high quantity of CTV and severe disease symptoms, making this rootstock very susceptible to CTV infection. This hybrid also showed a strong seedling yellows reaction in the companion greenhouse challenge. In contrast, a group of somatic hybrid rootstock candidates including somatic hybrids A+HBJL-1, A+4-3-99-2, and A+ Chandler #A1 showed zero to very low CTV titer and no disease symptoms, suggesting some resistance to CTV replication and QD disease. Many hybrids showed intermediate levels of CTV titer, but no disease symptoms. The results obtained from real-time PCR for quantifying CTV accumulation are very accurate and important for effective screening of new rootstock candidates. Moreover, the high efficiency of this technology allow the analyses of large numbers of samples in less than 3 h. qRT- PCR provided a fast, reliable and accurate method to determine the level of CTV tolerance in the pre-selected rootstock candidates.

A final group of new rootstock candidates including somatic hybrids A+4-3-99-2 and A+5-1-99-3, and the open-pollinated tetraploid 2247-OP-A2, were MCA13 negative and shown by the molecular analysis (MMM) to be resistant to CTV replication. In the qRT-PCR test these rootstock candidates showed very low CTV titer (0.002-0.019), respectively. Such low titers could be accounted for by virus movement alone, possibly with no replication. Thus, these hybrid rootstock candidates have potential to replace sour orange rootstock in Florida if they meet other required horticultural criteria. These rootstocks are among many top-worked rootstock candidates that are expected to begin fruiting next year. Seeds will be extracted from the fruits, counted, and tested by microsatellite analysis to determine if embryos are of nucellar or zygotic origin, with nucellar origin being required for standard nursery propagation. CTV-

resistant zygotic hybrids would still have value as rootstock breeding parents. In the future, qRT-PCR should be performed using the strain specific primers. This assay could have numerous potential applications for differentiation of CTV strains in the CTV complex at once, using the strain-specific primers. Quantitative multiplex TaqMan Assay can use up to four different probes simultaneously in the same reaction to differentiate and quantify the different CTV genotypes in isolate containing mixtures. Applying this technique to screen the rest of top-worked rootstocks for strain differentiation will be very useful for fast and reliable results.

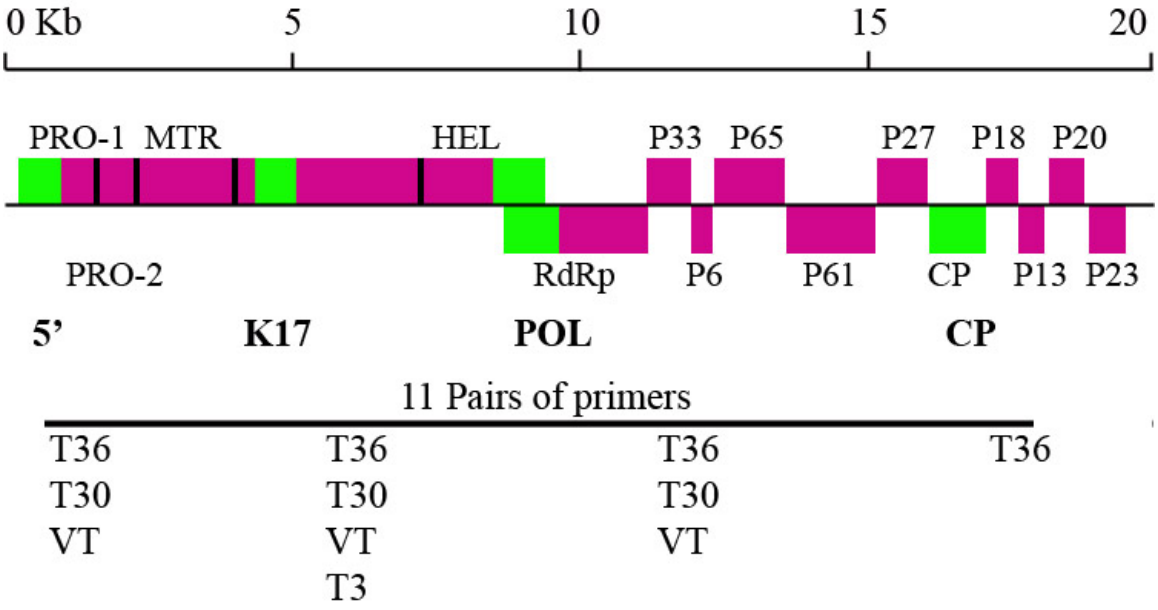

Figure 5-1. Citrus tristeza virus (CTV) genome indicating different ORFs and approximate portions of the genome amplified with genotype specific molecular markers by Hilf et al, 2000. The sequence- specific markers amplified are indicated by the lime green blocks and the name of the amplified marker underneath.

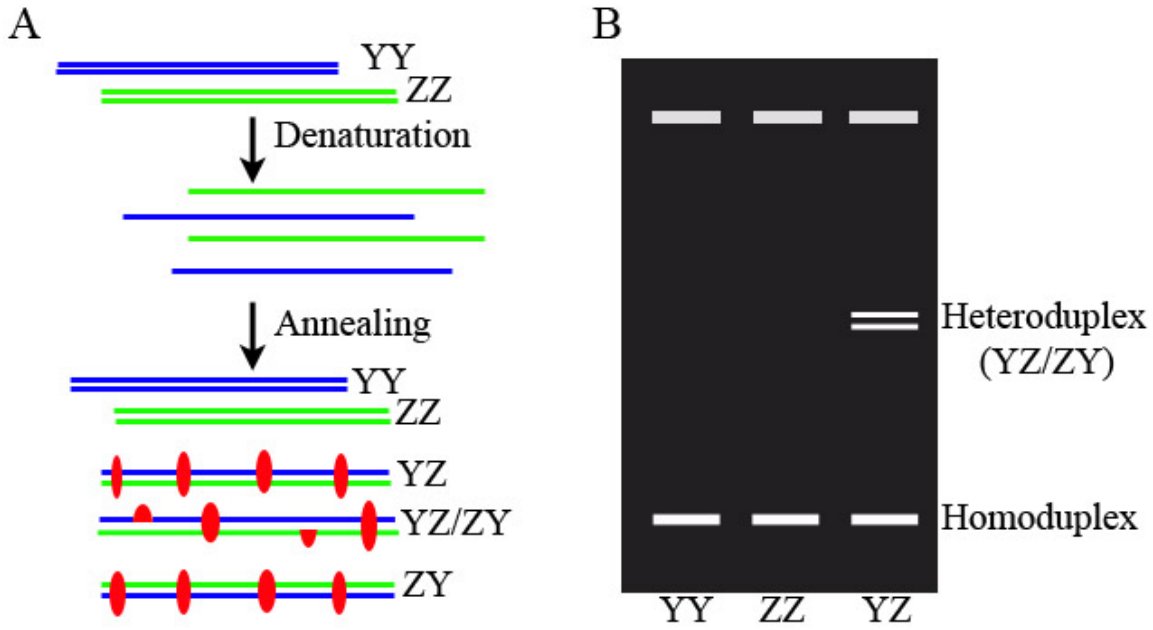

Figure 5-2. Heteroduplex Mobility Assay (HMA). A) The HMA reaction. B) The polyacrylamide gel of the HMA reaction.

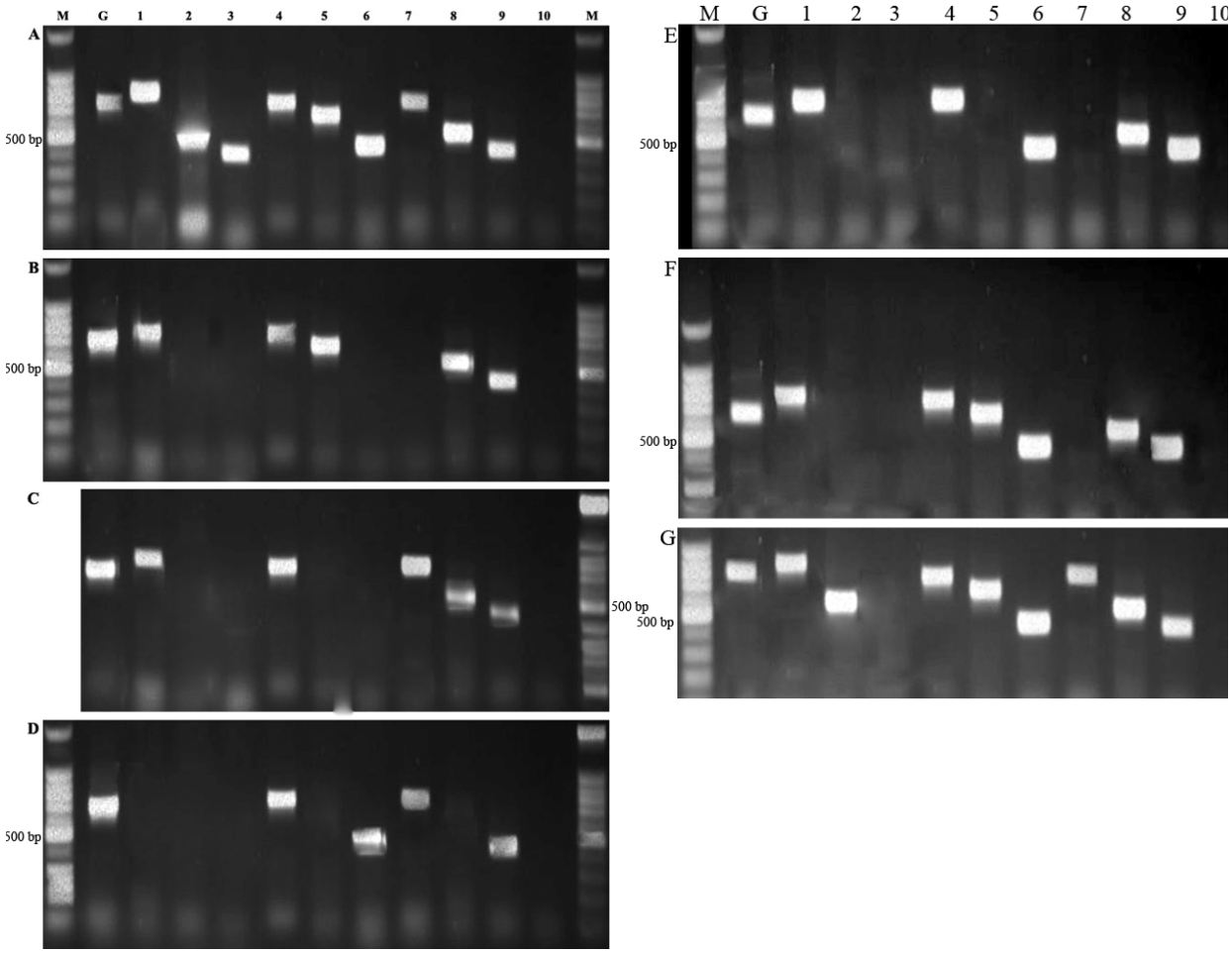

Figure 5-3. Multiple molecular marker (MMM) profiles of CTV source isolate and selected test rootstocks, created by PCR amplification using sequence–specific primers. A) Profile of CTV source isolate. B) Profile of CTV in rootstock A + Chandler #A-11. C) Profile of CTV in rootstock A+7-2-99-5. D) Profile of CTV in rootstock Page+HBJL-3. E) Profile of CTV in rootstock Page+ HBJL-7. F) Profile of CTV in rootstock A+ HBJL-5. G) Profile of CTV in rootstock sour orange. Ten µl of MMM-PCR product was loaded in lanes 1-10. Lanes (1-3) show amplification of T36 POL, T36 5' and T36 K17 markers, specific for T36 isolate from Florida. Lanes (4-6) show amplification of T30 POL, T30 5' and T30 K17 markers, specific for mild T30 isolate from Florida. Lanes 7-9 show amplification of VT POL, VT 5' and VT K17 markers, specific for VT isolate from Israel. Lane 10 show amplification of T3 K17 marker, specific for T3 isolate from Florida. Lane G shows amplification of general markers: T36 CP. M = 100pb DNA ladder.

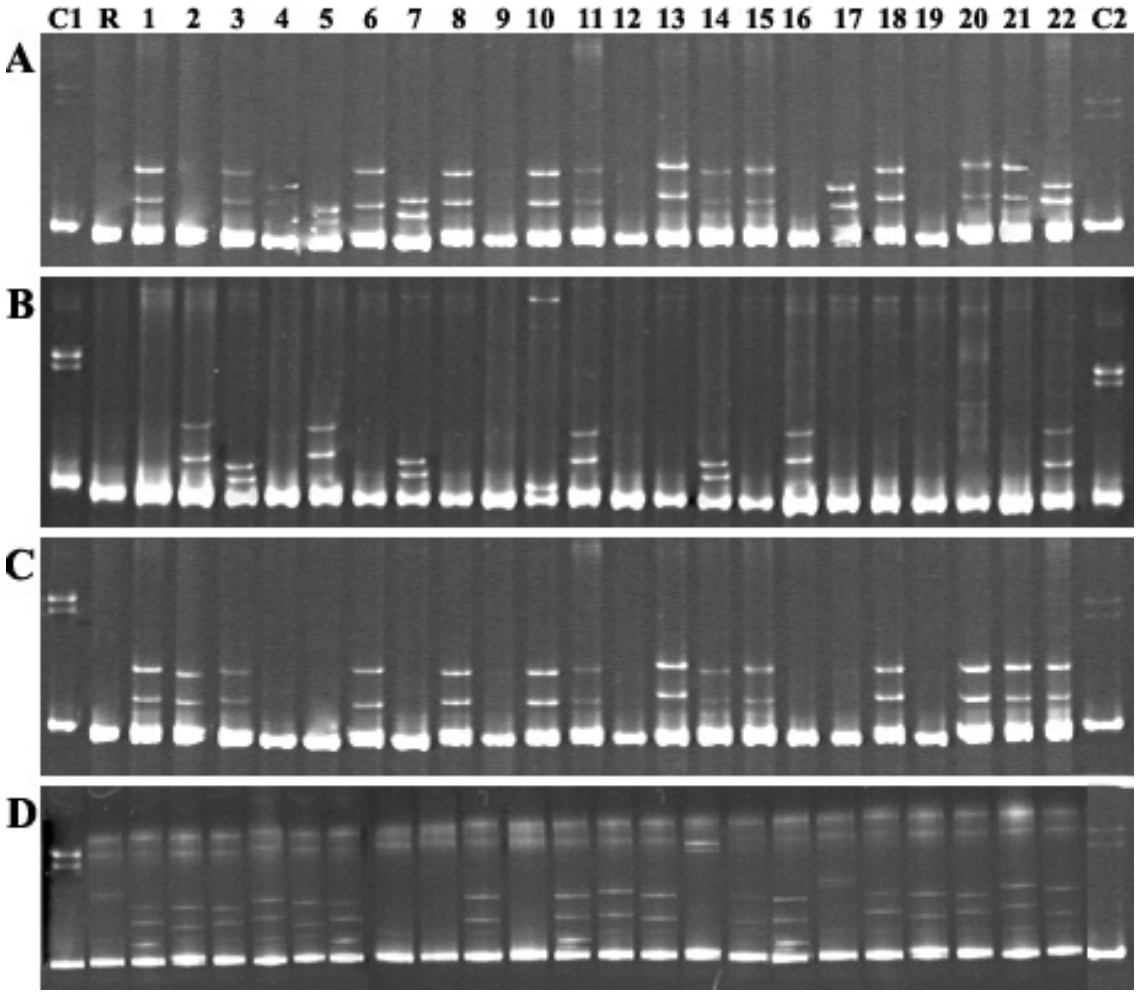

Figure 5-4. PAGE 1 showing the retarded mobility of heteroduplexes 1 (HtD2) formed due to the nucleotide sequence differences in the RT-PCR amplified cloned 403 bp region of ORF 1a. Each lane represents the homoduplex (HmD) or the HtD formed between the reference clone and each of the test clones. A) HtD profiles of CTV source isolate. B, C, and D) Profiles of CTV in tested rootstocks. B) Representative of group II; A+Chandler A1-11. C) Representative of group III; A+7-2-99-5. D) Representative of group IV; Page+HBJL-3. C1 and C2: positive control; R: Clone # 1 as a reference with the HmD band; Lanes 1-22 represent the tested clones showing either HmD or HtD formations.

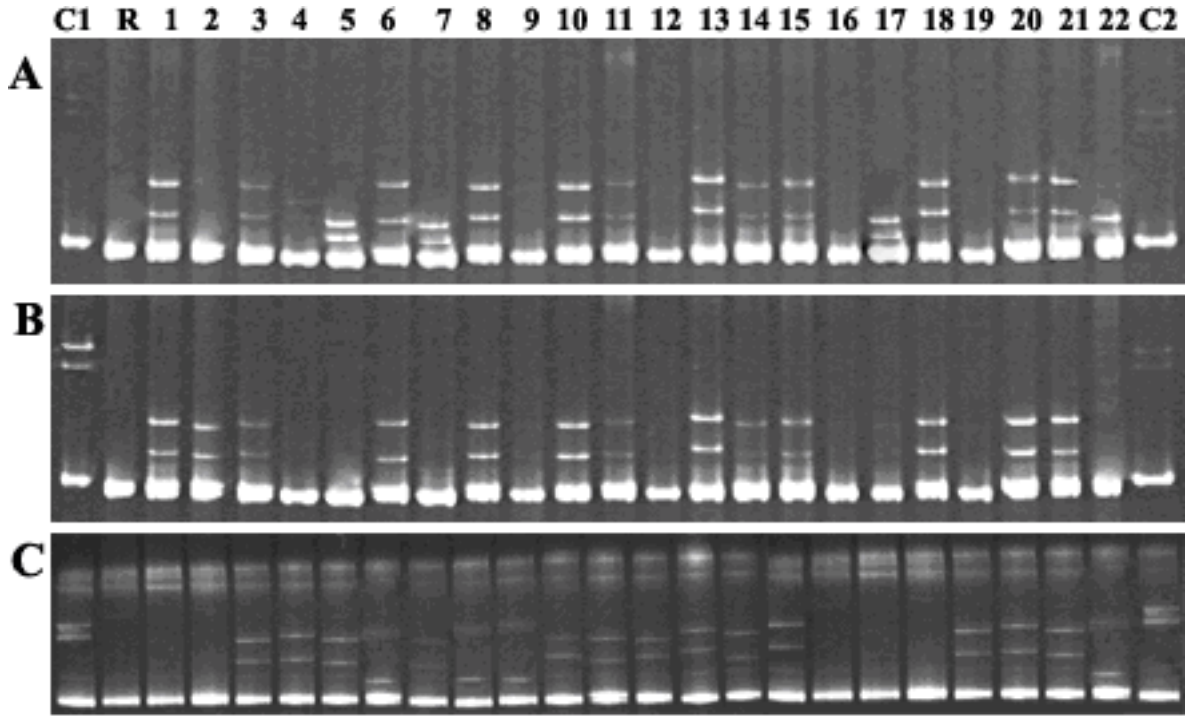

Figure 5-5. PAGE 2 showing the retarded mobility of heteroduplexes 2 (HtD2) formed due to the nucleotide sequence differences in the RT-PCR amplified cloned 403 bp region of ORF 1a. Each lane represents the homoduplex (HmD) or the HtD formed between the reference clone and each of the test clones. A) HtD profiles of CTV sour orange isolate. B) Profiles of CTV in tested rootstock A+ HBJL-5. C) Profiles of CTV in tested rootstock Page + HBJL-7. Lanes C1 and C2; positive control. Lane R; Clone # 1 as a reference with the HmD band; Lanes 1-22 represent the tested clones showing either HmD or HtD formations.

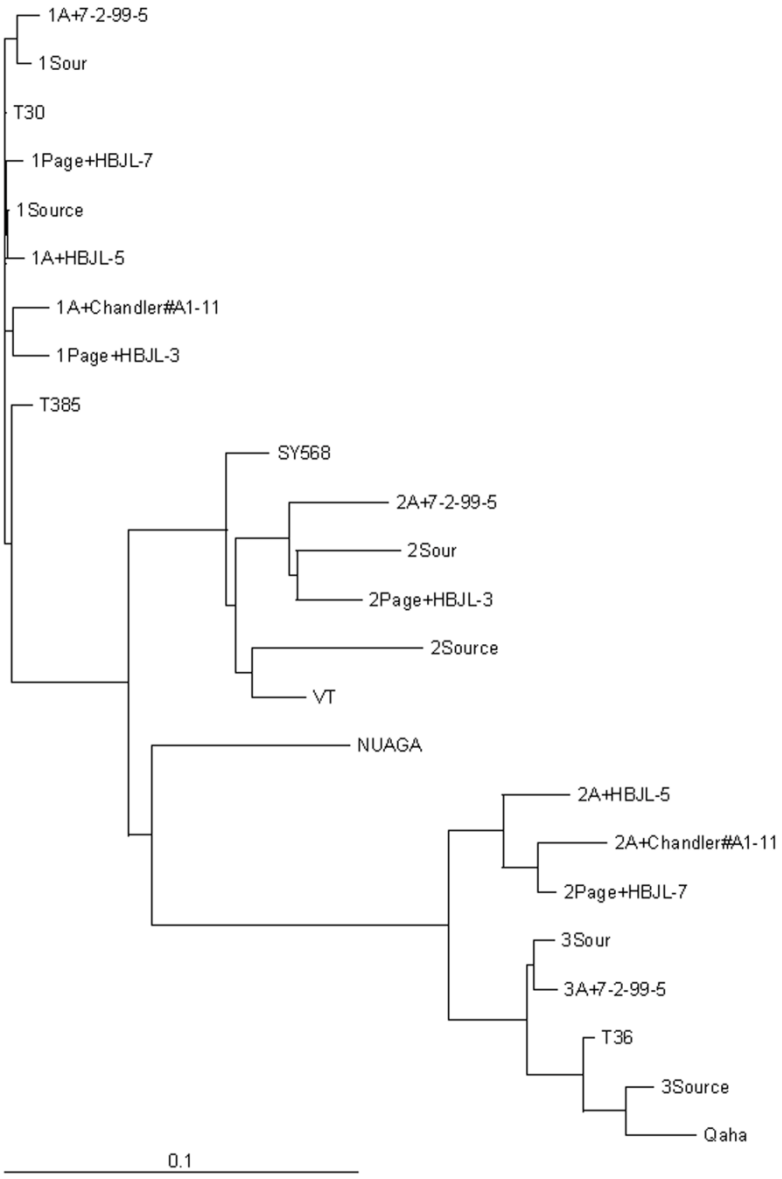

Figure 5-6. Phylogenetic tree showing genetic relationships of the CTV genotypes found in top-worked scions A+7-2-99-5, A+Chandler#A1-11, Page+HBJL-3, 4Page+HBJL-7, A+HBJL-5, sour orange and the interstock source obtained after heteroduplex analysis (HMA) of the 403 bp amplicon, with the already sequenced CTV isolates. The number before each rootstock or source indicated the colony number used for DNA sequencing from this specific sample. Sequence analysis was done by using CLUSTAL X (Thompson et al., 1997) the phylogenetic relationship of the sequences were generated using the program TreeView version 1.6.6.

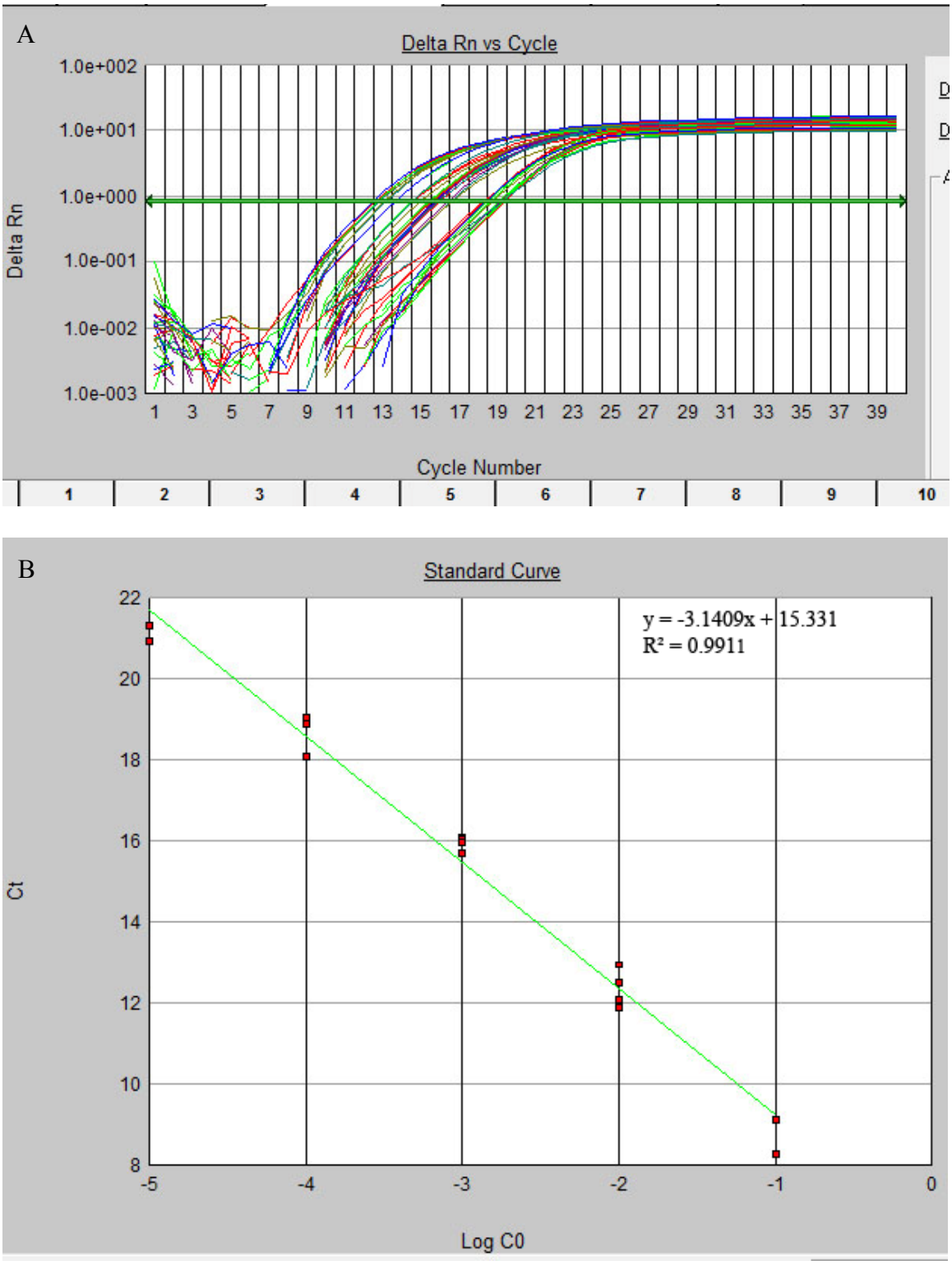

Figure 5-7. Q-RT-PCR amplification. A) Amplification curve. B) The standard curve.

Table 5-1. Sequence of Multiple Molecular Markers (MMM) primers (Hilf and Garnsey, 2000).

Primer		Primer sequence (5'-3')	Amplified size(bp)
*T36 CP	SENS	ATGGACGACGAAACAAAGAAATTG	672
	ANTISENSE	TCAACGTGTGTTGAATTTCCCA	
T36	SENS	GATGCTAGCGATGGTCAAAT	714
	ANTISENSE	CTCAGCTCGCTTTCTCGCAT	
T36 -5'	SENS	CTCAGCTCGCTTTCTCGCAT	500
	ANTISENSE	AATTTCACAAATTCAACCTG	
T36 K17	SENS	CTTTGCCTGACGGAGGGACC	409
	ANTISENSE	GTTTTCTCGTTTGAAGCGGAAA	
T30 POL	SENS	GATGCTAGCGATGGTCAAAT	696
	ANTISENSE	CTCAGCTCGCTTTCTCGCAT	
T30 5'	SENS	CGATTCAAATTCACCCGTATC	594
	ANTISENSE	TAGTTTCGCAACACGCCTGCG	
T30 K17	SENS	GTTGTCGCGCCTAAAGTTCGGCA	409
	ANTISENSE	TATGACATCAAAAATAGCTGAA	
VT POL	SENS	GACGCTAGCGATGGTCAAGC	695
	ANTISENSE	CTCGGCTCGCTTTCTTACGT	
VT 5'	SENS	AATTTCTCAAATTCACCCGTAC	492
	ANTISENSE	CTTCGCCTTGGCAATGGACTT	
VT K17	SENS	GTTGTCGCGCTTTAAGTTCGGTA	409
	ANTISENSE	TACGACGTTAAAAATGGCTGAA	
T3 K17	SENS	GTTATCACGCCTAAAGTTTGGT	409
	ANTISENSE	CATGACATCGAAGATAGCCGAA	

*Universal primer pair

Table 5-2. Genotype profiles of TW (top-worked scion) source isolates and sub-isolates, created by RT-PCR amplification of ten genotype-specific markers and one general marker. Ten genotype-specific markers are T36 POL, T36 5', T36 K17, T30 POL, T30 5', T30 K17, VT POL, VT 5', VT K17 and T3 K17 and the general marker, T36 CP.

Isolate / subisolate	MCA -13[a]	T36 CP	T36 POL	T36 5'	T36 K17	T30 POL	T30 5'	T30 K17	VT POL	VT 5'	VT K17	T3 K17
'Hamlin' interstock (TW) source	+	+	+	+	+	+	+	+	+	+	+	-
A + Chandler #A1-11	+	+	+	-	-	+	+	-	-	+	+	-
A+ 7-2-99-5	+	+	+	-	-	+	-	-	+	+	+	-
Page + HBJL-3	+	+	-	-	-	+	-	+	+	-	+	-
Page + HBJL-7	+	+	+	-	-	+	-	+	-	+	+	-
A+ HBJL-5	+	+	+	-	-	+	+	+	-	+	+	-
sour orange	+	+	+	+	-	+	+	+	+	+	+	-
A+4-3-99-2*	-	-	-	-	-	-	-	-	-	-	-	-
A+5-1-99-3*	-	-	-	-	-	-	-	-	-	-	-	-
2247-OP-A2*	-	-	-	-	-	-	-	-	-	-	-	-
HBJL-3* R10T20	-	-	-	-	-	-	-	-	-	-	-	-
HBJL-5*	-	-	-	-	-	-	-	-	-	-	-	-
8-1-99-2B*	-	-	-	-	-	-	-	-	-	-	-	-

a= Monoclonal antibody, MCA-13, Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)
*Rootstocks candidates collected from field shown no PCR amplification with all the MMM primers

Table 5-3. Summary of the multiple molecular markers (MMM) results showing differential movement of CTV genotypes from the sweet orange interstock into the top-worked test rootstock material.

	Citrus Germplasm	Group	CTV Resistance Category based on the performance	MCA13a	Sub-isolate	CTV genotypes identified
1	Source	I	susceptible	+	3	T36, T30, VT
2	A + Chandler #A1-11	II	tolerant	+	2	T36, T30
3	A+ HBJL-5		intermediate	+	2	T36, T30
4	Page + HBJL-7		intermediate	+	2	T36, T30
5	A+ 7-2-99-5	III	susceptible	+	3	T36, T30, VT
6	sour orange		susceptible	+	3	T36, T30, VT
7	Page + HBJL-3	IV	Slightly tolerant	+	2	T30, VT
8	A+4-3-99-2	V	resistant	-	-	Virtually no virus replication
9	A+5-1-99-3		resistant	-	-	Virtually no virus replication
10	2247-OP-A2		resistant	-	-	Virtually no virus replication
11	HBJL-3 R10T20		resistant	-	-	Virtually no virus replication
12	HBJL-5		resistant	-	-	Virtually no virus replication
13	8-1-99-2B		resistant	-	-	Virtually no virus replication

a= A Monoclonal antibody, MCA-13, Double-antibody sandwich enzyme-linked immunosorbent assay indirect (DASI-ELISA)

Table 5-4. The comparison of nucleotide sequence identities of the different genotypes from the rootstock candidate representatives (A+7-2-99-5, A+Chandler#A1-11, Page+HBJL-3, 4Page+HBJL-7, A+HBJL-5), sour orange, and the source, obtained after heteroduplex mobility assay (HMA) of the 403 bp amplicon from CTV genome (ORF1a) with sequenced CTV isolates from GenBank database. Nucleotide sequence analysis was done using CLUSTALX (Thompson et al., 1997) and GeneDoc version 2.6.002 (Nicholas and Nicholas, 1997).

Colony number		8Sour orange	1Source	4Page+HBJL-7	3A+HBJL-5	2A+Chandler#A1-11	6 Page + HBJL-3	T30*	T385*	13Sour orange	14Page+HBJL-3	15A+7-2-99-5	10Source	SY568 *	VT *	NUAGA*	7A+Chandler#A1-11	11Page+HBJL-7	16A+HBJL-5	9Sour orange	12A+7-2-99-5	17Source	QAHA *	T36*
1	A+7-2-99-5	99	98	98	98	97	97	98	98	86	88	87	86	91	85	89	83	83	82	84	82	80	77	81
1	Sour orange		99	98	98	98	98	99	98	87	88	88	86	91	85	89	83	83	82	83	83	80	78	82
1	Source			99	99	98	98	99	99	87	88	88	86	92	90	90	83	84	83	83	83	80	78	82
1	Page+HBJL-7				99	98	98	99	98	87	88	88	86	92	90	98	83	84	82	83	83	80	78	82
1	A+HBJL-5					98	98	99	98	87	88	88	86	92	89	89	83	84	82	83	83	80	78	82
1	A+Chandler#A1-11						98	98	97	86	87	87	85	91	89	89	82	83	82	82	82	80	77	81
1	Page + HBJL-3							98	97	86	87	87	85	91	89	89	83	83	82	83	82	79	77	81
	T30*								99	87	87	88	86	92	90	90	83	84	83	83	83	80	78	82
	T385*									87	88	88	86	92	89	89	83	84	82	83	82	80	77	81
2	Sour orange										88	93	88	93	92	84	80	80	81	80	81	80	75	79
2	Page+HBJL-3										93	93	88	94	92	84	81	81	82	81	82	78	76	80
2	A+7-2-99-5												88	94	92	85	80	81	82	81	81	78	76	80
2	Source													91	91	83	79	79	78	79	78	78	73	77
	SY568 *														96	88	83	84	83	84	84	76	79	83
	VT *															87	82	83	82	82	82	81	79	82
	NUAGA*																83	83	81	82	82	79	78	82
2	A+Chandler#A1-11																	99	95	93	92	80	87	91
2	Page+HBJL-7																		96	92	92	90	87	91
2	A+HBJL-5																			92	92	89	88	92
3	Sour orange																			92	92	90	92	96
3	A+7-2-99-5																				98	94	92	96
3	Source																					95	96	96
	QAHA *																							95

* The nucleotide sequences retrieved from GenBank [Accession number, AF260651 (T30), Y1842 0(T385), AB 046398 (NUAGA), EU937519 (VT), AF001623 (SY568), U16304 (T36)] and AY340974 (QAHA).

Table 5-5. Detection and relative quantification of CTV in selected test rootstock material using quantitative Real-time PCR.

Controls & Top-worked scion germplasm	Resistance classification	Ct ± S.D.[a]	CTV titer (ng/µl total RNA)	CV %[b]
Healthy tissue		Not detected		
Infected tissue		11.24 ± 0.150	20.020	1.34
Page+HBJL-3	Slightly tolerant	13.93 ± 0.235	2.786	1.68
A+SN7	Susceptible	13.31 ± 0.098	4.400	0.74
A+4-3-99-2	Resistant	23.55 ±0.007	0.002	0.03
A+HBJL-1	Resistant	25.33 ± 0.300	0.001	1.18
A+ HBJL-5	Intermediate	16.53 ± 0.211	0.415	1.27
A + HBJL-3	Intermediate	17.31 ± 0.143	0.235	0.38
A+7-2-99-5	Susceptible	13.14 ± 0.040	4.996	0.31
A + Chandler #A1-11	Tolerant	21.93 ± 0.569	0.008	2.59
A+4-4-99-6	Intermediate	15.15 ± 0.101	1.139	0.67
sour orange	Susceptible	11.55 ± 0.050	16.07	0.56
A+5-1-99-3*	Resistant	20.71±0.216	0.019	1.04
2247-OP-A2*	Resistant	21.67±1.318	0.010	1.38
HBJL-3 R10T20*	Resistant	19.97±0.212	0.033	1.06
HBJL-5*	Resistant	20.14±0.665	0.029	3.30
8-1-99-2B*	Resistant	18.63 ±0.136	0.089	0.73

* Top-worked field samples collected 16 months after top-working graft

[a] S.D.=Standard divion [b]CV= Coefficient of Variance

CHAPTER 6
CONCLUSIONS

Citrus is the most extensively grown fruit crop worldwide. Citrus tristeza is the most economically important viral pathogen in citrus. The primary diseases caused by citrus tristeza virus (CTV) are quick decline (QD) that kills commercial trees grafted to sour orange rootstock, and stem-pitting (SP), a non-lethal disease that reduces the fruit quality and productivity of sweet oranges and grapefruit. The introduction of the primary CTV vector, the brown citrus aphid, into Florida in 1995 resulted in the rapid spread of CTV-QD isolates. This immediately jeopardized millions of commercial citrus trees planted on sour orange rootstock in Florida, since it is highly susceptible to citrus tristeza quick decline disease. This eventually eliminated the use of sour orange (*Citrus aurantium* L.) rootstock, with a few minor exceptions. Currently there is no rootstock that provides an adequate replacement for sour orange. The primary problem is that the top rootstocks in Florida are trifoliate hybrids, and in general they are not adapted to high pH, calcareous soils.

A primary objective of citrus improvement programs has been the development of new rootstocks that perform similarly to sour orange, but are of course resistant to CTV-induced QD. Since sour orange has been shown to be a pummelo-mandarin hybrid, researchers have produced many diploid and tetraploid pummelo-mandarin hybrids with a goal of identifying QD resistant hybrids that can otherwise perform like sour orange. If successful, not only would citrus growers have an answer to QD, but also possibly to citrus blight, since pummelo-mandarin hybrids generally show a high tolerance to this malady. Citrus blight remains a serious problem in Florida and Brazil, where it kills an estimated one million and 10 million trees, respectively, on an annual basis. Screening new rootstock germplasms in the greenhouse has been hampered by a CTV-induced seedling yellows (SY) disease of no commercial importance. Inoculated trees in

greenhouse screening assays that show disease symptoms are generally considered susceptible to QD; however, such symptoms could be due only to SY, and it has not been proven whether or not there is a high correlation between QD and SY. Trees showing seedling yellows symptoms can often recover over time or following field planting. Thus, relying only on greenhouse screens could result in the discarding of QD resistant hybrids. A major objective of the present study was to determine if a field-screen that relied on the top-working of new candidate rootstock hybrids onto established CTV-infected field trees could bypass the confounding of SY encountered in greenhouse screening for CTV-induced QD.

A new field assay to assess the reaction of new hybrid rootstock candidates to CTV was developed by applying a top-working technique, using the hanging bud method. Seventy-two citrus genotypes, including pre-selected pummelo parents, and pummelo/mandarin hybrids including somatic hybrids, tetrazygs from controlled tetraploid crosses, diploid hybrids and open pollinated tetraploids, were included in this study. All selections were made based on advice from Dr. Grosser, based on availability, previous observations, and results from other screens for soil adaptation, insect and disease resistance. Several allotetraploid combinations of selected pummelo seedlings with Changsha and Amblycarpa mandarins; ‘Murcott’ and ‘W. Murcott’ tangors, and ‘Page’ tangelo, were developed using somatic hybridization. Pummelo zygotic seedlings were used as leaf parent in somatic hybridization experiments and were selected from a greenhouse screening for soil adaptation and Phytophthora resistance. Some of these pummelo selections also showed resistance/tolerance to CTV-induced quick decline after grafting with Valencia scion containing T36 CTV after 2 years in the field. The mandarin-type parents were chosen based on their performance in the protoplast system and general rootstock performance with wide soil adaptation. Therefore, these somatic hybrids are considered as good candidates to

replace sour orange rootstock. All of these new rootstock candidates, mostly developed via protoplast fusion were top-worked along with sour orange as a control onto 15 year old 'Hamlin' trees infected with three different strains of CTV common to Florida (T30, T36 and VT).

Although, some limitations were encountered in this experiment, including the bud availability of some of the test hybrids and the limited number of 'Hamlin' interstock trees available for top-working, the hanging bud method provided a highly efficient method (80-90% success) for top-working. In the present study, different scaffold branches on individual trees were used as replications for the individual hybrids. If available, it would be beneficial in future work if at least three replicate trees were used for each candidate rootstock selection. Careful management of irrigation, fertilization and pesticides is a necessity. Eighteen months after successful top-working, the shoot growth of the new hybrid shoots were measured. Overall, there were significant differences in the shoot growth among the tested selections and the CTV-susceptible sour orange control shoots, which were consistently stunted. The highest three shoot growth obtained from the parental pummelo seedlings were from seedlings 5-1-99-2, HBJL-3 and 8-1-99-2B. For the somatic hybrids, the highest three shoots growths were from Amb+ HBJL-3, Amb+ HBJL-1, Amb+HBJL-2B.

Examination of the top-worked test stems for stem pitting symptoms showed no stem pitting evidence, even after observation under the light microscope. In general, no seedling yellows-type symptoms were observed in any of the top-worked scions, even from the MCA13 positive materials, including the grafted sour orange. This result indicates that top-working overcame the seedling yellows (SY) effect that has previously caused problems with our greenhouse QD resistance assays. The only other clearly observed CTV symptom was the stunted growth in all top-worked sour orange. This method was proven to bypass the SY effect

that confounds CTV screening in the greenhouse. To support this foundation, a SY companion experiment in the greenhouse was conducted by inoculating representatives of the top-worked rootstock candidates with the T36, CTV quick decline isolate from Florida. After the successful T-budding, the buds were unwrapped and left to push. The plants were trimmed when the new buds pushed strong new flushes that were then monitored for the SY symptoms. The data was in contrast with data from the field top-working experiment. Some tested somatic hybrid rootstock candidates (A + 7-2-99-5 and A + Chandler #69) showed strong SY symptoms in the greenhouse study, and a high susceptibility to CTV in the top-working field study. However, several other tested somatic hybrid rootstocks (A + Chandler #A1-11, A+ HBJL-5, A+ 4-4-99-6 and Page+HBJL-3) showed a very strong SY reaction in the greenhouse study, but none of these showed any SY reaction or any disease symptoms in the field. In another experiment, the somatic hybrids A+ 5-1-99-3 and A + HBJL-5 showed a strong SY reaction in the greenhouse, but again in the current field study showed no symptoms whatsoever (J.W. Grosser, personal communication). Thus, there is clearly no strong correlation between the SY and QD diseases, and the top-working approach provides a more reliable screen for CTV-QD resistance in the new rootstock candidates. Another advantage of the top-working approach is to speed flowering and fruiting, allowing for a more rapid assessment of the hybrids for amenability to seed propagation, with the final result being a seed tree.

The virus infection was detected in the grafted materials by serological techniques including tissue blot immunoassay (TBIA), double antibody sandwich enzyme –linked immunosorbent assay (DAS-ELISA) and western analysis. MCA 13 monoclonal antibody provides a tool to screen for severe CTV infection, especially in the Florida budwood registration program to prevent propagation of budwood containing potentially damaging isolates. The

relatively quick tissue print method using the monoclonal antibody, MCA13 was determined to be a good method for high throughput and to validate traditional ELISA. Seventeen of the test genotypes were MCA13 negative in this study. The test hybrid candidates that showed negative results by MCA13 monoclonal antibody were pummelo seedlings: 5-1-99-2, 7-2-99-1, 8-1-99-2B, 8-1-99-4B set 2, Chandler #A1-11, HBJ-L3 R10T20 and HBJL-5; and somatic hybrids: Amb +4-3-99-2, Amb +5-1-99-3, Amb +Chandler, Amb + HBJL -1, Amb + HBJL -2B, Murcott + HBJL -1 and W. Murcott + HBJL -7. The tetrazygy 2247 x 6073-00-6 (GREEN 6), the diploid hybrid Volk x P, and the open pollinated tetraploid 2247-OP-A2 were also MCA-13 negative. These results suggest that these rootstock candidates should be resistant to CTV-induced QD. It was unfortunate that efforts to top-work pummelo seedlings HBJL-1 and HBJL-2B were unsuccessful, since somatic hybrids made with these parents were resistant (two somatic hybrids made with HBJL-1). Original 5-year old trees of pummelo seedlings HBJL-1, HBJL-2B and 5-1-99-2 exist in a grove adjacent to the top-worked trees, so we plan to run ELISA on these trees to determine if they have become infected by CTV. The tetrazyg Green 6 has Carrizo citrange parentage, and could possibly contain the trifoliate orange CTV resistance gene. The open-pollinated tetraploid 2247-OP-A2 came from the Nova mandarin +HBP zygotic pummelo somatic hybrid mother plant, and this test hybrid also performed extremely well in a Diaprepes/Phytopthhora screen (J.W. Grosser, personal communication). Although the pollen parent is unknown, the seed tree has a mandarin-type appearance with narrow leaves and petioles, suggesting some additional mandarin parentage. The data also revealed various degrees of CTV resistance/tolerance in the remaining tested genotypes. The rootstock candidates were divided into 5 categories based on the MCA13 –ELISA, resistant; highly tolerant, intermediate, slightly tolerant and susceptible.

The interstock ‘Hamlin’ field trees contain mixture of different genotypes and one of this study objectives was to determine the different CTV genotypes moved and replicate in the newly top-worked scions (test rootstock candidates and the corresponding sour orange control). Molecular techniques including multiple molecular markers (MMM) analysis, and heteroduplex mobility assay (HMA) coupled with the DNA sequencing of the amplified region were done to determine the population diversity and the differential movement of CTV genotypes from the interstock into the newly grafted test rootstock materials. The results of both MMM and HMA molecular techniques showed that a range of different genotype combinations moved to the tested materials and therefore, the new rootstock candidates were classified into four different groups based on the number of the detected genotypes (Table 5-3 and Figures5-3, 5-4, 5-5 and 5-6).

The population diversity of CTV may change due to several factors, such as grafting with a different citrus genotype. In some cases this can lead to the formation of new CTV genotypes (Ayllon et al., 1999b; Ayllon et al., 1999a). The molecular characterization of the CTV field isolates in the top-worked hybrid rootstock candidates using MMM and HMA conducted in this study also showed significant changes in the population structure of CTV genotypes moving from the ‘Hamlin’ sweet orange interstock (proved to be mixture of T30, T36 and VT) into the newly grafted top-worked hybrid material. The changes in the CTV genotype composition also suggest differential selection properties of these different rootstocks candidates.

As mentioned above, based on the MMM and the sequence analysis in the HMA, the hybrid rootstock candidates were divided into 4 groups (I-IV) in Table (5-3) based on the different combination of CTV genotypes that were observed, whereas group V was composed of hybrid rootstock candidates that didn’t show any amplification with the MMM, showing no CTV

replication from any of the viral genotypes, and indicating broad resistance to CTV. The resistant hybrids indicated by the MMM analysis were somatic hybrids A+4-3-99-2 and A+5-1-99-3, open-pollinated tetraploid 2247-OP-A2, and pummelo seedlings HBJL-3R10T20, HBJL-5, and 8-1-99-2B. The pummelo seedlings can be used as a leaf parent to develop more somatic hybrids via protoplast fusion system or can be crossed with other interesting varieties to produce diploids. The somatic hybrids: A+4-3-99-2, A+5-1-99-3 can be tested for use as direct roostocks (propagated by seeds or by rooted cuttings depending on amenability to seed propagation). These CTV resistant somatic hybrids may also have value as tetraploid breeding parents. The open pollinated tetraploid 224-OP-A2 could also be used as a breeding parent.

The HMA and the sequence information generated in this study provide very valuable information about the CTV population diversity. Furthermore, this study suggested the distant relationship of the VT isolate found in the field under this study and the VT isolate known as the stem pitting (SP) isolate from Israel. This raised the need for determining the complete sequence of the Florida field VT isolate as needed for comparison with the complete genome sequence of the VT isolate from Israel. It is possible that the common Florida field VT isolate may be a completely different isolate than the VT isolate from Israel, since no stem pitting symptoms developed in any of the VT infected materials in the current study. This information could be useful regarding current and future regulatory considerations of SP isolates.

None of the used serological or molecular methods provides a reliable estimation of the CTV accumulation, therefore quantification of CTV titer by using reverse transcriptase quantitative real time PCR (qRT-PCR) is important in evaluating the candidate rootstocks for CTV resistance. It is highly sensitive and the most accurate technique to quantify and compare virus infection such as CTV, and to determine the level of resistance/tolerance among the tested

rootstocks. Real Time PCR is rapid, reliable, quantitative, and a very accurate method. This technique will allow us to not only detect but also quantify and differentiate the different CTV genotypes in field samples in one single reaction if strain specific primers are used.

Based on qRT-PCR results, the test hybrid rootstock candidates including A+HBJL-1, A+4-3-99-2, A+ Chandler #A1, A+5-1-99-3 and 2247-OP-A2, showed zero to very low CTV titer, good growth and no disease symptoms, suggesting resistance to CTV replication and QD disease. Many hybrids showed intermediate levels of CTV titer, but good growth and no disease symptoms in the top-working study. The results obtained from real-time PCR for CTV quantification were very helpful in screening the rootstock candidates. The current study has identified a large pool of apparently QD resistant hybrids that have potential to replace sour orange rootstock, if they show adequate nursery and horticultural performance in ongoing studies. These candidate rootstocks are expected to fruit during the next year or two. As they fruit, seed will be extracted to determine seediness. Microsatellite analysis will be performed on germinated seedlings to determine if they are of zygotic or nucellar origin, as standard nursery propagation of rootstocks relies on nucellar seedlings for rootstock uniformity. Alternatively, good rootstock candidates producing predominantly zygotic seedlings could be propagated using a rooted cutting method. As mentioned, CTV-resistant pummelo seedlings producing zygotic seedlings could be used for additional breeding at the diploid level or as fusion parents in somatic hybridization experiments. CTV-resistant tetraploid hybrids producing zygotic seedlings should have value in the tetraploid rootstock breeding program.

There are a large number of traits needed to be packaged in order to develop an improved citrus rootstock. Although many of the tested rootstocks allowed for CTV replication, many exhibited no apparent disease symptoms, suggesting some level of tolerance to CTV-induced

QD. Several years of field testing will be required to determine if yield and fruit quality will be adequate for any of these rootstocks to replace sour orange. Many of the top-worked rootstock selections are growing well and are expected to become fruit bearing seed trees in the near future. Overall, this study has significantly advanced the efforts of the CREC variety improvement team regarding the development of a replacement for sour orange rootstock that will possess the good traits of sour orange but with resistance to CTV-induced QD. It is recommended that this approach be continued for screening additional promising diploid and tetraploid pummelo/mandarin hybrids being created by the CREC breeding team. Use of a professional top-working team could improve top-working efficiency. It should also be realized that regulatory considerations may hamper future use of this approach, as it is illegal to move CTV-infected budwood from one field location to another. Thus, new hybrids to be tested must come directly from certified production greenhouses.

APPENDIX A
ELISA BUFFERS AND STARCH SOLUTIONS

Table A-1. ELISA buffers

Coating buffer (CB)			
	1 L	2 L	4 L
N_2CO_3	1.59 g	3.18 g	6.36 g
$NaHC_3$	2.93 g	5.86 g	11.72 g
NaN_3	0.20 g	0.40 g	0.80 g
pH = 9.6			

Phosphate Buffer Saline (PBS)*			
NaCl	8.00 g	16.00 g	32.00 g
KH_2PO_4	0.20 g	0.40 g	0.80 g
Na_2HPO_4-$12H_2O$ (anhydrous)	2.90 g (1.15 g)	5.80 g (2.30 g)	11.60 g (4.60 g)
KCl	0.20 g	0.40 g	0.80 g
pH = 7.2 to 7.4			

Conjugate Buffer: (Prepared Fresh)			
PBST	1 L	2 L	4 L
BSA	2.00 g	4.00 g	8.00 g
pH = 7.4			

Substrate Buffer (SB): (Prepared Fresh)			
Diethanolamine	97 mL	194 mL	388 mL
pH = 9.8 by HCl			

Reaction Stopping Solution			
NaOH	120 g	240 g	480 g

*Tween-Phosphate Buffer Saline (TPBS) (Washing Buffer): 1 L PBS + 0.5 ml Tween-2
Extraction Buffer (EB): 1 L PBST

Table A-2. Starch determination solutions

Reagent A	1 L	Reagent B	1 L
Potassium Sodium Tartrate	12 g	Ammonium Molybdate	50 g
Na_2CO_3 Anhydrous	24 g	H_2SO_4 (96%)	42 mL
$CuSO_4.5H_2O$	4 g	Disodium-hydrogen Arsenate Heptahydrate	6 g
$NaHCO_3$	16 g		
Na_2SO_4	180 g		

APPENDIX B
WESTERN BLOT ANALYSIS

Table B-1. Western blot analysis buffers and solutions.

Tris Buffered Saline (TBS)*				
	1 L	4 L	8 L	10 L
Tris base	12.11 g	48.44 g	96.88 g	121.1 g
NaCl	8.775 g	35.1 g	70.2 g	87.75 g
pH = 7.9				
Autoclave				

5 X Transfer Buffer			
	1 L	2 L	Final for 1X
Tris base	15.1 g	30.2 g	24.9 mM
Glycine	72.0 g	144.0 g	191.8 mM

5 X Running Buffer	
	1 L
Glycine	72 g
Tris base	15 g
10% SDS	50 mL

Loading Dye			
	2X 1 mL	4X 1 mL	Final
Tris-HCl pH 6.8	125 µL	250 µL	62.5 mM
Glycerol	200 µL	400 µL	10%
SDS	200 µL of 20%	20 mg	2%
5% β-ME	100 µL	200 µL	0.5%
Bromophenol blue	2 mg	4 mg	0.1%
H_2O	To 1 mL	To 1 mL	

*Tween-Tris Buffered Saline (TTBS): 1 L TBS + 1 ml Tween-20

APPENDIX C
PCR REACTION MIX AND PROGRAM

PCR reaction mixture

Reagents	Volume
GoTaq® Green Master Mix 2X	12.5 µL
5 µM F primer	1.5 µL
5 µM R primer	1.5 µL
DNA template (100 ng/µL)	2.5 µL
Nuclease-Free Water	7.0 µL
Total	25.0 µL

PCR program

Step 1	2 minute at 94º C	Denaturation
Step 2	30 second at 94º C	Denaturation
Step 3	30 second at 56º C	Annealing
Step 4	45seconds at 72º C	Elongation
Step 5	Repeat steps 2-4	30 times
Step 6	10 minute at 72º C	Elongation
Step 7	4º C forever	
Step 8	End	

APPENDIX D
QUANTITATIVE REAL TIME-PCR

Table D-1. Primers pairs used for quantitative real-time PCR assay.

Name	Orientation	Sequence (5′-3′)	Length	Position
Forward primer		TGCCGAGTCTTCTTTCA	16	69
Reverse primer		TGTTCAAAGCAGCGTTC	16	172

Table D-2. Real-time PCR reaction**.**

Number of reactions	1 X (µL)	50X
SYBR GREEN PCR Master Mix (2X)	12.5	625
Multiscribe (50u/ul)	0.125	6.25
RNase inhibitor (20U/Ul)	0.5	25
F primer (5 mM)	1.5	75
R primer (5 mM)	1.5	75
Free Nuclease Water	7.875	318.75
RNA	1	125
Total	25	1250

LIST OF REFERENCES

Agranovsky, A.A., Lesemann, D.E., Maiss, E., Hull, R., and Atabekov, J.G. (1995). 'Rattlesnake' structure of a filamentous plant RNA virus built of two capsid proteins. Proceedings of the National Academy of Sciences-USA **92,** 2470-2473.

Al-Senan, A., Bonsi, C.K., and Basiouny, F.M. (1997). Indexing of *citrus tristeza virus* using serological and biological tests. Proceeding of Florida State Horticultural Society **110,** 77-79.

Albiach-Marti, M., Grosser, J.W., Hilf, M.E., Gowda, S.M., M. , Satyanarayana, T., Garnsey, S.M., and Dawson, W.O. (1999). *Citrus tristeza virus* (CTV) resistant plants are not immune at the cellular level. In The18th Annual Meeting of the American Society for Virology (University of Massachusetts, Amherst, Mass), pp. 192.

Albiach-Marti, M.R., Guerri, J., de Mendoza, A.H., Laigret, F., Ballester-Olmos, J.F., and Moreno, P. (2000). Aphid transmission alters the genomic and defective RNA populations of *citrus tristeza virus* isolates. Phytopathology **90,** 134-138.

Ananthakrishnan, G., Calovic, M., Serrano, P., and Grosser, J.W. (2006). Production of additional allotetraploid somatic hybrids combining mandarins and sweet orange with pre-selected pummelos as potential candidates to replace sour orange rootstock. In Vitro Cellular & Developmental Biology-Plant **42,** 367-371.

Annual Report. (2003). Bureau of Citrus Budwood Registration (Winter Haven, Florida).

Annual Report. (2007). Bureau of Citrus Budwood Registration (Winter Haven, Florida).

Audy, P., Palukaitis, P., Slack, S.A., and Zaitlin, M. (1994). Replicase-mediated resistance to potato virus Y in transgenic tobacco plants. Molecular Plant-Microbe Interactions **7,** 15-22.

Ayllon, M.A., Rubio, L., Moya, A., Guerri, J., and Moreno, P. (1999a). The haplotype distribution of two genes of *citrus tristeza virus i*s altered after host change or aphid transmission. Virology **255,** 32-39.

Ayllon, M.A., Lopez, C., Navas-Castillo, J., Garnsey, S.M., Guerri, J., Flores, R., and Moreno, P. (2001). Polymorphism of the 5' terminal region of *citrus tristeza virus* (CTV) RNA: incidence of three sequence types in isolates of different origin and pathogenicity. Archives of Virology **146,** 27-40.

Ayllon, M.A., Lopez, C., Navas-Castillo, J., Mawassi, M., Dawson, W.O., Guerri, J., Flores, R., and Moreno, P. (1999b). New defective RNAs from *citrus tristeza virus*: evidence for a replicase-driven template switching mechanism in their generation. Journal of General Virology **80,** 817-821.

Bar-Joseph, M., and Lee, R.F. (1989). *Citrus tristeza virus*. In Description of Plant Viruses (Kew, Surrey, UK: Commonwealth Mycological Institute/Association of Applied Biology.

Bar-Joseph, M., Loebenstein, G., and Cohen, J. (1972). Partial purification of virus-like particles associated with *citrus tristeza virus*. Phytopathology **60,** 75-78.

Bar-Joseph, M., Raccha, B., and Loebenstein, G. (1977). Evaluation of main variables that affect *citrus tristeza virus* transmission by aphids. In Proceedings of the International Society of Citriculture, pp. 958-961.

Bar-Joseph, M., Garnsey, S.M., and Gonsalves, D. (1979a). The closteroviruses: a distinct group of elongated plant viruses. Advances in Virus Research **25,** 93-168.

Bar-Joseph, M., Marcus, R., and Lee, R.F. (1989). The continuous challenge of *citrus tristeza virus* control. Annual Review of Phytopathology **27,** 291-316.

Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F., and Loebenstein, G. (1979b). The use of enzym-linked immunosorbent assay for detection of citrus tristeza. Phytopathology **69,** 190-194.

Bar-Joseph, M., Che, X., Mawassi, M., Gowda, S., Satyanarayana, T., Ayllon, M.A., Albiach, M., Garnsey, S.M., and Dawson, W.O. (2002). The continuous challenge of *citrus tristeza virus* molecular reseach. In Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 1-7.

Batuman, O., Mawassi, M., and Bar-Joseph, M. (2006). Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3 ' UTR provide resistance to *citrus tristeza virus* sequences in Nicotiana benthamiana but not in citrus. Virus Genes **33,** 319-327.

Bauer, M., Castle, W.S., Boman, B.J., and Obreza, T.A. (2005). Economic longevity of citrus trees on swingle citromelo rootstock and their suitbility for soils in the Indian River region. Proceeding of Florida State Horticultural Society **118,** 24-27.

Beachy, R.N. (1994). Mechanisms and applications of pathogen-derived resistance in transgenic plants. Current Opinion in Biotechnology **8,** 215-220.

Berry, S., and C., R.M.E. (2001). Differentiation of cassava-infecting begomoviruses using heteroduplex mobility assays. Journal of Virological Methods **92,** 151-163.

Biswas, K.K., Manjunath, K.L., Marais, L.J., and Lee, R.F. (2004). Single aphids transmit multiple genotypes of *citrus tristeza virus*, but often with changed population dynamics. Phytopathology **94:S8**.

Blackman, R.L., and Eastop, V.F. (1984). Aphids on world crops. (John Wiley & Sons Chichester, United Kingdom).

Blazek, J. (2002). Prediction of profitability of topworking in older apple orchards under contemporary economic conditions of the Czech Republic. Horticultural Science **29,** 85-91.

Boonham, N., Smith, P., Walsh, K., Tame, J., Morris, J., Spence, N., Bennison, J., and Barker, I. (2002). The detection of tomato spotted wilt virus (TSWV) in individual thrips using real-time fluorescent RT-PCR (TaqMan). Journal of Virological Methods **101,** 37-48.

Bove, J.M., and Ayres, A.J. (2007). Etiology of three recent diseases of citrus in Sao Paulo State: Sudden death, variegated chlorosis and huanglongbing. Lubmb Life **59,** 346-354.

Bowman, K.D. (2000). New hybrid citrus developed by U.S. Department of Agriculture. In 9th International Citrus Congress (Orlando, Florida), pp. 51.

Bowman, K.D. (2007). Raising Rootstocks : USDA researchers are working hard to develop new disease-resistanct rootstocks. Florida Grower.

Bowman, K.D., and Roman, M.F. (1999). New rootstocks for orange and mandarin. Proceeding of Caribbean Food Crops Society **35,** 119-130.

Bowman, K.D., and Garnsey, S.M. (2001). A comparison of five sour orange rootstocks and their response to *citrus tristeza virus*. Proceeding of Florida State Horticultural Society **114,** 73-77.

Bowman, K.D., and Rouse, R.E. (2006). US-812 citrus rootstock. HortScience **41,** 832-836.

Bowman, K.D., Albano, J.P., and Graham, J.H. (2002). Greenhouse testing of rootstocks for resistance to *Phytophthora* species in flatwoods soil. Proceeding of Florida State Horticultural Society **115,** 10-13.

Bradford, M.M. (1979). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72,** 248-254.

Bramlett, D.L., and Burris, L.C. (1995). Topworking young scions into reproductively-mature Loblolly pine. In 23rd Southern Forestry Tree Improvement Conference (Event Asheville, NC), pp. 234-241.

Brlansky, R.H. (1987). Inclusion bodies produced in citrus by *citrus tristeza virus*. Phytophylactica **19,** 211-213.

Brlansky, R.H., and Lee, R.F. (1990). Numbers of inclusion bodies produced by mild and severe strains of *citrus tristeza virus i*n seven citrus hosts. Plant Disease **74,** 297-299.

Brlansky, R.H., Lee, R.F., and Garnsey, S.M. (1988). In situ immunofluorescence for the detection of citrus tristeza inclusion bodies. Plant Disease **72,** 1039-1041.

Brlansky, R.H., Garnsey, S.M., Lee, R.F., and Purcifull, D.E. (1984). Application of *citrus tristeza virus* antisera for use in labeled antibody, immuno-electron microscopical, sodium dodecyl sulphate immunodiffusion tests. In Proceedings of the 9th Conference of the International Organization of Citrus Virologists (IOCV), S.M. Garnsey, L.V. Timmer, and J.A. Dodds, eds (Riverside, CA), pp. 342-345.

Brlansky, R.H., Howd, D.S., Broadbent, P., and Damsteegt, V.D. (2002). Histology of sweet orange stem pitting caused by Australian isolate of *citrus tristeza virus*. Plant Disease **86,** 1169-1174.

Brlansky, R.H., Damsteegt, V.D., Roy, A., and Howd, D.S. (2003). Molecular analyses of *citrus tristeza virus* subisolates seperated by aphid transmissions. Plant Disease **87,** 397-401.

Brlansky, R.H., Hilf, M.E., Sieburth, P.J., Dawson, W.O., Roberts, P.D., and Timmer, L.W. (2008). 2008 Florida citrus pest management guide: tristeza1 (Gainesville, FL: University of Florida, IFAS).

Broadbent, P., Brlansky, R.H., and Indsto, J. (1996). Biological characterization of Australian isolates of *citrus tristeza virus* and separation of subisolates by single aphid transmissions. Plant Disease **80,** 329-333.

Broadbent, P., Dephoff, C.M., Franks, N., Gillings, M., and Industo, J. (1995). Preimmunization of grapefruit with a mild protective isolate of Citrus tristeza in Australia. In Proceedings of the 3rd International Workshop on Citrus tristeza virus and Brown citrus aphid in Central America and the Caribbean, R.L. Lee, M. Rocha-Peña, C.L. Niblett, F. Ochoa, S.M. Garnsey, R.K. Yokomi, and R. Lastra, eds (FAO-USDA-OICD-University of Florida, Lake Alfred, Florida), pp. 163-168.

Brown, M.G., and Spreen, T.H. (2000). An economic assessment of the impact of the *citrus tristeza virus* on the Florida grapefruit industry. Proceeding of Florida State Horticultural Society **113,** 79-82.

Brunt, A., Crabtree, K., and Gibbs, A. (1990). Viruses of tropical plants. (Wallingford, Oxon, UK: CAB International).

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L., and Zurcher, E.J. (1996). Plant viruses online: descriptions and lists from the VIDE database.Version: 20th August 1996.

Button, J. (1975). The topworking of citrus trees. Citrus and Subtropical Fruit Journal**,** 5-8.

Cai, S.P., Eng, B., Kan, Y.W., and Chui, D.H.K. (1991). A rapid and simple electrophoretic method for detection of mutations involving small insertions and deletions: applications to β-thalassemia. Human Genetics **87,** 720-728.

Cambra, M., Serra, J., Vilalba, D., and Moreno, P. (1988). Present situation of *citrus tristeza* virus in the Valencian community. In Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV), L.V. Timmer, S.M. Garnsey, and L. Nararro, eds (Riverside, CA), pp. 1-7.

Cambra, M., Camarasa, E., Gorris, M.T., Garnsey, S.M., and Carbonell, E. (1991). Comparison of different immunosorbent assays for *citrus tristeza virus* (CTV). In Proceedings of the 11th Conference of the International Organization of Citrus Virologists (IOCV), R.H. Brlansky, R.F. Lee, and L.V. Timmer, eds (Riverside, CA), pp. 38-45.

Cambra, M., Gorris, M.T., Olmos, A., Martinez, M.C., Roman, M.P., Bertolini, E., Lopez, A., and Carbonell, E.A. (2002). European diagnostic protocols (DIAGPRO) for *citrus tristeza virus* in adult trees. In Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 69-78.

Cambra, M., Olmos, A., Gorris, M.T., Marroquın, C., Esteban, O., Garnsey, S.M., Llauger, R., Batista, L., Pe˜na, I., and Hermoso de Mendoza, A. (2000). Detection of c*itrus tristeza virus* by print capture and squash capture-PCR in plant tissue and single aphids. In Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV), J.V. da Graca, R.F. Lee, and R.K. Yokomi, eds (Riverside, CA), pp. 42-49.

Castle, B., and Stover, E. (2001). Update on use of Swingle citrumelo rootstock. In University of Florida, Extention: Fact Sheet (Gainesville, FL: University of Florida).

Castle, W.S. (1987). Citrus rootstocks. In Rootstocks for Fruit Crops, R.C. Rom and R. Carlson, eds (Wiley J and Sons, New York.

Castle, W.S., and Tucker, D.P.A. (1998). Florida citrus rootstocks selection guide. In University of Florida Corporation Extension (Gainesville, FL: University of Florida).

Castle, W.S., Tucker, D.P.H., Krezdorn, A.H., and Youtsey, C.O. (1993). Rootstocks for Florida citrus. (Gainesville, FL: University of Florida, IFAS).

Castle, W.S., Bowman, K.D., Grham, J.H., and Tucker, D.P.H. (2006). Forida citrus rootstock selection guide U.o. Florida, ed (Lake Alfred, FL: University of Florida, IFAS, CREC).

Cevik, B. (1995). Molecular differentiation of strains of *citrus tristeza virus* using the coat protein gene sequences. In Department of Plant Pathology (Gainesville: University of Florida.), pp. 112.

Cevik, B. (2001). Characterization of the RNA-dependent RNA polymerase gene of citrus tristeza closterovirus (Gainesville, FL: University of Florida).

Cevik, B., Pappu, S.S., Pappu, H.R., Benscher, D., Irey, M., Lee, R.F., and Niblett, C.L. (1996a). Application of bi-directional PCR to *citrus tristeza virus*: detection and strain differentiation. In Proceedings of the 13th Conference of the International Organization of Citrus Virologists (IOCV), J.V. da Grac¸a, P. Moreno, and R.K. Yokomi, eds (Riverside, CA), pp. 17-24.

Cevik, B., Pappu, S.S., Pappu, H.R., Tight, D., Benscher, D., Futch, S.H., Rucks, P., Lee, R.F., and Niblett, C.L. (1996b). Molecular cloning and sequencing of coat protein genes of *citrus tristeza virus* isolates from Meyer lemon and Homely tangor trees in Florida. In Proceedings of the 13th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 47–53.

Che, X., Piestun, D., Mawassi, M., Yang, G., Satyanarayana, T., Gowda, S., Dawson, W.O., and Bar-Joseph, M. (2001). 5' Coterminal subgenomic RNAs in *citrus tristeza virus* infected cells. Virology **283,** 374-381.

Chen, C., Grosser, J.W., Calovic´, M., Serrano, P., Pasquali, G., Gmitter, J., and Gmitter, F.G. (2008). Verification of Mandarin and Pummelo Somatic Hybrids by Expressed Sequence Tag–Simple Sequence Repeat Marker Analysis. Journal of the American Society for Horticultural Science **133,** 794–800.

Clark, M.F., Lister, R.M., and Bar-Joseph, M. (1988). ELISA techniques. In Methods for Plant Molecular Biology, A.a.W. Weissbach, H, ed (San Diago, CA), pp. 507-530.

Costa, A.S., and Grant, T.J. (1951). Studies on the transmission of the tristeza virus by the vector Aphid citricidus. Phytopathology **41,** 105-122.

d'Urso, F., Ayllon, M.A., Rubio, L., Sambade, A., de Mendoza, A.H., Guerri, J., Moreno, P., and Moreno, P. (2000). Contribution of uneven distribution of genomic RNA variants of *citrus tristeza virus* (CTV) within the plant to changes in the viral population following aphid transmission. Plant Pathology **49,** 288-294.

Da Graca, J.V., Marais, L.J., and Von Broembsen, L.A. (1984). Severe tristeza stem pitting decline of young grape fruits in South Africa. In Proceedings of the 9th Conference of the International Organization of Citrus Virologists (IOCV), S.M. Garnsey, L.V. Timmer, and J.A. Dodds, eds (Riverside, CA), pp. 62-65.

Davies, F.S., and Albrigo, L.G. (1994). Citrus. (Wallingford: CAB International).

Davino, S., Davino, M., Sambade, A., Guardo, M., and Caruso, A. (2003). The first *citrus tristeza virus* outbreak found in a relevant citrus producing area of Sicily, Italy. Plant Disease **87,** 314.

Delwart, L.E., Shpaer, G.E., Louwagie, J., McCutchan, E.F., Grez, M., Rubsamen-Waigmann, H., and Millins, J.I. (1993). Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 env genes. Science **262,** 1257-1261.

Deng, Z., S., H., S., X., and Gmitter, F.G. (1996). Development and characterization of scar markers linked to the *citrus tristeza virus resistance* gene from *Poncirus trifoliata* Genome **40,** 697-704.

Deng, Z., Huang, S., Ling, P., Chen, C., Yu, C., Weber, C.A., Moore, G.A., and Gmitter, F.G. (2000). Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. Theoretical and Applied Genetics **101,** 814-822.

Deng, Z., Tao, Q., Chang, Y.L., Huang, S., Ling, P., Yu, C., Chen, C., Gmitter, F.G., and Zhang, H.B. (2001a). Construction of a bacterial artificial chromosome (BAC) library for citrus and identification of BAC contigs containing resistance gene candidates. Theoretical and Applied Genetics **102,** 1177-1184.

Deng, Z., Huang, S., Ling, P., Yu, C., Tao, Q., Chen, C., Wendell, M.K., Zhang, H.B., and Gmitter, F.G. (2001b). Fine genetic mapping and BAC contig development for the *citrus tristeza virus* resistance gene locus in *Poncirus trifoliata* (Raf.). Molecular Genetics and Genomics **265,** 739-747.

Dolja, V.V., Karasev, A.V., and Koonin, E.V. (1994). Molecular biology and evaluation of closteroviruses: sophisticated build-up of large RNA genomes. Annual Review of Phytopathology **32,** 261-285.

Dominguez, A., Hermoso de Mendoza, A., Guerri, J., Cambra, M., Navarro, L., Moreno, P., and Pena, L. (2002). Pathogen derived resistance to *citrus tristeza virus* (CTV) in transgenic Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) plants expressing its p25 coat protein gene. Molecular Breeding **10,** 1-10.

Domínguez, A., Guerri, J., Cambra, M., Navarro, L., Moreno, P., and Peña, L. (2000). Efficient production of transgenic citrus plants expressing the coat protein gene of *citrus tristeza virus*. Plant Cell Reports **19,** 427-433.

Esau, K. (1960). Cytological and histological symptoms of beet yellows. Virology **10,** 73-85.

Fabre, F., Kervarrec, C., Mieuzet, L., Riault, G., Vialatte, A., and Jacquot, E. (2003). Improvement of barley yellow dwarf virus-PAV detection in single aphids using a fluorescent real-time RT-PCR. Journal of Virological Methods **110,** 51-60.

Fagoaga, C., Lopez, C., Moreno, P., Navarro, L., Flores, R., and Pena, L. (2005). Viral-like symptoms induced by the ectopic expression of the p23 gene of *citrus tristeza virus* are citrus specific and do not correlate with the pathogenicity of the virus strain. Molecular Plant-Microbe Interactions **18,** 435-445.

Fallahi, E., Moon, J., J. W., and Ross, D.R. (1989). Yield and quality of 'Redblush' grapefruit on twelve rootstocks. Journal of the American Society for Horticultural Science **114,** 187-190.

Fang, D.Q., Federici, C.T., and Roose, M.L. (1998). A high-resolution linkage map of the *citrus tristeza virus* resistance gene region in Poncirus trifoliata (L.) Raf. Genetics **150,** 883-890.

FAOSTAT. (2007). FAOSTAT Database on Agriculture: Citrus Production (Rome, Italy: Food and Agriculture Organization of the United Nations).

Fawcett, H.S., and Wallace, J.M. (1946). Evidence of the virus nature of citrus quick decline. California Citrograph **32,** 88-89.

Febres , V.J., Lee, R.F., and Moore, G.A. (2008). Transgenic resistance to Citrus tristeza virus in grapefruit. Plant Cell Rep **27,** 93–104.

Febres, V.J., Niblett, C.L., Lee, R.F., and Moore, G.A. (2003). Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with Citrus tristeza closterovirus genes. Plant Cell Reports **21,** 421-428.

Febres, V.J., Pappu, H.R., Anderson, E.J., Pappu, S.S., Lee, R.F., and Niblett, C.L. (1994). The diverged copy of the *citrus tristeza virus* coat protein is expressed in vivo. Virology **201,** 178-181.

Febres, V.J., Ashoulin, L., Mawassi, M., Frank, A., Bar-Joseph, M., Manjunath, K.L., Lee, R.F., and Niblett, C.L. (1996). The p27 protein is present at one end of *citrus tristeza virus* particles. Phytopathology **86,** 1331-1335.

Fraser, L.R. (1952). Seedling yellows, an unreported virus disease of citrus. Agricultural Gazette of New South Wales **63,** 125-131.

Freeman, W.M., Walker, S.J., and Vrana, S.J. (1999). Quantitative RT-PCR: Pitfalls and potential. Biotechniques **26,** 112-1125.

Fuchs, M., and Gonsalves, D. (1997). Environmentally safe approaches to crop disease control In Genetic Engineering (CRC Press), pp. 333-368

Fulton, R.W. (1986). Practices and precautions in the use of cross protection for plant virus disease control. Annual Review of Phytopathology **67,** 965-968.

Futch, S.H., and Brlansky, R.H. (2008). Field diagnosis of *citrus tristeza virus*1 (Gainesville, FL: University of Florida, IFAS).

Garnsey, S.M. (1990). Seedling yellows isolates of *citrus tristeza virus* in commercial citrus in Florida. Proceeding of Florida State Horticultural Society **103,** 83-87.

Garnsey, S.M., and Young, R.H. (1975). Water flow rates and starch reserves in roots from citrus trees affected by blight and tristeza. Proceeding of Florida State Horticultural Society **4,** 79-84.

Garnsey, S.M., and Lee, R.F. (1988). Tristeza. In Compendium of Citrus Diseases, J.O. Whiteside, S.M. Garnsey, and L.W. Timmer, eds (APS Press, St. Paul), pp. 48-50.

Garnsey, S.M., and Cambra, M. (1991). Enzyme-Linked immunosorbent assay (ELISA) for cirrus pathogens. In Graft-Transmissible Diseases of Citrus: Handbook for Detection and Diagnosis, C.N. Roistacher, ed, pp. 193-216. .

Garnsey, S.M., Gonsalves, D., and purcifull, D.E. (1979). Rapid diagnosis of *citrus tristeza virus* infections by sodium dodecyl sulfate immunodiffusion procedures. Phytopathology **69,** 88-95.

Garnsey, S.M., Christie, R.G., and Derrick, K.S. (1980). Detection of *citrus tristeza virus* II. Light and electron microscopy of inclusions and virus particles. In Proceedings of the 8th Conference of the International Organization of Citrus Virologists (IOCV), E.C. Calavan, S.M. Garnsey, and L.W. Timmer, eds (Riverside, CA), pp. 9-16.

Garnsey, S.M., Barrett, H.C., and Hutchison, D.J. (1987a). Identification of *citrus tristeza virus* resistance in citrus relatives and potential applications. Phytophylactica **19,** 187-191.

Garnsey, S.M., Su, H., and Tsai, M. (1997). Differential susceptibility of pummelo and Swingle citrumelo to isolates of *citrus tristeza virus*. In Proceedings of the 3rd Conference of the International Organization of Citrus Virologists (IOCV), J. Da Graca, P. Moreno, and R. Yokomi, eds (Riverside, CA), pp. 38-146.

Garnsey, S.M., Permar, T.A., Cambra, M., and Henderson, C.T. (1993). Direct tissue blot Immunoassay (DTBIA) for detection of *citrus tristeza virus* (CTV). In Proceedings of the 12th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 39-50.

Garnsey, S.M., Gumpf, D.J., Roistacher, C.N., Civerolo, E.L., Lee, R.F., Yokomi, R.K., and Bar-Joseph, M. (1987b). Toward a standard evaluation of the biologically properties of c*itrus tristeza virus*. Phytophylactica **19,** 151-157.

Ghorbel, R., Domínguez, A., Navarro, L., and Peña, L. (2000). High efficiency genetic transformation of sour orange (Citrus aurantium) and production of transgenic trees containing the coat protein gene of *citrus tristeza virus*. Tree Physiology **20,** 1183-1189.

Ghorbel, R., López, C., Fagoaga, C., Moreno, P., Navarro, L., Flores, R., and Peña, L. (2001). Transgenic citrus plants expressing the *citrus tristeza virus* p23 protein exhibit viral-like symptoms. Molecular Plant Pathology **2,** 27-36.

Gibson, U.E., Heid, C.A., and Williams, P.M. (1996). A novel method for real time quantitative RT-PCR Genome Research **6** 995-1001.

Gillings, M., Broadbent, P., Indsto, J., and Lee, R.F. (1993). Characterisation of isolates and strains of citrus tristeza closterovirus using restriction analysis of the coat protein gene amplified by the polymerase chain reaction. Journal of Virological Methods **44,** 305-317.

Ginzinger, D.G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Experimental Hematology **30,** 503-512.

Gmitter, F.G., Lee , R.F., powell, A.C., and Hu, X.L. (1992). Rootstocks similar to sour orange for Florida citrus trees. Proceeding of Florida State Horticultural Society **105,** 56-60.

Gmitter, F.G., Xiao, S.Y., Huang, S., Hu, X.L., Garnsey, S.M., and Deng, Z. (1996). A localized linkage map of the *citrus tristeza virus* resistance gene region. Theoretical and Applied Genetics **92,** 688-695.

Gonsalves, D., purcifull, D.E., and Garnsey, S.M. (1978). Purification and serology of citrus tristese virus. Phytopathology **68,** 553-559.

Gowda, S., Satyanarayana, T., Davis, C.L., Navas-Castillo, J., Albiach-Martí, M., Mawassi, M., Valkov, N., Bar-Joseph, M., Moreno, P., and Dawson, W.O. (2000). The p20 gene product of *citrus tristeza virus* accumulates in the amorphous inclusion bodies. Virology **274,** 246-254.

Grant, T.J. (1952). Evidence of tristeza, or quick decline, virus in Florida. . Proceeding of Florida State Horticultural Society **65,** 28-31.

Grant, T.J., Costa, A.S., Moreira, S., and (1951). Tristeza disease of citrus in Brazil-other citrus disease may be variation of more spectacular tristeza or quick decline. Citrus Leave **31,** 36-37.

Grosser, J.W., and Gmitter, F.G. (1990). Protoplast fusion and citrus improvement Plant Breeding Reviews **8,** 339-374.

Grosser, J.W., and Chandler, J.L. (2000). Somatic hybridization of high yield, cold hardy and disease resistanct parents for citrus rootstock improvement. Journal of Horticultural Science & Biotechnology **75,** 641-644.

Grosser, J.W., and Chandler, J.l. (2002). Somatic hybridization for citrus rootstock improvement. In Proceedings of the 7th International Citrus Seminar (Estacao Experimental De Citricultura De Bebedouro, SP, Brazil).

Grosser, J.W., Gmitter, F.G., and Castle, W.S. (1995). Production and evaluation of citrus somatic hybrid rootstocks: Progress report. In Proceeding of Florida State Horticultural Society, pp. 140-143.

Grosser, J.W., Garnsey, S.M., and Halliday, C. (1996). Assay of sour orange somatic hybrid rootstocks for quick decline disease caused by *citrus tristeza virus*. In Proceedings of the International Society of Citriculture, pp. 353-356.

Grosser, J.W., Ollitrault, P., and Olivares-Fuster, O. (2000). Somatic hybridization in citrus: An effective tool to facilitate variety improvement. In Vitro Cellular & Developmental Biology-Plant **36,** 434-449.

Grosser, J.W., Chandler, J.L., and Duncan, L.W. (2007a). Production of mandarin + pummelo somatic hybrid citrus rootstocks with potential for improved tolerence/ resistance to sting nematode. Scientia Horticulturae **113**.

Grosser, J.W., Chen, C., and Gmitter, F.G. (2007b). Microsatellite genotyping of seedlings from somatic hybrid and 'Tetrazygy' citrus rootstock candidates to determine maternal or zygotic origin. Hortscience **42,** 904.

Grosser, J.W., Louzada, E.S., Gmitter, F.G., and Chandler, J.L. (1994). Somatic hybridization of complimentary citrus rootstocks: Five new hybrids. HortScience **29,** 812-813.

Grosser, J.W., Medina-Urrutia, V., Govindarajulu, A., and Serrano, P. (2004a). Building a Replacement Sour Orange Rootstock: Somatic Hybridization of Selected Mandarin + Pummelo Combination. Journal of the American Society for Horticultural Science **129,** 530-534.

Grosser, J.W., Medina-Urrutia, V., Ananthakrishnan, G., and Serrano, P. (2004b). Building a replacement sour orange rootstock: Somatic hybridization of selected mandarin plus pummelo combinations. Journal of the American Society for Horticultural Science **129,** 530-534.

Grosser, J.W., Jiang, J., Louzada, E.S., Chandler, J.L., and GmittterJr, F.G. (1998). Somatic hybridization an integral component of citrus cultivar improvement: II. Rootstock improvement. HortScience **33,** 1060-1061.

Grosser, J.W., Graham, J.H., McCoy, C.W., Hoyte, A., Rubio, H.M., Bright, D.B., and Chandler, J.L. (2003). Development of "Tetrazyg" rootstocks tolerant of the diaprepes/phytophthora complex under greenhouse conditions. Proceeding of Florida State Horticultural Society **116,** 262-267.

Gutiérrez, E.M.A., Luth, D., and Moore, G.A. (1997). Factors affecting the *agrobacterium*-mediated transformation in citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of *citrus tristeza virus*. Plant Cell Reports **16,** 745-753.

Halbert, S.E., Gene, H., Cevic, B., Brown, L.G., Rosales, I.M., Manjunath, K.L., Pomerinke, M., Davison, D.A., Lee, R.F., and Niblett, C.L. (2004). Distribution and characterization of *citrus tristeza virus* in South Florida following establishment of Toxoptera citricida Plant Disease **88,** 935–941

Halbert, S.E.H., and Brown, L. (1996). Toxoptera citricidae (Kirkaldy), Brown citrus aphid-identification, biology and management stratigies. In Florida Deptartment of Agriculture and Consumer Servce, Entomology, pp. 6.

Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. (1996). Real time quantitative PCR Genome Research **6,** 986-994.

Hermosa de Mendoza, A., Ballester-Olmos, J.F., and Pina-Lorca, J.A. (1984). Transmission of *citrus tristeza virus* by aphids (*Homoptera, Aphididae*) in spain. In Proceedings of the 9th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 68-70.

Herron, C.M. (2003). *Citrus tristeza virus*: Characterization of texas isolates, studies on aphid transmission and pathologen-derived control strategies (Texas: Texas A&M University).

Herron, C.M., Yang, Z.N., Molina, J.J., da Graça, J.V., van Vuuren, J.P., and Mirkov, T.E. (2002). Assessments of Rio Red grapefruit scions with a *citrus tristeza virus* untranslatable coat protein transgene for resistance to the virus. Phytopathology **92,** S36.

Hilf, M.E., and Garnsey, S.M. (2000). Characterization and classification of *citrus tristeza virus* isolates by amplification of multiple molecular markers. In Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV), J.V. da Graca, R.F. Lee, and R.K. Yokomi, eds (Riverside, CA), pp. 18-27.

Hilf, M.E., and Garnsey, S.M. (2002). *Citrus tristeza virus* in Florida: A synthesis of historical and contemporary biological, serological and genetic data. In Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 13-20.

Hilf, M.E., Karasev, A.V., Pappu, H.R., Gumpf, D.J., Niblett, C.L., and Garnsey, S.M. (1995). Characterization of *citrus tristeza virus* subgenomic RNAs in infected tissue. Virology **208,** 576-582.

Hilf, M.E., Karasev, A., Maria, R., Albiach, M., Dawson, W.O., and Garnsey, S.M. (1999). Two paths of seuence divergence in the *citrus tristeza virus* complex. Phytopathology **89,** 336-342.

Hong, L.T., and Truc, N.T.N. (2003). Iodine reaction quick detection of huanglongbing disease In Proceedings of the 2003 Annual Workshop of JIRCAS Mekong Delta Project.

Huang, Z., Rundell, A.P., Guan, X., and Powell, A.C. (2004). Detection and isolate differentiation of *citrus tristeza virus* in infected field trees based on reverse transcription-polymerase chain reaction. Plant Disease **88,** 625-629.

Hubert, G.M., and Niesters. (2001). Quantitation of Viral Load Using Real –Time Amplification techniques. Methods **25,** 419-429.

Hutchson, D.J. (1985). Rootstock development screening and selection for disease tolerance and horticulture characteristics. Fruit Varieties Journal **39,** 1-25.

Iglesias, N.G., Marengo, J., Reiquelme, K., Costa, N., Plata, M.I., and Semorile, L. (2005). Characterization of the population structure of a grapefruit isolate of *citrus tristeza virus* (CTV) selected for pre-immunization assays in Argentina. In Proceedings of the 16th Conference of the International Organization of Citrus Virologists (IOCV), M.E. Hilf, N. Duran-Vila, and M.A. Rocha-Peña, eds (Riverside, CA), pp. 150-158.

Karasev, A.V. (2000). Genetic diversity, and evolution of closteroviruses. Annual Review of Phytopathology **38,** 293-324.

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O.V., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., and Lee, R.F. (1995). Complete sequence of the *citrus tristeza virus* RNA genome. Virology **208,** 511-520.

Kitajima, E.W., Silva, D.M., Oliveira, A.R., Muller, G.W., and Costa, A.S. (1994). Threadlike particles associated with tristeza disease of citrus. Nature **201,** 1011-1012.

Klotz, L.J. (1978). Fungal, bacterial and non-parasitic diseases and injuries in the seed bed nursery and orchard. In The Citrus Industry, E.C. Calavan and G.E. Carman, eds (Berkeley, CA: University of California Press.

Koonin, E.V., and Dolija, V.V. (1993). Evolution and taxonomy of positive-strand RNAviruses: implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Molecular Biology **28,** 375-430.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680-685.

Lee, R.F., and Rocha-Pena, M.A. (1992). *Citrus tristeza virus*. In Plant Disease of International Importance Disease of Fruit Crops, J. Kumar, H.S. Chaube, U.S. Singh, and A.N. Mukhopadhya, eds (NJ: Prentice Hall, Englewood Cliffs), pp. 226-249.

Lee, R.F., Baker, P.S., and Rocha-Peña, M.A. (1994). The *citrus tristeza virus* (CTV): an introduction to current priorities, with special reference to the worsening situation in Central America and the Caribbean. (Ascot, Berks. UK: International Institute of Biological Control).

Lee , R.F., Dekkers, M.G.H., and Bar-Joseph, M. (2005). Development of stable, uniform antigen controls for use in ELISA for *citrus tristeza virus*. In Proceedings of the 16th Conference of the International Organization of Citrus Virologists (IOCV), M.E. Hilf, N. Duran-Vila, and M.A. Rocha-Peña, eds (Riverside, CA), pp. 127-136.

Lee, R.F., Garnsey, S.M., Brlansky, R.H., and Goheen, A.C. (1987). A purification procedure for the enhancement of *citrus tristeza virus* yields and its application to other phloem-limited viruses. Phytopathology **77** 543-549.

Lee, R.F., Calvert, L.A., Nagel, J., and Hubbard, J.D. (1988). *Citrus tristeza virus*: characterization of coat proteins. Phytopathology **78,** 1221-1226.

Lee , R.F., Garnsey, S.M., Marais, L.J., Moll, J.N., and Youtsey, C.O. (1988). Distribution of *citrus tristeza virus* in grapefruit and sweet orange in Florida and South Africa. In Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV), L.V. Timmer, S.M. Garnsey, and L. Nararro, eds (Riverside, CA), pp. 33-38.

Lee, R.F., McConnell, P., Manjunath, K.L., Cevik, B., Nikolaeva, O.V., Dekkers, M.G.H., and Niblett, C.L. (2002). The *citrus tristeza virus* epidemic in Bog Walk valley, Jamaica. In Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA.), pp. 95-101.

Lee, R.F., Pappu, H.R., Pappu, S.S., Rocha-Pena, M.A., Febres, V.J., Manjunath, K.L., Nikolaeva, O.V., Karasev, A., Cevik, B., Akbulut, M., Bencher, D., Anderson, E.J., Price, M., Ochoa-Corona, F.M., and Niblett, C.L. (1996). Progress on strain differentiation of *citrus tristeza virus*. Phytopathology **14,** 79-87.

Lin, S.S., Hou, R.F., and Yeh, S.D. (2000). Heteroduplex Mobility and Sequence Analyses for assessment of variability of *Zucchini yellow mosaic virus*. Phytopathology **90,** 228-235.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., and Deetz, K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization,. PCR: . Methods and Applications **4,** 357-362.

Louzada, E.S., Grosser, J.W., Gmitter, F.G., Deng, X.X., Tusa, N., Nielson, B., and Chandler, J.L. (1992). Eight new somatic hybrid citrus rootstocks with potential for improved disease resistance. HortScience **27,** 1033-1036.

Lu, R., Folimonov, A., Li, W.-X., Choi, Y.G., Shintaku, M., Falk, B.W., Dawson, W.O., and Ding, S.W. (2003). *Citrus tristeza virus* genome encodes two distinct suppressors of RNA silencing. In American Society of Virology Meeting (Davis, CA).

Mackinney, G. (1941). Absorption of light by chlorophyll solutions Journal of Biological Chemistry **140,** 315-322.

Marais, L.J., and Breytenbach, J.H.J. (1996). The effect of tristeza stem pitting on the Star Ruby grapefruit industry in southern Africa. In Proceedings of the International Society of Citriculture, pp. 357-365.

Marbot, S., Salmon, M., Vandrame, M., Huwaert, A., Kummert, J., Dutrecq, O., and Lepoivre, P. (2003). Development of real-time RT-PCR assay for detection of prunus necrotic virus in fruit trees. Plant Disease **87,** 1344-1348.

Mathews, D.M., Riley, K., and Dodds, J.A. (1997). Comparison of detection methods for *citrus tristeza virus* in field trees during months of nonoptimal titer. Plant Disease **81,** 525-529.

Mawassi, M., Mietkiewska, E., Gofman, R., Yang, G., and Bar-Joseph, M. (1996). Unusual sequence relationships between two isolates of *citrus tristeza virus*. Journal of General Virology **77,** 2359-2364.

Mawassi, M., Karasev, A., Mietkiewska, E., Gafny, R., Lee, R.F., Dawson, W.O., and Bar-Joseph, M. (1995a). Defective RNA molecules associated with *citrus tristeza virus*. Virology **208,** 383-387.

Mawassi, M., Mietkiewska, E., Hilf, M.E., Ashoulin, L., Karasev, A.V., Gafny, R., Lee, R.F., Garnsey, S.M., Dawson, W.O., and Bar-Joseph, M. (1995b). Multiplespecies of defective RNAs in plants infected with *citrus tristeza virus*. Virology **214,** 264-268.

McClean, A.P.D. (1957). Tristeza virus of citrus; Evidence for absence of seed transmission. Plant Disease Reporter **41,** 821.

McClean, A.P.D. (1975). Stem pitting disease (tristeza virus) on limes in field plantings in South Africa. Phytophylactica **7,** 75-80.

McClean, A.P.D. (1950). Possible identity of three citrus diseases. . Nature **165,** 767-768.

McGovern, R.J., Lee , R.F., and Niblett, C.L. (1994). Ttristeza (Gainesville, FL: Florida Coopration Extention Servce, University of Florida), pp. 1-4.

McLaughlin, M.R., Barnett, O.W., Burrows, P.M., and Baum, R.H. (1981). Improved ELISA conditions for detection of plant viruses. Journal of Virological Methods **3,** 13-25.

Medina-Urrutia, V., F., M.K.L., Serrano, P., and Guo, W. (2004). New intergeneric somatic hybrids combining amplycarpa mandarin with six trifoliate/ trifoliate hybrid selections for lime rootstock improvement. HortScience **39,** 355-360.

Meneghini, M. (1946). Sobre a natureza e transmissibilidade do doencia "tristeza" dos citrus. Biologico **12,** 285-287.

Mestre, P.F., Asins, M.J., Pina, J.A., and Navarro, L. (1997a). Efficient search for new resistant genotypes to the citrus tristeza closterovirus in the orange subfamily Aurantioideae. Theoretical and Applied Genetics **95,** 1282-1288.

Mestre, P.F., Asins, M.J., Carbonell, E.A., and Navarro, L. (1997b). New gene(s) involved in the resistance of Poncirus trifoliata (L.) Raf. To *citrus tristeza virus*. Theoretical and Applied Genetics **95,** 691-695.

Mestre, P.F., Asins, M.J., Pina, J.A., Carbonell, E.A., and Navarro, L. (1997c). Molecular markers flanking *citrus tristeza virus* resistance gene from *Poncirus trifoliata* (L) Raf. Theoretical and Applied Genetics **94,** 458-464.

Metha, P., Brlansky, R.H., Gowda, S., and Yokomi, R.K. (1997). Reverse transcription polymerase chain reaction detection of *citrus tristeza virus* in aphids. Plant Disease **81,** 1066-1069.

Moore, G.A., Gutiérrez, E.M.A., Jacano, C., McCaffery, M., and Cline, K. (1993). Production of transgenic citrus plants expressing the citrus tristeza coat protein gene. HortScience **28,** 152.

Moreno, P., and Guerri, J. (1997). Variability of citrus tristeza closterovirus (CTV): methods to differentiate isolates. In Filamentous Viruses of Woody Plants, P. Monette, ed (Trivandrum, India: Research Signpost), pp. 97-107.

Moreno, P., Guerri, J., Ballesterolmos, J.F., Albiach, R., and Martinez, M.E. (1993). Separation and interference of strains from a *citrus tristeza virus* isolate evidenced by biological-activity and double-stranded-RNA (dsRNA) analysis. Plant Pathology **42,** 35-41.

Moreno, P., Ambros, S., Albiach-Marti, M.R., Guerri, J., and Pena, L. (2008). *Citrus tristeza virus* : a pathogen that changed the course of the citrus industry. Molecular Plant Pathology **9,** 251–268.

Muller, G.W., and Garnsey, S.M. (1984). Susceptibility of citrus varities, species, citrus relatives, and non-rutecous plants to slash-cut machanical inoculation with *citrus tristeza virus*. In Proceedings of the 8th Conference of the International Organization of Citrus Virologists (IOCV), S.M.T. Garnsey, L. V. And Didds, J. A, ed (Riverside, CA), pp. 62-65.

Muller, G.W., Rodriguez, O., and Costa, A.S. (1968). A tristeza virus complex severe to sweet orange cultivars. In Proceedings of the 4th Conference of the International Organization of Citrus Virologists (IOCV), J.F.L. Childs, ed (Gainesville: University of Florida Press), pp. 64-71.

Muller, G.W., Costa, A.S., Kitajima, E.W., and Camorgo, J.B. (1974). Additional evidence that tristeza multipleis in Passiflora spp. In Proceedings of the 6th Conference of the International Organization of Citrus Virologists (IOCV), L.G. Weathers and M. Cohen, eds (Riverside, CA), pp. 75-77.

Nagy, P.D., and Simon, A.E. (1997). New insights into the mechanisms of RNA recombination. Virology **235,** 1-9.

Navas-Castillo, J., Albiach-Marti, M.R., Gowda, S., Hilf, M.E., Garnsey, S.M., and Dawson, W.O. (1997). Kinetics of accumulation of *citrus tristeza virus* RNAs. Virology **228,** 92-97.

Nelson, N., J. . (1944). A photometric adaptation of the Somogy method for the determination of glucose Journal of Biological Chemistry **153** 375-379.

Niblett, C.L., Genc, H., Cevik, B., Halbert, S., Brown, L., Nolasco, G., Bonacalza, B., Manjunath, K.L., Febres, V.J., Pappu, H.R., and Lee, R.F. (2000). Progress on strain differentiation of *citrus tristeza virus* and its application to the epidemiology of citrus tristeza disease. Virus Research **71,** 97-106.

Nicholas, K.B., and Nicholas, H.B. (1997). GeneDoc a tool for editing and annotating multiple sequence alignments. .

Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G., and Tribulato, E. (2000). Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theoretical and Applied Genetics **100,** 1155-1166.

Olmos, A., Bertolini, E., Gil, M., and Cambra, M. (2005). Real-time assay for quantitative detection of non-persistently transmitted Plum pox virus RNA targets in single aphids. Journal of Virological Methods **128,** 151-155.

Opitz, K.W. (1961). Guide to top-working. California Citrograph **46,** 320.

Osman, F., and Rowhani, A. (2006). Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). Journal of Virological Methods **133,** 130-136.

Osman, F., Leutenegger, C., Golino, D., and Rowhani, A. (2007). Real-time RT-PCR (TaqMan®) assays for the detection of Grapevine Leafroll associated viruses 1–5 and 9. Journal of Virological Methods **141,** 22-29.

Palukaitis, P., and Zaitlin, M. (1997). Replicase-mediated resistance to plant virus disease Advances in Virus Research **48,** 349-377.

Papic, T., Santos, C., and Nolasco, G. (2005). First report of *Citrus tristeza virus* in the State Union of Serbia and Montenegro. Plant Disease **89,** 434.

Pappu, H.R., pappu, S.S., Manjunath, K.L., Lee , R.F., and Niblett, C.L. (1993). Molecular charecterization of a structural epitope that is largely conserved among severe isolates of a plant virus In Proceedings of the National Academy of Sciences-USA (USA), pp. 3641-3644.

Pappu, H.R., Karasev, A.V., Anderson, E.J., Pappu, S.S., Hilf, M.E., Febres, V.J., Eckloff, R.M., McCaffery, M., Boyko, V., and Gowda, S. (1994). Nucleotide sequence and organization of eight 3' open reading frames of the citrus tristeza Closterovirus genome. Virology **199,** 35-46.

Pappu, S.S., Febres, V.J., Pappu, H.R., Lee, R.F., and Civerolo, E.L. (1997). Characterization of the 3' proximal gene of citrus tristeza closterovirus genome. Virus Research **47,** 51-57.

Permar, T.A., Garnsey, S.M., Gumpf, D.J., and Lee, R.F. (1990). A monoclonal antibody that discriminates strains of *citrus tristeza virus*. Phytopathology **80,** 224-228.

Petersen, Y. (2003). Pokeweed antiviral protein-mediated resistance to citrus pathogens (Gainesville: University of Florida).

Pina, J.A., Moreno, P., Juarez, J., Guerri, J., Cambra, M., Gorris, T., and Vavarro, L. (2005). A new procedure to index for *citrus tristeza virus*-induced decline on sour orange rootstock. In Proceedings of the 16th Conference of the International Organization of Citrus Virologists (IOCV), M.E. Hilf, N. Duran-Vila, and M.A. Rocha Peña, eds (Riverside, CA), pp. 491.

Platt, R.G., and Opitz, K.W. (1973). Production technology:The propagation of citrus. In The Citrus Industry, W. Reuther, ed, pp. 1-45.

Powell, C.A., Pelosi, R.R., and Cohen, M. (1992). Superinfection of orange trees containing mild isolates of Citrus tristeza virus and severe Florida isolates of *citrus tristeza virus*. Plant Disease **76,** 141-144.

Powell, C.A., Pelosi, R.R., Rundell, P.A., and Cohen, H. (2003). Breakdown of crossprotection of grapefruit from decline-inducing isolates of *citrus tristeza virus* following introduction of brown citrus aphid. Plant Disease **87,** 1116-1118.

Price, M., Schell, J., Grosser, J.W., Pappu, S.S., Pappu, H.R., Febres, V.J., Manjunath, K.L., Niblett, C.L., Derrick, K.S., and Lee, R.F. (1996). Replication of citrus tristeza closterovirus in citrus protoplasts. Phytopathology **86,** 830-833.

Rai, M. (2006). Refinement of the *citrus tristeza virus* resistance gene (CTV) positional map in Poncirus trifoliata and generation of transgenic grapefruit (Citrus paradisi) plant lines with candidate resistance genes in this region. Plant Molecular Biology **61,** 399-414.

Reed, J.C., Kasschau, K.D., Prokhnevsky, A.I., Gopinath, K., Pogue, G.P., Carrington, J.C., and Dolja, V.V. (2003). Suppressor of RNA silencing encoded by Beet yellows virus. Virology **306,** 203-209.

Rezaee, R. (2008). Introducing a simple and efficient procedure for topworking Persian walnut trees. Journal of the American Pomological Society **62,** 21-26.

Rocha-Pena, M.A., Lee, R.F., Lastra, R., Niblett, C.L., Ochoa-Corona, F.M., Garnsey, S.M., and Yokomi, R.K. (1995). *Citrus tristeza virus* and its aphid vector *Toxoptera citricida*: threats to citrus production in the Caribbean and Central and North America. Plant Disease **79,** 437-445.

Rocha-Peña, M.A., and Lee, R.F. (1991). Serological techniques for the detection of *citrus tristeza virus*. Journal of Virological Methods **34,** 311-331.

Rocha Peña, M.A., Lee , R.F., permar, T.A., Yokomi, R., and Garnsey, S.M. (1991). Use of Enzyme-Linked Immunosorbent and Dot- Immunobinding Assay to evaluate two mild-strain cross protection experiments after challenging with a severe *citrus tristeza virus* isolate In Proceedings of the 11th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 93-102.

Roistacher, C.N. (1976). Detection of *citrus tristeza virus* by graft transmission. In Proceedings of the 7th Conference of the International Organization of Citrus Virologists (IOCV), E.C. Calavan, ed (Riverside, CA), pp. 175-184.

Roistacher, C.N. (1982). A blueprint of disaster I: The history of seedling yellows disease. California Citrograph **67,** 48-53.

Roistacher, C.N., and Bar-Joseph, M. (1984). Aphid transmission of citrus tristeza and seedling yellows tristeza by small population of Aphis gossypii. Plant Disease **68,** 494-499.

Roistacher, C.N., and Bar-Joseph, M. (1987a). Aphid transmission of *citrus tristeza virus*: a rewiew. Phytophylactica **19,** 163-167.

Roistacher, C.N., and Bar-Joseph, M. (1987b). Transmission of *citrus tristeza virus* (CTV) by Aphis gossypii and by graft inoculation to and from Passiflora spp. Phytophylactica **19,** 179-182.

Roistacher, C.N., Blue, R.L., Nauer, E.M., and Calavan, E.C. (1974). Suppression of tristeza virus symptoms in Mexican Lime seedlings grown at warm temperatures. Plant Disease Reporter **58,** 757-760.

Roistacher, R.N., and Moreno, P. (1991). The worldwide threat from destructive isolatesf *citrus tristeza virus*-a review. In Proceedings of the 11th Conference of the International Organization of Citrus Virologists (IOCV), R.H. Brlansky, R.F. Lee, and L.V. Timmer, eds (Riverside, CA), pp. 7-19.

Roy, A., Fayad, A., Barthe, G., and Brlansky, R.H. (2005). A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. Journal of Virological Methods **129,** 47-55.

Rubio, L., Guerri, J., and Moreno, P. (2000). Characterization of *citrus tristeza virus* isolates by single-strand conformation polymorphism analysis of DNA complemetary to their RNA population. In Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV), J.V. da Graca, R.F. Lee, and R.K. Yokomi, eds (Riverside, CA), pp. 12-17.

Rubio, L., Ayllon, M.A., Guerri, J., Pappu, H.R., Niblett, C.L., and Moreno, P. (1996). Differentiation of citrus tristeza closterovirus (CTV) isolates by single-strand conformation polymorphism analysis of coat protein gene. Annals of Applied Biology **129,** 479-489.

Rubio, L., Ayllon, M.A., Kong, P., Fernandez, A., Polek, M., Guerri, J., Moreno, P., and Falk, B.W. (2001). Genetic variation of *citrus tristeza virus* isolates from California and Spain: evidence for mixed infections and recombination. Journal of Virology **75,** 8054-8062.

Ruiz-Ruiz, S., Moreno, P., Guerri, J., and Ambros, S. (2007). A real-time RT-PCR assay for detection and absolute quantitation of *citrus tristeza virus* in different plant tissues. Journal of Virological Methods **145,** 96-105.

Saito, W., Ohgawara, T., Shimizu, J., and Ishii, S. (1991). Acid citrus somatic hybrids between Sudachi (*Citrus sudachi* Hort. ex Shirai) and lime (*C. aurantifolia* Swing.) produced by electrofusion. HortScience **77,** 125-130.

Salibe, A.A. (1977). The stem-pitting effects of tristeza on different citrus hosts and their economic significance. In Proceedings of the International Society of Horticulture pp. 953-955.

Saponari, M., Keremane, M., and Yokomia, R.K. (2008). Quantitative detection of *citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan®). Journal of Virological Methods **147,** 43–53.

Sasaki, A. (1974). Studies on hasaku dwarf: Special bulletin of fruit tree experimental station of hiroshima prefecture 2, pp. 106.

Satyanarayana, T., Gowda, S., Ayllon, M.A., and Dawson, W.O. (2004). Closterovirus bipolar virion: Evidence for initiation of assembly by minor coat protein and itsrestriction to the genomic RNA 5' region. Proceedings of the National Academy of Sciences-USA **101,** 799-804.

Satyanarayana, T., Gowda, S., Mawassi, M., Albiach-Martí, M.R., and Dawson, W.O. (2000). HSP70 homolog and p61 in addition to the two coat protein genes of Citrus tristeza closterovirus are required for efficient assembly of infectious virions. Phytopathology **90,** S69.

Satyanarayana, T., Gowda, S., Ayllon, M.A., Albiach-Marti, M.R., and Dawson, W.O. (2002a). Mutational analysis of the replication signals in the 3'-nontranslated region of *citrus tristeza virus* virus. Virology **300,** 140-152.

Satyanarayana, T., Gowda, S., Ayllon, M.A., Albiach-Marti, M.R., Rabindran, S., and Dawson, W.O. (2002b). The p23 protein of *citrus tristeza virus* controls asymmetrical RNA accumulation. Virology **76,** 473-483.

Satyanarayana, T., Robertson, C.J., Garnsey, S.M., Bar-Joseph, M., Gowda, S., and Dawson, W.O. (2008). Three genes of *citrus tristeza virus* are dispensable for infection and movement throughout some varieties of citrus trees. Virology **376** 297–307.

Satyanarayana, T., Gowda, S., Boyko, V.P., Albiach-Marti, M.R., Mawassi, M., Navas-Castillo, J., Karasev, A.V., Dolja, V., Hilf, M.E., Lewandowski, D.J., Moreno, P., Bar-Joseph, M., Garnsey, S.M., and Dawson, W.O. (1999). An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. Proceedings of the National Academy of Sciences-USA **96,** 7433-7438.

Saunt, J. (1990). Citrus varieties of the world. (Norwich, England: Sinclair International Limited).

Schneider, H. (1959). The anatomy of tristeza virus-infected citrus. In Citrus Virus Diseases, J.M. Wallace, ed (Berkeley: University of California Press), pp. 73-84.

Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D., and Frederick, R.D. (2004). Specific detection and quantification of plum pox virus by real-time fluorescent reverse transcription-PCR. Journal of Virological Methods **120,** 97-105.

Sieburth, P.J. (2000). Pathogen testing in the Florida mandatory citrus budwood protection program. In Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV), J.V. a Graca, R.F. Lee, and R.K. Yokomi, eds (Riverside, CA), pp. 408-410.

Sieburth, P.J., Nolan, K.G., Hilf, M.E., Lee, R.F., Moreno, P., and Garnsey, S.M. (2005). Discrimination of stem-pitting from other isolates of *citrus tristeza virus*. In Proceedings of the 16th Conference of the International Organization of Citrus Virologists (IOCV), M.E. Hilf, N. Duran-Vila, and M.A. Rocha-Peña, eds (Riverside, CA), pp. 1-10.

Somogy, M. (1952). Notes on sugar determination Journal of Biological Chemistry. **195,** 19-23.

Soost, R.K., and Roose, M.L. (1996). Citrus. In Fruit Breeding J. Janick and J.N. Moore, eds (John Wiley, New York.), pp. 257-323

Stover, E., and Castle, B. (2002). Citrus rootstock usage, characteristics, and selection in the Florida Indian River region. HortTechnology **12,** 143-147.

Suastika, G., Natsuaki, T., Terui, H., Kano, T., Ieki, H., and Okuda, S. (2001). Nucleotide sequence of *citrus tristeza virus* seedling yellows isolate. Journal of General Plant Pathology **67,** 73-77.

Tanaka, H. (1969). Virus of citrus fruits in Japan. Agriculture Horticulture **44,** 455-459.

Tanaka, H., Yamada, S., and Nakanishi, J. (1971). Approach to eliminating tristeza virus from from citrus trees by using trifoliate orange seedlings. Bulletin Horticultural Research Station, Japan **11,** 157-165.

Tanaka, M. (1981). Citrus interstock-scion combinations and topworking procedures in the Wakayama region of Japan. International Society of Citriculture, 1981. Volume 1**,** 127-130.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nuclic Acids Research. **25,** 4876-4882.

Toxopeus, H.J. (1937). Stock-action in-compatibility in citrus and its cause. Journal of Pomology and Horticultural Science **14,** 360-367.

Tsai, J.H., Liu, Y.H., Wang, J.J., and Lee, R.F. (2000). Recovery of orange stem pitting strains of *citrus tristeza virus* (CTV) following single aphid transmission with Toxoptera citricida from a Florida decline isolate of CTV. Proceeding of Florida State Horticultural Society **113,** 75-78.

USDA-Natural Resources Conservation Service. (1987). Soil survey of Indian River County, Florida. USDA/UF/FDACS National Cooperative Soil Survey.

Van Vuuren, S.P., and da Graça, J.V. (2000). Evaluation of graft-transmissible isolates from dwarfed citrus trees as dwarfing agents. Plant Disease **84,** 239-242.

Van Vuuren, S.P., and van der Vyver, J.B. (2000). Comparison of South African preimmunizing *citrus tristeza virus* virus isolates with foreign isolates in three grapefruit selections. In Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV), J.V. da Graça, R.F. Lee, and R.K. Yokomi, eds (Riverside, CA), pp. 50-56.

Van Vuuren, S.P., Collins, R.P., and Da Graca, J.V. (1993). Evaluation of *citrus tristeza virus* isolates for cross protection of grapefruit in South Africa. Plant Disease **77,** 24-28.

Varga, A., and James, D. (2005). Detection and differentiation of plum pox virus using real-time multiplex PCR with SYBR green and melting curve analysis: a rapid method for strain typing. Journal of Virological Methods **123,** 213- 220.

Varga, A., and James, D. (2006). Real-time RT-PCR and SYBR green I melting curve analysis for the identification of plum pox virus strains C, EA, andW: effect of amplicon size, melt rate, and dye translocation. Journal of Virological Methods **132,** 146-153.

Vela, C., Cambra, M., Sanz, A., and Moreno, P. (1988). Use of the specific monoclonal antibodies for diagnosis of *citrus tristeza virus*. In Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV), L.W. Timmer, S.M. Garnsey, and L. Navarro, eds (Riverside, CA), pp. 55-61.

Viggiani, G. (1988). Citrus pests in mediterranean basin. In Proceedings of the 6th Conference of the International Organization of Citrus Virologists (IOCV) (Riverside, CA), pp. 1067-1073.

Vives, M.C., Rubio, L., Lopez, C., Navas-Castillo, J., Albiach-Marti, M.R., Dawson, W.O., Guerri, J., Flores, R., and Moreno, P. (1999). The complete genome sequence of the major component of a mild *citrus tristeza virus* isolate. Journal of General Virology **80,** 811-816.

Von Broembsen, L.A., and Lee, A.T.C. (1988). South Africa's Citrus Improvement Program. In Proceedings of the 10th Conference of the International Organization of Citrus Virologists (IOCV), L.W. Timmer, S.M. Garnsey, and L. Navarro, eds (Riverside, CA), pp. 407-416.

Wallace, J.M. (1956). Tristeza disease of citrus, with special reference to its situation in the United States. FAO Plant Protection Bulletin **4,** 77-94.

Wallace, J.M., and Drake, R.J. (1955). The trsiteza virus in Meyer lemon. Citrus Leaves **35,** 8-9.

Webber, H.J. (1925). A comparative study of citrus industry in South Africa. Union South Africa: Department of Agriculture **6** 106.

Webber, H.J. (1943). The 'Tristeza' disease of sour-orange rootstock. Proceeding of the American Society for Horticultural Science Journal **43** 360-364.

Wheaton, T.A., Castle, W.S., Whitny, J.D., and Tucker, D.P.A. (1991). Performance of citrus scion cultivars and rootstocks in a high density planting. HortScience **26,** 837-840.

Wutscher, H.K., and Bowman, K.D. (1999). Performance of Valencia orange in 21 rootstocks in central Florida. HortScience **33,** 622-624.

XinZhong, H., ChangHe, Z., and HongLong, L. (2005). Study on the techniques of topworking methods for Pears. South China Fruits**,** 45-47.

Yang, G., Mawassi, M., Gofman, R., Gafny, R., and Bar-Joseph, M. (1997). Involvement of a subgenomic mRNA in the generation of a variable population of defective *citrus tristeza virus* molecules. Journal of Virology **71,** 9800-9802.

Yang, G., Che, X., Gofman, R., Ben-Shalom, Y., Piestun, D., Gafny, R., Mawassi, M., and Bar-Joseph, M. (1999). D-RNA molecules associated with subisolates of the VT strain of *citrus tristeza virus* which induce different seedling-yellows reactions. Virus Genes **19,** 5-13.

Yang, Z.N., Ingelbrecht, I.L., Louzada, E., Skaria, M., and Mirkov, T.E. (2000). *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). Plant Cell Reports **19,** 1203-1211.

Yang, Z.N., Ye, X.R., Molina, J., Roose, M.L., and Mirkov, T.E. (2003). Sequence analysis of a 282-kilobase region surrounding the *citrus tristeza virus* resistance gene (CTV) locus in *Poncirus trifoliata* L. Raf. Plant physiology **131,** 482-492.

Yang, Z.N., Ye, X.R., Choi, S.D., Molina, J., Moonan, F., Wing, R.A., Roose, M.L., and Mirkov, T.E. (2001). Construction of a 1.2-Mb contig including the *citrus tristeza virus* resistance gene locus using a bacterial artificial chromosome library of Poncirus trifoliata (L.) Raf. Genome **44,** 382-393.

Yokomi, R.K., Lastra, R., Stoetzel, M.B., Amgsteet, V.D., Lee, R.F., Garnsey, S.M., Rocha-Pena, M.A., and Niblett, C.L. (1994). Establismnet of brown citrus aphid Toxoptera citricida (Kirkaldy) (Homoptera: Aphididae) in Central America and the Caribbien Basin, and its transmission of *citrus tristeza virus*. Journal of Economic Entomology **87,** 1078-1085.

Zaitlin, M., Anderson, J.M., Perry, K.L., Zhang, L., and Palukaitis, P. (1994). Specificity of replicase-mediated resistance to *cucumber mosaic virus*. Virology **201,** 200-205.

BIOGRAPHICAL SKETCH

Azza Hosni Ibrahim Mohamed was born in Altahera, Sharkia, Egypt, in 1971. She earned a Bachelor of Science degree in agriculture chemistry in June 1993 from the Biochemistry Department, Zagazig University, Egypt. Azza was appointed to a position as a research assistant at the Biochemistry Department, Mansoura University, Egypt, where she received the Master of Science in biochemistry in 1999. She is married to Ahmad Omar who also recently completed his Ph.D. from the University of Florida. They have one daughter, Aala. Azza is getting her degree from the Horticultural Science Department under the supervision of Dr Jude W. Grosser, professor of plant cell genetics at the University of Florida. After Azza graduation, she will return to Egypt to resume her position as an assistant professor in the Biochemistry Department, Faculty of Agriculture, Mansoura University, Egypt. Her work will include teaching several biochemistry and molecular biology courses and research that will feature techniques she has learned during her Ph.D. program.