UNIVERSITY OF CATANIA

DEPARTMENT OF AGRI-FOOD AND ENVIRONMENTAL SYSTEMS MANAGEMENT

SECTION OF PLANT PATHOLOGY

INTERNATIONAL Ph. D. PROGRAM IN PLANT HEALTH TECHNOLOGIES AND PROTECTION OF AGRO-ECOSYSTEMS

XXV-Cycle 2009-2012

Characterization of Moroccan *Citrus Tristeza Virus* (CTV) isolates and study of their genomic variability after aphid transmission

This thesis is presented for the degree of

Doctor of Philosophy

By

Mohamed AFECHTAL

COORDINATOR:

Prof. Carmelo RAPISARDA

SUPERVISORS:

Prof. Giuseppe COCUZZA

Dr. Anna Maria D'ONGHIA

Dr. Khaled DJELOUAH

Academic years 2009-2012

BIOGRAPHY

Mohamed AFECHTAL was born in Ouaouizerth, Morocco, on February 2nd 1983. He attended Ouaouizerth High School and graduated in October 2007 at the Hassan II Institute of Agronomy and Veterinary Medicine (IAV-Hassan II), Rabat, Morocco, as an Agronomic Engineer in Plant Pathology. In 2005, he got a scholarship from CIHEAM to pursue a Master of Science degree in Integrated Pest Management at the Mediterranean Agronomic Institute of Bari (MAI-Bari), Italy - in collaboration with IAV-Hassan II - which he completed in October 2007. Two months later, he became a researcher in virology of citrus and fruit trees at the National Institute of Agricultural Research (INRA-Morocco). In the same year, 2008, he reported the largest outbreak ever reported of *Citrus tristeza virus* in Morocco, which led him to perform his Ph.D. thesis on the same topic at the University of Catania, Italy - in collaboration with the MAI-Bari - under the supervision of Prof. G. Cocuzza, Dr. A.M. D'Onghia and Dr. K. Djelouah.

ACKNOWLEDGEMENTS

It is with great pleasure that I write these words to express my deep gratitude to everyone who helped, guided and contributed directly or indirectly for completing this Ph.D. thesis.

INRA-Morocco

Well, this work was only possible because of Prof. M. Badraoui, the Director of INRA-Morocco, and I will always be indebted to him for giving me this chance to make my Ph.D. thesis. It is my utmost pleasure to express my deep sense of heartfelt gratitude towards Mr. A. F. Andaloussi, the Chief of the Plant Protection Department in INRA; Mr. H. Benaouda, the Chief of CRRA-Kénitra; and Dr. M. El Guilli, the Coordinator of the Plant Protection Unit in CRRA-Kénitra for their help and encouragement.

UNICT

Sincere gratitude goes to my supervisor Prof. G. Cocuzza, for blessing me with his kind supervision, guidance and encouragement. I am very much thankful to our kind coordinator Prof. C. Rapisarda, for his friendly guidance and coordination. I would also like to take the opportunity to thank Prof. G. Cirvilleri for her valuable advice and help.

CIHEAM / MAI-Bari

My deep gratitude to all the staff of the Mediterranean Agronomic Institute of Bari, represented by the Director Prof. C. Lacirignola, for their support, hospitality and for permitting me to use all available facilities in the Institute.

My deepest thanks and appreciation go to my supervisor Dr. A.M. D'Onghia, Head of IPM department, for blessing me with her kind guidance and for her enlightening knowledge which helped me to complete my project and allowed me to achieve this work. I enjoyed being here and am enriched by many dimensions being a student of the department.

But also, my appreciation and my deep gratitude to my kind supervisor Dr. K. Djelouah, who always helped me with promptness and kindness and for all the scientific information he provided me. Since my working with him, not a single day has been spent without exploring and experiencing his new aspects of expertise in teaching, research, maintaining human relations and resources management. Without him, this work would not have been accomplished.

My special thanks to all the staff of IPM department, Dr. M. Digiaro, Dr. T. Elbeaino, Dr. T. Yaseen, Dr. D. Frasheri, Dr. F. Valentini, Mme. S. Santoro, Mr. F. Santoro, Mr. G. Santoro and Mr. G. Cavallo for their kind support and their scientific information shared with me.

I have a great pleasure in extending my sincere thanks to my lab-mates, some of them left earlier and some are still around, for their help and kind co-operation during all these years. Special and cordial thanks to my friends Dr. H. El Bilali, Dorsaf Yahyaoui and Yosra Ahmed for their help and for the moments we shared together.

At last but not least I would like to thank my parents and my brothers Ahmed and Ali. To them I dedicate this thesis.

Above all, thanks to the ALMIGHTY GOD for his blessings.

Mohamed AFECHTAL

TABLE OF CONTENTS

ABSTRACT

A survey of *Citrus tristeza virus* (CTV) was carried out in the main Moroccan citrus growing areas (Souss, Gharb, Moulouya, Tadla, Haouz and Loukkos), analyzing by DTBIA a total of 5192 plants. About 20% of the tested trees proved to be CTV-infected in the groves, mainly in Loukkos region with a 60% infection rate. On the contrary, only three CTV infected plants were found in Souss region and one plant in Gharb region. No CTV infection was detected in the surveyed nurseries, plots of budwood sources and varietal collections.

CTV vectors were monitored in Gharb and Loukkos regions. *Aphis spiraecola* (56%) and *A. gossypii* (32%) were the most abundant aphid species, while no evidence of *Toxoptera citricidus* was reported.

Ten out of 12 selected CTV isolates showed, by coat protein gene sequencing, the highest nucleotide similarity with the T30 mild reference strain (Florida) and the Spanish mild isolate T385, with a sequence homology ranging from 98.6% to 99.4%. The remaining isolates from Loukkos clustered close to the Portuguese 19-21 severe reference strain and to the Puerto Rican *Poncirus trifoliata* resistance breaking (RB) severe isolate B301 (98.5% to 99.2% homology); these two Moroccan isolates induced mild stem pitting symptoms on Duncan grapefruit by biological indexing and reacted positively to the MCA13 monoclonal antibodies. This study confirmed high CTV genetic diversity in some Moroccan citrus groves. The finding of the CTV stem pitting represents a serious threat to the Moroccan citrus industry, mainly if *T. citricidus* is introduced in the country.

Meanwhile, aphid transmission experiments by *A. gossypii* and *A. spiraecola* on two local CTV isolates (mild and severe), were carried out in laboratory. The obtained results showed that *A. gossypii* was able to transmit efficiently the L-Clem2-Mor mild isolate (40%), while less was the transmission rate (13.33%) for the L-Clem1-Mor severe isolate whereas, the mild isolate was transmitted at a rate of 6.67% by *A. spiraecola,* which was not able to transmit the severe one. The transmission trials showed that the mild CTV isolate was more transmissible than the severe one; this may explain the prevalence of the mild isolate in the region of Loukkos.

The field CTV isolates were compared to their correspondent aphid derived sub-isolates using SSCP and sequencing analysis of the p18, p23 and p25 genes. SSCP analysis showed

that the CTV isolates had the same migration patterns as their correspondent aphid derived sub-isolates, whereas, sequencing analysis of the p18, p23 and p25 genes revealed minor changes in the sequences obtained from aphid derived sub-isolates compared to their parental field isolates. Consequently, very limited changes where registered in the corresponding predicted amino acids sequences of the studied genes.

Finally, a Tissue Print (TP) real time RT-PCR assay for a rapid differentiation and identification of potential severe strains of CTV was developed and validated for testing a panel of 15 CTV isolates from the Mediterranean area; the obtained results confirmed their corresponding genotypes. This assay was equally used to test 12 CTV sources from Morocco; interestingly, the two severe isolates that induced previously mild stem pitting on Duncan grapefruit were assigned as a T36-like genotype through this assay.

Keywords: Citrus, *Citrus tristeza virus*, aphids, survey, characterization, transmission, Morocco.

RESUME

Des prospections à large échelle sur le virus de la tristeza, *Citrus tristeza virus* (CTV), ont été conduites dans les principales régions agrumicoles du pays (Souss, Gharb, Moulouya, Tadla, Haouz et Loukkos) et analysant par le test DTBIA un total de 5192 échantillons. Environ 20% des arbres testés se sont avérés infectés, particulièrement dans la région du Loukkos qui a montré un taux d'infection de 60%. Par contre, seuls trois arbres infectés ont été détectés dans la région du Souss et un seul arbre infecté dans la région du Gharb. Aucune infection par le CTV n'a été décelée dans toutes les pépinières, parcs à bois et collections variétales prospectés.

D'autre part, une enquête effectuée sur les pucerons vecteurs du CTV dans les régions du Gharb et Loukkos, a montré une prédominance de *Aphis spiraecola* (56%) et *A. gossypii* (32%) alors que *Toxoptera citricidus* n'as pas été signalé.

Dix des 12 isolats de CTV sélectionnés pour une caractérisation ont montré, par séquençage du gène de la protéine capsidique, une similarité nucléotidique variant de 98.6% à 99.4% avec les isolats modérés T30 (Floride) et T385 (Espagne). Par contre, les deux autres isolats provenant de la région du Loukkos, ont été classés phylogénétiquement proche (homologie de 98.5% à 99.2%) des isolats sévères 19-21 (Portugal) et B301 (Porto Rico), ce dernier est responsable de l'interruption de la résistance du *Poncirus trifoliata* au CTV. Ces deux derniers isolats Marocains ont induit l'apparition de symptômes modérés de bois strié (stem pitting) sur le pamplemoussier Duncan et ont réagi positivement aux anticorps monoclonaux MCA13. Cette étude a révélé la présence d'une grande diversité génétique du CTV dans les vergers d'agrumes au Maroc. La découverte de souches sévères (stem-pitting) présenterait une menace importante pour l'industrie agrumicole nationale surtout si *T. citricidus*, le vecteur le plus efficace du CTV, serait introduit au pays.

Parallèlement, des essais de transmission de deux isolats locaux de CTV (modéré et sévère) par *A. gossypii* et *A. spiraecola* ont été effectués au laboratoire. Les résultats obtenus ont montré que *A. gossypii* a transmis efficacement l'isolat modéré L-Clem2-Mor (40%), mais moins pour l'isolat sévère L-Clem1-Mor (13,33%). D'autre part, la transmission par *A. spiraecola* a été basse, de (6.67%) pour l'isolat modéré mais n'a pas pu être effectuée avec l'isolat sévère.

En général, l'isolat modéré a montré un taux de transmissibilité plus important par rapport au sévère, ce qui pourrait expliquer son abondance dans la région du Loukkos. Une étude comparative des deux isolats avant et après transmission par pucerons a été réalisée par SSCP et le séquençage de trois gènes p18, p23 et p25. La SSCP a montré une similarité des profils de migration (avant et après la transmission), alors que le séquençage génomique a révélé des changements très contenus au niveau de la séquence nucléotidique des trois gènes étudiés, induisant une petite différence au niveau des séquences polypeptidiques respectives.

Finalement, un test de différenciation rapide et d'identification des souches potentiellement sévères de CTV a été développé. 15 souches de CTV originaires de la Méditerranée ont été testées en vue de valider cette nouvelle technique; les résultats obtenus ont parfaitement confirmé leurs génotypes respectifs. Cette technique a également été employée pour tester 12 sources locales de CTV; et a montré que les deux isolats, qui ont précédemment induit les symptômes de stem-pitting sur le pamplemoussier Duncan, ont été assignés au génotype T36-like.

Mots-clés: Agrumes, *Citrus tristeza virus*, pucerons, caractérisation, transmission, Maroc.

RIASSUNTO

Un'indagine sul *Citrus tristeza virus* (CTV) è stata condotta nelle principali aree agrumicole del Marocco (Souss, Gharb, Moulouya, Tadla, Haouz e Loukkos), analizzando con il DTBIA una totalità di 5192 piante di agrumi. Nei campi commerciali, soprattutto nell'area di Loukkos, sono risultate infette al CTV circa il 20% delle piante saggiate, con un'incidenza d'infezione pari al 60%. Al contrario, solo tre piante sono risultate infette nella regione di Souss ed una in quella di Gharb. Nessuna infezione del virus è stata riscontrata nei vivai, nei campi di piante madri e nelle collezioni varietali.

Dalle indagini condotte sui vettori del CTV nelle regioni di Gharb e Loukkos è emerso che *A. spiraecola* (56%) e *A. gossypii* (32%) sono le specie afidiche più presenti, mentre non è stata evidenziata la presenza della *T. citricidus*.

Attraverso il sequenziamento della proteina, dieci dei 12 isolati selezionati di CTV hanno mostrato una omologia variabile dal 98.6 al 99.4% con i ceppi tenui di riferimento T30 (Florida) e T385 (Spagna). Gli isolati rimanenti provenienti da Loukkos sono stati raggruppati con il ceppo severo Portoghese 19-21 e con quello del Porto Rico B301 (con un'omologia variabile dal 98.5 al 99.2%); questi due isolati Marocchini hanno indotto, durante i saggi per innesto, sintomi di lieve butteratura del legno sul pompelmo Duncan ed hanno reagito positivamente agli anticorpi monoclonali MCA13. Questo studio conferma l'alta diversità genetica degli isolati di CTV provenienti da alcuni agrumeti del Marocco. Il ritrovamento del ceppo di CTV-butteratura del legno rappresenta un serio rischio per l'industria agrumicola del Marocco, soprattutto se nel Paese farà il suo ingresso la *T. citricidus*.

Contestualmente, sono state condotte prove di trasmissione di due isolati locali di CTV (uno tenue ed uno severo) attraverso *A. gossypii* e *A. spiraecola.* I risultati ottenuti hanno mostrato una trasmissione attraverso *A. gossypii* del 40% dell'isolato tenue di CTV rispetto al 13,33% dell'isolato severo, mentre attraverso *A. spiraecola* è stato trasmesso solo l'isolato tenue di CTV (6.67%) e non quello severo; questo risultato spiega la prevalenza dell'isolato tenue di CTV nella regione di Loukkos.

Gli isolati di CTV selezionati in campo sono stati comparati con il loro corrispondenti sub-isolati derivati dalla trasmissione per afide attraverso le analisi SSCP ed il sequenziamento

dei geni p18, p23 e p25. Le analisi SSCP hanno evidenziato che gli isolati di CTV hanno gli stessi profili di migrazione dei loro corrispondenti sub-isolati derivati dall'afide, mentre il sequenziamento dei geni p18, p23 e p25 ha mostrato minori cambiamenti nelle sequenze ottenute dai sub-isolati derivati dagli afidi rispetto agli isolati originari dal campo. Pochissimi cambiamenti sono stati quindi registrati nelle corrispondenti sequenze degli amminoacidi dei geni studiati.

In fine, è stata sviluppata la tecnica di Tissue Print (TP) real time RT-PCR per la rapida differenzazione ed identificazione dei potenziali ceppi severi di CTV. Questa tecnica è stata utilizzata per analizzare un gruppo di 15 isolati di CTV dell'area Mediterranea ed i risultati ottenuti hanno confermato la loro corrispondenza genotipica. Questo saggio è stato egualmente utilizzato per analizzare 12 fonti di CTV del Marocco ed ha permesso di attribuire ai due isolati severi, che avevano indotto la formazione di una lieve butteratura del legno nel pompelmo Duncan, una similitudine al genotipo T36.

Parole chiave: Agrumi, *Citrus tristeza virus*, aphidi, charatterizzazione, trasmissione, Marocco.

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
APS	Amonium Persulfate
BCIP-NBT	5-Cromo-4-Chloro-3-Indolyl-Phosphate/Nitro Blue Tetrazolium
bp	Base pair
BrCA	Brown citrus aphid
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary Deoxyribonucleic Acid
CE	Controller element
CLAM	Comité de Liaison de l'Agrumiculture Méditerranéenne
CH	Leaf chlorosis
cm	Centimeter
CP	Coat Protein
CPm	Minor coat protein
CSD	Citrus sudden death
CTV	Citrus tristeza virus
CTV-QD	Citrus tristeza virus-quick decline strain
CTV-SP	Citrus tristeza virus-stem pitting strain
CTV-SY	Citrus tristeza virus-seedling yellows strain
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
D-RNA	Defective RNA
dsRNA	Double stranded RNA
DTBIA	Direct Tissue Blot Immunoassay
DTT	Dithiothreitol
e.g	For example
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization
Fig.	Figure
FL	Flowering
g	Gram
GFP	Green Fluorescent Protein
gRNA	Genomic RNA
h	Hour
H_2O	Water
Ha	Hectare
HEL	Helicase
HCl	Hydrochloric Acid

HS	Hierarchical Scheme
HSP70	Heat Shock Protein 70
INRA	National Institute of Agricultural Research
Kbp	Kilo Base Pair
KDa	Kilo Dalton
KOH	Potassium Hydroxide
LC	Leaf cupping
LB	Luria Bertani
LMTs	Low-molecular-weight- tristeza sub-genomic RNAs
m	Meter
M	Molar
MAb	Monoclonal Antibody
MEGA	Molecular Evolutionary Genetics Analysis Software
mg	Milligram
mg/ml	Milligram per millilitr
MGB	Minor Groove Binding
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitr
M-MLV RT	Moloney-Murine Leukemia Virus Reverse Transcriptase
MMM	Multiple Molecular Markers
MT	Methyl Transferase
NaCl	Sodium Chloride
NaI	Sodium Iodide
nm	Nanometr
No.	Number
nt	Nucleotide
NTP	Nucleotide Triphosphate
O.D	Optical Density
ONSSA	National Office for Food Safety
ORF	Open Reading Frame
PAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pmol	Picomole
PVP	Polyvinylpyrrolidone
RdRp	RNA dependent RNA Polymerase
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNAase	Ribonuclease
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SLS	Sodium Lauryl Sarkosyl
S	Second

sgRNA	Sub-genomic RNA
spp.	Species
ST	stunting
SSCP	Single Strand Conformation polymorphism
ssRNA	Single Stranded RNA
STET	Sodium chloride Ethylenediaminetetraacetic acid Tris
TAE	Tris-acetate-EDTA
Taq	*Thermophilus aquaticus*
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TNA	Total Nucleic Acid
TP	Tissue print
U	Enzymatic Unit
USA	United Sates of America
UTR	Untranslated Region
UV	Ultraviolet
V	Volt
V/V	Volume per Volume
VC	Vein clearing
W/V	Weight per Volume

Chapter I. INTRODUCTION

Citrus is one of the most important cultivated fruit crops in the world; among ca. 60 species belonging to the genus *Citrus*, only ten are cultivated for their edible fruits and all are believed to be native of the subtropical and tropical regions of Asia and the Malay Archipelago, prior to be widely spread around the world (Webber, 1948). Citriculture is mainly concentrated in the tropical and subtropical regions between 40° north and south latitude in over 137 countries (Ismail and Zhang, 2004).

Citrus fruits are the highest value fruit crop in terms of international trade; current annual worldwide citrus production is estimated at over 122 million tons, with more than half of this being oranges. The main citrus fruit producing countries are, the United States of America, China, Brazil although the Mediterranean basin accounts for about 20% of the world citrus production and about 60% of the world fresh citrus trade (CLAM, 2007).

In Morocco, the citrus orchards cover about 101.000 Ha with an annual production of 1.76 million Tons (Anonymous, 2011). Citrus cultivation represents an important source of employment for thousands of families at various levels of the chain (production, processing trade and farming consumable suppliers); as over 40% of the production volume is exported, it is considered the first crop oriented to foreign markets. The European Union markets are by far the main destination of Moroccan export; however, in the last years, there was a significant diversification towards Russia and Canada. Hence, citrus plays an invaluable role as a driving force to the Moroccan economy.

Citrus trees in Morocco are grown mainly in the coastal areas of the Mediterranean Sea and Atlantic Ocean, where the climate is mild and rainfall abundant. The most important citrus production areas are Souss Valley (40% of the area dedicated to citrus production), Gharb (20%), Moulouya (16%), Tadla (14%) and Haouz (6%) (Anonymous, 2011).

Although more than 20 varieties of oranges and mandarins are produced in the country, oranges, which include Valencia late (commonly named Maroc late), Washington navel, Salustiana and blood types, are dominant in term of areas planted. Small citrus fruits are mainly represented by Common clementine and late mandarins.

Sour orange [*Citrus aurantium* L.] is the dominant rootstock in over 95% of grafted citrus trees and is well adapted to the calcareous soils of the country. This universal rootstock is

also tolerant to Phytophtora gummosis, salinity and drought, which are considered the prevalent biotic and abiotic stresses of citrus in the country. However, it is highly susceptible to citrus tristeza disease, which is the most severe virus disease affecting these species (Lacirignola and D'Onghia, 2009). Other rootstocks, nowadays used in the Morocco, include citrange types ([*C. sinensis* Osbeck] x [*Poncirus trifoliata* (L.) Raf.]) (C35, Troyer and Carrizo), alemow [*C. macrophylla* Wester], ''Volkamer'' lemon [*C. volkameriana* Tan. and Pasq], Citrumelo Sacaton and Swingle ([*P. trifoliata* (L.) Raf.] x [*C. paradisi* Macf.]).

The citrus certification program had a considerable impact on the development of the citrus industry at the national level; the yearly production of registered nurseries in the country has reached a production of about 4 million certified plants. The impact of certified material on plant vigour and development and the impressive yields obtained have greatly raised the interest of growers, and the demand for certified plants is constantly growing especially that they are subsidize by the government.

Citrus tristeza virus (CTV), one of the most important and devastating pathogens affecting citrus species worldwide, was reported several times, as isolated foci, over the years in Morocco. Tristeza represents the major threat to the Moroccan citrus industry, since the CTV-sensitive sour orange is still the major rootstock used. Furthermore, the most efficient vector *Toxoptera citricidus* (Kirkaldy), which is named the brown citrus aphid (BrCA), became recently established in Spain and Portugal (Ilharco *et al.,* 2005); it is probably only a matter of time before this aphid reaches the Moroccan citrus growing areas. The situation experienced in other regions of the world, after the establishment of this aphid, is marked by a massive destruction irrespective of rootstocks. Therefore, the rapid identification of the CTV presence and its severity became critical for the management and control of the disease. As well, monitoring of *T. citricidus* and evaluation of the transmission efficiency of local CTV isolates by the present vectors are of utmost importance for predicting the disease dynamics, thus preventing further CTV outbreaks and/or maintaining the infection within acceptable limits.

Chapter II. BIBLIOGRAPHIC REVIEW

Tristeza is globally a devastating disease of citrus, thus representing a limiting factor of citrus production worldwide. Many millions of trees on sour orange have been destroyed in Argentina, Brazil, California, Florida and Spain. The disease continues to spread into new areas destroying plantings where sour orange is the predominant rootstock. The first reference of the disease was the Portuguese ''A Podridão des radicelas'' (Bitancourt, 1940) and the Spanish ''Podredumbre de las raicillas'' (Valiela, 1948), literately translated as ''rotting of the rootlets''; it was appropriately named Tristeza, which means ''Sadness'' in order to describe the declining aspect of citrus trees in the lack of any scientific explanation. In 1942, this name was also used by Moreira in Brazil where the infection was known by ''Podridão das radicelas'', but also it was designated as ''budunion decline'' in California (Davino *et al.*, 1998).

2.1. Origins of the tristeza disease

Tristeza apparently originated in Asia where it subsisted for many years in tolerant cultivars which were propagated vegetatively as cuttings or by seed. In 1836, a foot rot epidemic caused by oomycetes of the genus Phytophthora started in the Azores and later affected Mediterranean countries, decimating citrus plantings established from seeds or bud propagated on sweet orange seedlings or on rooted citron cuttings. This disaster forced bud propagation of citrus varieties on sour orange, a foot-rot-resistant rootstock, highly adaptable to all soil types that induce good bearing and excellent fruit quality.

The use of sour orange as a rootstock gained popularity for its resistance to root diseases and its ability to promote a vigorous fast growing scion with desirable fruit qualities under certain soil conditions (Fawcett, 1936; Rocha-Peña *et al.*, 1995). Sour orange soon became almost the exclusive rootstock in the Mediterranean area, then in America. This rootstock unfortunately created the conditions for tristeza-decline epidemics in South Africa (1910), Java (1928), Brazil (1937) and Argentine (1938) (Herron, 2003).

Davis (1924) reported sweet oranges and mandarins died on sour orange rootstock whereas lemons did not. Webber (1943) reporting on his comments from South Africa in 1924-5, had difficulty finding any trees on sour orange rootstocks under 20 years old in various locations. Toxopeus in Java (1937) described disease symptoms on sour orange similar to those in

South Africa and ruled out environmental factors as the cause of the problem. Bitancourt (1940) described a ''Podridão das radicelas'' or a rooting of citrus feeder roots; he tested numerous scion-rootstock combinations in Brazil and suggested a viral origin of the syndrome: a latent virus in sour orange could be transmitted via the bud union to sweet orange. Webber (1943) published the first comprehensive report on citrus diseases in the absence of any mention of tristeza-like symptoms. Moreira (1942) used the term ''tristeza'', Portuguese sadness or melancholy, to describe the symptoms of canopy decline of scions grafted on sour orange in Brazil. Meneghini (1946) was yet to confirm the aphid transmission of the tristeza agent, and Fawcett and Wallace (1946) were yet to prove the viral nature of the disease.

Concerning the Mediterranean, epidemics have occurred in Spain, Israel and Italy, while CTV has been reported also in some citrus groves as isolated foci in Albania, Algeria, Egypt, France, Greece, Jordan, Lebanon, Libya, Morocco, Palestine, Portugal, Syria, Tunisia and Turkey (Djelouah and D'Onghia, 2001a). The spread and movement of tristeza depends upon distribution of infected budwood, type of aphid vectors, virus strains and temperatures (Roistacher, 1991).

2.2. Brief history of *Citrus tristeza virus* in Morocco

CTV was probably introduced in Morocco early in the 1930's with the citriculture development in the country. Furthermore, commercial germoplasm repositories were established in the 1950s, with material introduced from abroad, when the aetiology of tristeza disease was still unknown (Zemzami *et al.,* 2009).

In 1963, Cassin detected CTV, by biological indexing on Mexican lime, in Meyer lemon and eight other accessions in a stock of old citrus trees introduced to Morocco; the infected trees were eliminated. Later Chapot and Delucchi (1964) reported infection of some exotic varieties with CTV, including all Meyer lemons, Satsumas and Kumquats in the germoplasm repositories as well as in commercial plantings. In 1967, Bové confirmed the introduction of tristeza into Morocco in budwood of exotic varieties including Owari Satsuma and King Mandarin imported from Florida, between 1945 and 1948. He adverted against the risks of having CTV-infected Meyer lemon trees planted to a large extent in the Marrakech valley. One year later, the Citrus Committee of the Franc Zone (CAZF) stressed out the urgent need to eradicate all known sources in the country. A governmental decree was issued for the

mandatory eradication of Meyer lemons in the region of Marrakech; professionals and farmers cooperated fully with the authorities to launch an eradication campaign of Meyer lemon that included 18 farms. In the 1980s, the *Sociéte de Développement Agricole* (SODEA), conducted a survey for the sanitation of SODEA farms, with special consideration of Meyer lemons; seven infected Meyer lemon trees were found and eradicated in three farms (Nhami, 1981).

In 1983, a certification program for citrus nursery plants was set up; the relative scheme was inspired by the Californian and Spanish certification programs (Anonymous, 1984). The first quick declining tree was found in the citrus germoplasm block of INRA-Station of El Menzeh, in the Gharb Valley, in which a tree of Pan American mandarin declined suddenly (Zemzami *et al.,* 2009).

In 1987, a survey was undertaken to test the citrus germoplasm block of INRA-Souihla, near Marrakech. Among the 1749 tested trees, 67 belonging to 18 varieties were CTV positive and were eradicated (Nadori and Zemzami, 1987). A similar survey was carried out in INRA-Station El Menzeh germplasm block where 21 out of 2130 tested trees were found to be CTV positive; the infected trees, which were immediately eradicated, represented 6 different varieties (Nadori and Zemzami, 1992). Other surveys were accomplished to control other INRA citrus germoplasm collections: Aïn Chaïb and Melk-Zhar in the Souss valley near Agadir and Aïn Taoujdate in the Saïss valley near Fès. Nine positives were found all among duplicate trees of Pan American Mandarin at Aïn Chaïb and Aïn Taoujdate (Zemzami and Nadori, 1989).

In 1988, the first massive importation of certified citrus nursery plants from Spain to Morocco was made, it concerned 60 000 plants of Fortuna, Nova and Marisol varieties. The plants had the label of the Spanish certification authority; nevertheless all plants were systematically tested through ELISA test by the Moroccan Plant Protection Services and evidenced several hundreds of CTV positive cases, representing an average of 5% to 10% depending on batches and varieties, all the infected material was eradicated (Zemzami *et al.,* 2009).

In 1999, the plant protection service was informed by the SODEA about abnormal dwarfing of 12 trees of Nova mandarin and Lane Late navel in a topworked orchard in the Souss Valley. Presence of CTV in samples collected from suspected trees was confirmed by ELISA.

The owner of the orchard cooperated fully with authorities and eradicated the whole plot composed of 900 trees.

In 1998 and 2000, CTV infected material was intercepted in citrus material introduced legally from Spain by the Plant Quarantine Service (Lbida *et al.,* 2004, 2005).

In 2008, a large scale survey on CTV was conducted in the main citrus growing regions of the country, in addition to the varietal collections of INRA-Station El Menzeh and INRA-Station of Tadla. The CTV was detected in 29 samples in the region of Loukkos (Northern Morocco) using direct tissue blot immunoassay (DTBIA). Fifteen samples were collected in an orchard of common Clementine grafted on sour orange and fourteen came from another orchard of Clementine Peau Fine grafted on Citrange (Afechtal *et al.,* 2010). After being informed, the National Office for Food Safety (ONSSA) organized several meetings for national experts and professionals for an open debate about CTV control strategy to be adopted in Morocco in the light of these new findings of CTV in the region of Loukkos. Thus, additional CTV surveys were accomplished in this region by ONSSA using ELISA assays. The obtained results showed that more than 720 ha were found to be infected (ONSSA, personal communication). This was the largest outbreak of CTV ever reported in Morocco.

2.3. Causal agent

Citrus tristeza virus (CTV), the causal agent of the most important viral diseases of citrus, belongs to the genus *closterovirus* in the plant virus family *closteroviridae* (Karasev *et al.,* 1995). Viruses in the family have flexible elongated virus particles of 1250 to 2200 nm (Bar-Joseph *et al.,* 1979a); they are transmitted in nature semi persistently by insects, tend to replicate in phloem-associated cells where they cause a characteristic cytopathic effect, and have a complex and a very distinct genome organization. Within this family, three viral genera have been described based on their RNA genome and their vector transmission (Fig. 1). The genus *Closterovirus*, type species *Beet yellows virus* (BYV), has a monopartite RNA genome of up to 20 Kb and is transmissible by aphid species (Homoptera: Aphididae), as well as the *Ampelovirus*, type species *Grapevine leafroll-associated virus 3* (GLRaV-3), which are vectored by mealybugs (Homoptera: Pseudococcae); while, the genus *Crinivirus,* type species *Lettuce infectious yellow virus* (LIYV), has bipartite genomes and is spread by whiteflies (Homoptera: Alyrididae) (Klaassen *et al.,* 1995; Martelli *et al.,* 2002). Currently, more than 30 viruses are included in the family *Closteroviridae*; and two to four viruses are added to this list every year

(Karasev and Bar-Joseph, 2010). The evolution of Closteroviruses has been suggested on the basis of phylogenetic analysis of their replicative genes and the HSP70 homolog (HSP70h) (Karasev, 2000).

The genus *Closterovirus* represents a group of emerging and re-emerging economically important plant pathogens. Members of this group affect several crops of major economic importance, such as sugar beet, citrus, tomato, lettuce, potato, sweet potato, grapevine, pineapple, cherry, and some ornamentals. However slow, compared to other plant viruses, the life styles of closteroviruses proved evolutionarily successful given the long and rapidly growing list of closteroviruses that infect a broad range of hosts from cucumbers to strawberries, grapevine and citrus (Martelli *et al.*, 2002; Tzanetakis *et al.*, 2005a, b).

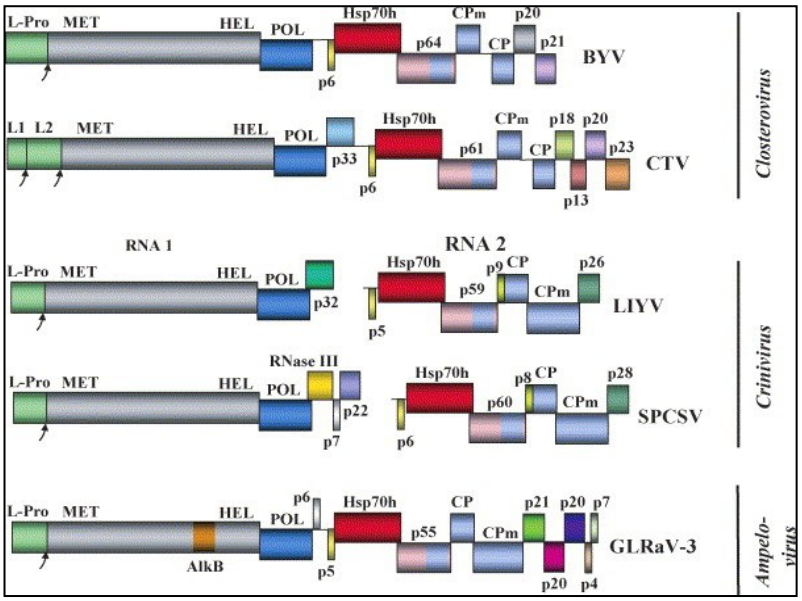


Figure 1. Genome maps of the family *Closteroviridae* genera. The genera names are shown on the right. BYV, *Beet yellows virus* (Agranovsky *et al.*, 1994); CTV, *Citrus tristeza virus* (Karasev *et al.*, 1995); LIYV, *Lettuce infectious yellows virus* (Klaassen *et al.*, 1995); SPCSV, *Sweet potato chlorotic stunt virus* (Kreuze *et al.*, 2002); GLRaV-3, *Grapevine leafroll-associated virus-3* (Ling *et al.*, 2004).

2.4. Host-virus relationships

CTV occurs in phloem cells in most species and hybrids of the genus *Citrus* and large number of the *Rutaceae*. Other hosts, which have been experimentally infected either by aphid, graft, or mechanical inoculation, include: *Aegle marmelos* (L.) Corr. Serr.; *Aeglopsis chevalieri* Swingle; *Afraegle paniculata* (Schum.) Engl.; *Citropsis gilletiana* Swingle and M.

Kellerman; *Microcitrus australis* (Planch.) Swingle; *Pamburus missionis* (Wight) Swingle (Müller and Garnsey, 1984); *Passiflora gracilis* Jacq.; *P. caerulea* L.; *P. incense* and *P. incarnate* (Roistacher and Bar-Joseph, 1987). CTV can replicate in protoplasts of *Nicotiana benthamiana* (Navas-Castillo *et al.,* 1995).

Some of the more important economic hosts are: sweet orange [*C. sinensis* L.], grapefruit [*C. paradisi* Macf.], mandarins [*C. reticulata* Blanco], limes [*C. aurantifolia* (Christm.) Swing. and *C. latifolia* Tan.], lemons [*C. limon* Burn. F.], pummelo [*C. grandis* (L.) Osbeck], tangelos [*C. reticulata* x *C. paradisi*], tangors [*C.reticulata* x *C. sinensis* (L.)], calamondin [*C. madurensis* Lour.], and kumquat [*Fortunella margarita* (Lour.) Swing.]. Sweet lime [*C. limettoides* Tan.], citron [*C. medica* L.] and combava [*C. hystrix* (DC) Swing.] are also infected. Many natural hosts of CTV remain essentially symptomless when infected by most of CTV isolates. Some citrus species show a selective susceptibility and are readily infected by some CTV isolates and not by others (Garnsey *et al.,* 1996b).

2.5. Symptoms of CTV

Virulence is affected by the CTV isolate and the environmental conditions. Since there are hundreds of citrus species, hybrids and citrus relatives, an isolate's virulence should be defined in terms of specific hosts. Being phloem-limited, most CTV symptoms are associated with damage of phloem and its function. Some isolates may cause few symptoms, even in plants that are normally reactive such as Mexican lime (Bové *et al.,* 1988). Most CTV strains causes vein flecking, leaf cupping, a transient leaf epinasty in young leaves, and some stem pitting on CTV-sensitive plants such as Mexican lime, *C. macrophylla* or *C. hystrix* (Fig. 2). Mild CTV infections can occur latently for long period of time and generally incite barely noticeable symptoms of vein clearing and flecking only on leaves of Mexican lime (Roistacher, 1991). There are three economically devastating field symptoms caused by CTV depending on virus strains and on the species or scion-rootstock combinations denoted: Quick decline (QD), Stem pitting (SP), and Seedling yellows (SY).

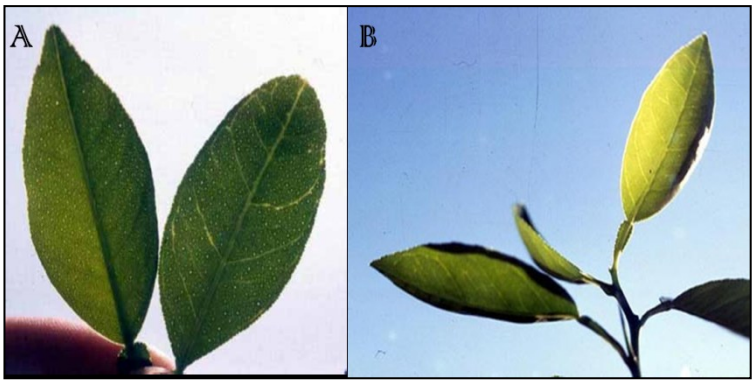


Figure 2. Greenhouse symptoms induced by Citrus tristeza virus (Roistacher, 2006). (A) Vein clearing and (B) Leaf cupping in Mexican lime leaves in glasshouse conditions at 18-25°C.

2.5.1. Quick decline (QD) syndrome of trees on sour orange rootstock

The most dramatic reaction of Tristeza disease is a decline syndrome caused by CTV infection of different citrus species (sweet oranges, mandarins, grapefruits, kumquats or limes) propagated on sour orange rootstock (Moreno *et al.*, 2008).

Tristeza quick decline is a syndrome in which a tree with normal appearance starts showing wilt symptoms and completely collapses in few weeks (Fig. 3A). Commonly, affected trees show dull green or yellow thin foliage, leaf shedding and twig dieback, small chlorotic leaves resembling the effect of nitrogen deficiency, and small pale-colored fruits that are unmarketable (Fig. 3B). CTV induces obliteration, collapse and necrosis of sieve tubes and companion cells close to the bud union, producing an excessive amount of non-functional phloem (Schneider, 1959). Starch movement from the canopy to the root system is blocked and starved roots are compromised in water uptake. This causes progressive dying and reduction of the root system with deficient supply of water and minerals, which results in wilting, chlorosis and dieback symptoms. During latent infections, the inner scion-rootstock interface shows typical 'pinholes' in the stem with corresponding 'pins' in the bark over the sour orange rootstock, which is called ''honeycombing'' or ''inverse pitting'' (Fig. 3C); it is generated by hyperplastic and/or hypertrophic medullar rays in the bark of sour orange species (Garnsey and Lee, 1988; Rocha-Peña *et al.*, 1995; Moreno and Garnsey, 2010). However, the most common reaction of CTV infected trees on sour orange rootstock is the slow decline, a process that may take from 2 to more than 10 years, along which, infection may occur symptomless or shows mild stunting. As this specific interaction does not occur with many other citrus species, the tristeza QD syndrome can be avoided using decline-

tolerant species as rootstocks (Moreno *et al.*, 2008). Tristeza QD epidemics have forced many countries to rebuilt new citrus industries based on the use of tristeza-tolerant rootstocks; these include species like trifoliate orange and its hybrids, such as Troyer and Carrizo citranges, which are resistant to most CTV strains, and other like rough lemon, Rangpur lime and Cleopatra mandarin, in which CTV replicates without causing obvious symptoms (Moreno and Garnsey, 2010).

Recently, a new disease called citrus sudden death (CSD) has been detected in Minas Gerais and São Paulo (Brazil) that causes decline and death of trees propagated on the tristeza-tolerant rootstocks Rangpur lime and Volkamer lemon, whereas trees grafted on Cleopatra mandarin or *Swingle citrumelo* (also tolerant to tristeza) remain symptomless. CSD is a bud union disease and affected trees can recover when they are grafted above the bud union with seedlings of the tolerant cultivars Cleopatra mandarin or *Swingle citrumelo* (Román *et al.*, 2004). A new marifivirus, *Citrus sudden death-associated virus* (family *Tymoviridae*), has been found associated with the disease and characterized, but its role in the etiology of CSD has not yet been demonstrated (Maccheroni *et al.*, 2005).

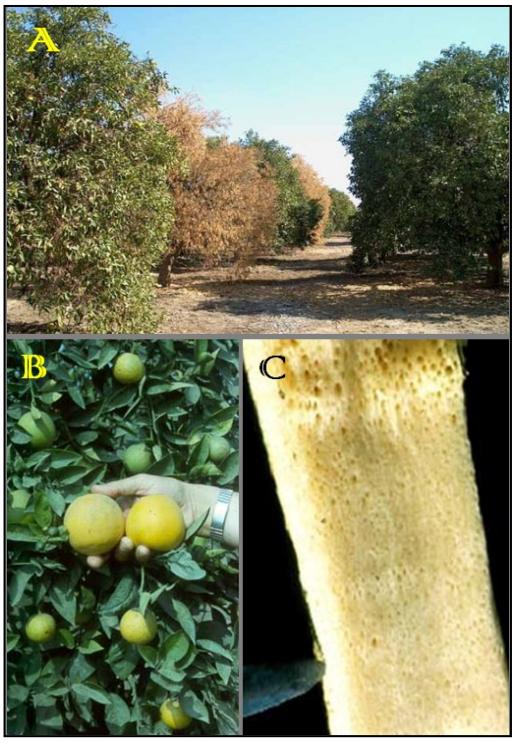


Figure 3. (A) Tristeza quick decline (QD) syndrome of sweet orange trees grafted on sour orange rootstock. (B) Reduction of fruit size of a navel orange by severe CTV isolates in Peru. (C) Honeycombing or inverse pitting induced by tristeza in the sour orange bark, below the bud union, of a sweet orange tree grafted on sour orange (Roistacher, 2006).

2.5.2. Stem pitting (SP) syndrome

Stem pitting symptom is likely initiated by the interruption of meristematic activity at limited areas of the cambium layer between the bark and the wood, which in turn results in irregular radial growth with local depression at the inactivated points (Schneider, 1959). SP may consist of isolated pits in vigorous shoots or very frequent pits that make the wood channeled and ropy in appearance. In some cases, extensive but shallow pitting occurs that gives the wood a porous appearance when the bark is removed. The SP syndrome, particularly in its more severe expression, causes variable stunting of the tree, thin foliage with small yellow leaves, low fruit set and a high ratio of small-sized fruits (Figs. 4A, B, C) with low juice content that are unmarketable (Rocha-Peña *et al.,* 1995; Roistacher and Moreno, 1991).

SP results from a direct interaction of CTV with a citrus cultivar, regardless of whether it is a seedling or used in graft combination as a rootstock or a scion. The effects of this disease depend on the CTV strain, on the cultivar affected and on the environmental conditions. The most sensitive species include acid limes, alemow, yuzu, *C. excelsa*, *C. hystrix*, Etrog citron and Palestinian sweet lime. A second group of intermediate sensitivity includes grapefruits, hassaku, Tahiti lime, some kumquats, pummelos, tangors and some citranges (Morton, Rusk, Savage), and sweet oranges cultivars, such as Pera in Brazil and Berna in Spain (Roistacher and Moreno, 1991; Timmer *et al.,* 2000; Wallace, 1978). The most tolerant species are mandarins, albeit CTV isolates causing SP in some mandarins have been reported from locations of Argentina, Japan, Inda, Indonesia or Central Africa (Du Charme *et al.,* 1951; Koizumi, 1991; Muharam and Whittle, 1991; Roistacher and Moreno, 1991).

The SP syndrome does not usually cause tree death, but induces unthrifty growth and chronic losses in yield that, even when moderate, may in the long-term cause greater cumulative losses than tristeza decline on trees propagated on sour orange rootstock; in the latter production is restored once damaged trees are replaced by those on tristeza-tolerant rootstocks. Moreover, the SP syndrome in susceptible scions cannot be avoided by propagating on a different rootstock. Thus areas invaded by these isolates may suffer severe permanent limitations for growing sensitive cultivars that may also be some of the most lucrative for the local market or for export.

SP is a limiting factor for production in parts of Brazil, South Africa and Australia (Garnsey *et al.,* 1987b; Bar-Joseph and Lee, 1990; Lee and Rocha-Peña, 1992). Severe SP destroyed the

important navel orange industry in the coastal regions of Peru (Roistacher, 1988; Bederski *et al.,* 2005). Presently, the only possibility to control SP damage is cross protection with selected mild CTV isolates. Cross protection has been used with variable success in different countries where SP strains were widespread (Moreno and Garnsey, 2010).

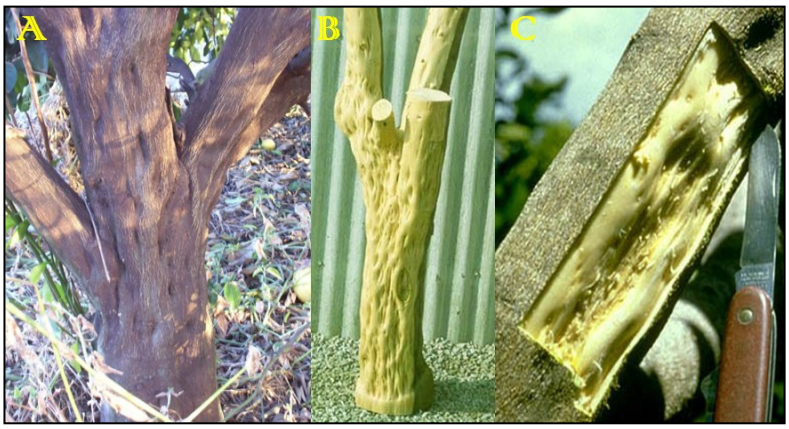


Figure 4. Stem pitting in grapefruit (A, B) and pera sweet orange (C) (Roistacher, 2006).

2.5.3. Seedling yellows (SY) syndrome

Some CTV isolates are able to induce in sour orange, grapefruit or lemon seedlings a syndrome called seedling yellows (SY), which is characterized by stunting, production of small and pale leaves, reduced root system, and sometimes a complete cessation of growth (Fig. 5) (Fraser, 1952; McClean, 1960). In some cases, severe CTV-SY isolates can be destructive and cause the death of tree in five months (Roistacher, 2006). The SY syndrome is often associated with severe isolates that cause SP in many citrus species, but it may be found also in isolates that do not cause SP, even in sensitive species. Conversely, some SP isolates are unable to induce SY, suggesting that both syndromes are likely produced by different pathogenicity determinants (McClean, 1963). In contrast to tristeza decline, which is typically a field syndrome and often difficult to produce in potted plants grown under controlled conditions, SY is essentially a syndrome observed in greenhouse-grown plants but rarely in the field (Moreno and Garnsey, 2010).

Figure 5. A close-up of the seedling-yellows reaction in a sour orange seedling: smaller pointed leaves and the yellows reaction; the non inoculated control plant is on the right (Roistacher, 2006).

2.6. The CTV genome

CTV is a member of the complex *Closteroviridae* family, which contains the largest positive-stranded RNA viruses of higher plants (Bar-Joseph *et al.*, 1979a; Dolja *et al.*, 1994; Karasev, 2000). The long flexuous virions of CTV are filamentous particles approximately 12 nm in diameter and about 2.000 nm in length and are extremely fragile (Bar-Joseph *et al.*, 1979a). The virions have helical symmetry with a pitch of 3-4 nm, about eight to nine capsids per helix turn, and a central cavity of 3-4 nm. The organization of the CTV genome was extrapolated from its sequence (Sekiya *et al.*, 1991; Karasev *et al.*, 1994, 1995; Pappu *et al.*, 1994). The 19.3-kb RNA is divided into 12 ORFs, potentially encoding at least 17 protein products, and two untranslated regions (UTRs) of about 107 and 273 nt at the 5′ and 3′ termini, respectively (Fig. 6A). ORFs 1a and 1b encode proteins of the replicase complex, whereas ORFs 2-11, spanning the 3′ terminal half of the gRNA, encode proteins p33, p6, p65, p61, p27, p25, p18, p13, p20 and p23 (Moreno *et al.*, 2008).

Sequences of the 5′-UTR have been unequivocally classified into three types (I, II and III), but their predicted secondary structure of minimum energy always consisted of two stem-loops separated by a short spacer region (López *et al.*, 1998). However, some sequences of the 5′-UTR were necessary for both replication and virion assembly (Gowda *et al.*, 2003b; Satyanarayana *et al.*, 2004). The 3′-UTR, a highly conserved region (López *et al.*, 1998), is critical for recognition by the replicase complex.

The coding region of the gRNA comprises the replication module (ORFs 1a and 1b), a five-gene module (p6, p65, p61, p27 and p25) encoding proteins involved in virion assembly and transport, which is conserved in all members of the family *Closteroviridae*, the p20 gene, a

homologue of the p21 gene of *Beet yellow virus* (BYV), and four genes encoding proteins with no homologue in other closteroviruses (p33, p18, p13 and p23) (Dolja *et al.,* 2006).

ORF 1a encodes a ~349-kDa polyprotein with two papain-like protease domains, a type I methyltransferase-like domain, and a helicase-like domain bearing the motifs of the superfamily I helycase domain. ORF 1b, via a frameshift, extends the 1a proteins by 54 kDa, which contain the RNA-dependent RNA polymerase (RdRp) domains (Karasev *et al.,* 1995) (Fig. 6A). Proteins encoded by the five-gene module include a transmembrane protein (p6), a homologue of the HSP70 plant heat-shock proteins (p65), two diverged copies of the capsid protein (p25 and p27) (Febres *et al.,* 1996; Pappu *et al.,* 1994), and an additional protein (p61) also regarded as a diverged CP copy (Dolja *et al.,* 2006). The p6 homologue in BYV has been shown to be a movement protein (Peremyslov *et al.,* 2004). The coordinate action of p65 and p61, in addition to the CP and CPm coat proteins, is required for proper virion assembly (Satyanarayana *et al.,* 2000).

In normal CTV virions, CP coats most of the gRNA and CPm only ~630 nt at the 5′-terminus, but in the absence of p65 or p61, CPm may coat larger segments or even the complete gRNA. These two proteins probably bind to the transition zone between CP and CPm and restrict CPm to the virion tail (Satyanarayana *et al.,* 2004). In other closteroviruses the homologues of p65 and p61 are coordinately assembled with CPm in the virion structure and remain attached to the virions (Tian *et al.,* 1999; Alzhanova *et al.,* 2007).

The p20 protein is a major component of CTV-induced amorphous inclusion bodies (Gowda *et al.,* 2000), and p23, an RNA binding protein with a Zn finger domain (López *et al.,* 2000), regulates asymmetrical accumulation of the positive and negative strands during RNA replication (Satyanarayana *et al.,* 2002b). Both p20 and p23, in addition to the CP, act as RNA silencing suppressors in *N. benthamiana* and *N. tabacum* plants, with p23 inhibiting intercellular silencing, CP intracellular silencing, and p20 both inter- and intracellular silencing (Lu *et al.,* 2004). Deletion mutants lacking genes p33, p18 and p13 were capable of replication and assembly (Satyanarayana *et al.,* 1999, 2000), indicating that they are not required for these functions. Their role in CTV biology remains unknown. By contrast, a construct carrying the green fluorescent protein (GFP) gene as an extra gene controlled either by a duplicated controller element (CE) of the CTV CP, or by a heterologous CE from BYV, stably replicated and moved in citrus plants and expressed GFP, suggesting that CTV can be reliably used as a virus vector for citrus (Folimonov *et al.,* 2007).

Additionally, other RNA molecules have been also distinguished from CTV, the defective RNAs (D-RNAs), which are virion RNA units that contain the 5′ and 3′ termini of normal gRNA in the lack of different internal portions (Mawassi *et al.,* 1995a). D-RNAs are variable between isolates and are thought to be correlated with SY symptom expression (G. Yang *et al.,* 1999). CTV isolates are frequently occurring as a complex of different genomic RNA populations associated with different properties, accompanied by multiple D-RNAs that vary in size, abundance and sequence (Lutting *et al.,* 2002). Therefore, a short 0.8 kb non-encapsidated ssRNA molecule has been exhibited from infected plants encompassing the 5′ terminal part of the CTV genome (Mawassi *et al.,* 1995a).

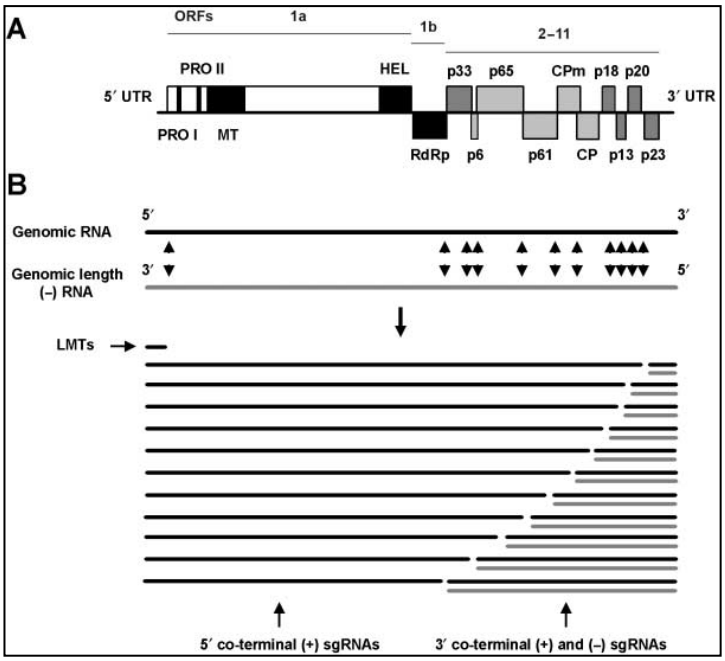


Figure 6. Organization and expression of the Citrus tristeza virus (CTV) genomic RNA (Moreno *et al.,* 2008). (A) Outline of the CTV genome. Boxes indicate ORFs, and proteins encoded by different ORFs are indicated. In ORFs 1a and 1b: PRO-I and PRO-II are papain-like proteases; MT, methyl transferase; HEL, helicase; and Rdrp, RNA-dependent RNA polymerase domains. CPm and CP are the minor and major coat proteins, and UTR indicates untranslated region. (B) Main RNA species produced for replication and expression of the genome. Black lines indicate positive stranded, and grey lines negative stranded, genomic or subgenomic (sg) RNAs. LMTs are low-molecular-weight tristeza sgRNAs. Arrowheads mark approximate positions of the controller elements inducing formation of sgRNAs.

2.7. Expression, replication strategies and translation of CTV

The CTV genome expression includes at least three mechanisms widely used by positive-stranded RNA viruses: proteolytic processing, ribosomal frameshifting and formation of a

nested set of 3'-coterminal subgenomic (sg) RNAs (Hilf *et al.*, 1995; Karasev *et al.*, 1995, 1997; Gowda *et al.*, 2001, 2003a, b; Satyanarayana *et al.*, 2002a, b; Ayllón *et al.*, 2003, 2004). Replication of the CTV gRNA involves synthesis of negative strands that serve as template for the generation of new positive strands, although the latter accumulate 10-20 times more than the negative strands (Satyanarayana *et al.*, 2002b).

ORFs 1a and 1b are directly translated from the gRNA. The first produces a ~349-kDa polyprotein, whereas translation of ORF 1b occurs by occasional +1 ribosomal frameshifting to yield a ~400-kDa polyprotein that is later proteolytically processed (Karasev *et al.*, 1995). The ten genes in the 3' half of the CTV gRNA are expressed by the synthesis of 3' coterminal sgRNAs that act as messenger RNAs from which the 5' proximal ORF is translated (Hilf *et al.*, 1995). A similar set of negative-stranded sgRNAs is also produced in infected cells, but these accumulate about 40-50 times less than positive-stranded sgRNAs (Satyanarayana *et al.*, 2002b).

Expression of the ten 3' proximal ORFs is regulated independently both in amount and in timing (Hilf *et al.*, 1995; Navas-Castillo *et al.*, 1997) by individual CEs that might serve for internal promotion from the minus strand gRNA template, or for the synthesis of negative-stranded sgRNAs by premature termination of some negative-stranded gRNAs (Ayllón *et al.*, 2003; Gowda *et al.*, 2001).

A set of less abundant 5'-coterminal positive-stranded sgRNAs is also generated, probably by premature termination of the gRNA at the CEs (Gowda *et al.*, 2001). Finally, two abundant positive-stranded 5'-coterminal sgRNAs of about 800 nt (LMT1 and LMT2) are produced by premature termination of the gRNA synthesis at a completely different CE, thus totaling more than 30 sgRNAs in infected cells (Che *et al.*, 2001; Mawassi *et al.*, 1995a; Ayllón *et al.*, 2004) (Fig. 6B). The viral function of this novel CE and of the two LMTs remains unknown (Gowda *et al.*, 2003b).

Frequently, CTV-infected tissues also accumulate large amounts of positive- and negative-stranded defective RNAs (D-RNAs) that contain the 3' and 5' termini of the gRNA but lack variable portions of the central region. These viral RNAs, which are easily observed by electrophoretic analysis of plant extracts enriched in double-stranded RNA (dsRNA), are very common in field isolates (Dodds *et al.*, 1987; Moreno *et al.*, 1990; Guerri *et al.*, 1991; Mawassi *et al.*, 1995a, b). Most D-RNAs are 2.0-5.0 kb in size, but large D-RNAs comprising ORFs 1a and 1b in their 5' proximal moiety, or ORFs 2-11 in their 3' terminal moiety, have

been reported (Che *et al.,* 2002, 2003). Those large D-RNAs resemble RNAs 1 and 2 of criniviruses. Sequence analysis of the junction site and flanking regions suggest that most D-RNAs must be generated by a template-switching mechanism induced by different factors (Yang *et al.,* 1997; Ayllón *et al.,* 1999a). The minimal replication signals required for D-RNA replication in trans are located in the 5′ proximal 1 kb and at the 3′-UTR of the D-RNA sequence (Mawassi *et al.,* 2000a, b). Although the biological role of D-RNAs is presently unknown, their wide occurrence in CTV isolates suggests that they may provide some advantage. At least in one case, the presence of a D-RNA was reported to modulate CTV symptom expression (G. Yang *et al.,* 1999).

The interaction of CTV with host factors is largely unknown, but analysis of the Mexican lime transcriptome using a citrus microarray showed altered expression of 334 genes after infection with a severe CTV isolate, about half of them without significant similarity to other known proteins (Gandía *et al.,* 2007).

2.8. Detection and strain characterization of CTV

For a virus like CTV, which manifests itself differently on different citrus cultivars and with different scion/rootstock combinations and which gives rise to many differentially reacting isolates and combination of isolates and to defective RNAs, the availability of an effective means of specific diagnosis is of the utmost importance. Strains of CTV have been identified primarily on the basis of their biological activities on a defined group of citrus indicator species (Garnsey *et al.,* 1987b). However, with the recent progress on serology and knowledge of the CTV nucleotide sequence, evolution on detection techniques has been shown for reliable differentiation of CTV strains (Niblett *et al.,* 2000).

2.8.1. Biological techniques

Diagnosis of CTV infection was performed for years by biological indexing on sensitive indicator hosts, mainly Mexican lime [*C. aurantifolia* (Christm.) Swing.] or *C. macrophylla* seedlings that upon CTV infection show vein clearing in young leaves, leaf cupping, short internodes and stem pitting (Roistacher, 1991).

Variations in biological characteristics of CTV isolates, including the type and intensity of symptoms induced or aphid transmissibility, have been observed since the first epidemics. Comparison of CTV isolates to provide a pathogenicity profile is still performed by indexing

on a panel of specific citrus indicators different indicator plants to define biogroups (Garnsey *et al.,* 1987b; Garnsey *et al.,* 1991; Ballester-Olmos *et al.,* 1993). A panel of five citrus cultivars or species combinations (Table 1) is generally used for the strain characterization (Garnsey *et al.,* 1987b). For the detection of more severe strains, the seedlings of grapefruit, lemon and the sour orange are highly sensitive to CTV seedling yellows; whereas, the Duncan grapefruit and the Madame vinous sweet orange seedlings provide satisfactory response to grapefruit and sweet orange stem pitting strains, respectively (Roistacher, 1991; Lee *et al.,* 1996). Additionally, the Madame vinous sweet orange is also an efficient indicator to the wood gumming induced by particularly severe CTV strains. Moreover, biological indexing still a time consuming technique (2-15 months), requires technical skills and high costs related to the greenhouse space, indexing facilities, plant and pest management, (Niblett *et al.,* 2000).

Table 1. Citrus indexing of CTV by Garnsey *et al.* (1991).

Citrus receptor	**Symptoms***	**Evaluation period** *Months post-inoculation*			
		2	**4**	**6**	**12**
Mexican lime	VC, LC	x	x	x	
Mexican lime	SP			x	x
Sweet/sour orange	CH, FL, ST		x	x	x
Sour orange seedlings	CH, ST	x	x	x	
Duncan grapefruit	CH, ST	x	x	x	
Duncan grapefruit	SP				x
Madame Vinous	SP, ST				x

* VC: leaf vein clearing; LC: leaf cupping; CH: leaf chlorosis; FL: flowering; ST: stunting, SP: stem pitting.

2.8.2. Serological techniques

Serological techniques have been used since the 1970s for routine detection of CTV. Enzyme-Linked Imunosorbent Assay (ELISA) and Direct Tissue Blot Immunoassay (DTBIA) are routinely used for CTV detection (Bar-Joseph *et al.,* 1979b; Garnsey *et al.,* 1993; Cambra *et al.,* 2000b). In Apulia region (Italy), a faster diagnosis by DTBIA is used instead of DAS-ELISA to carry out the CTV monitoring activities; through the use of this technique, several foci of the disease were identified.

Several monoclonal antisera have been produced to a number of different isolates and these detect nearly all isolates. Monoclonal antibodies (MAbs) have also been produced (Vela *et al.,*

1986; Permar *et al.,* 1990); some reacts to epitopes which are widely conserved among diverse CTV isolates and these also provide nearly universal detection, especially if two are used in combination (Cambra *et al.,* 1991); whereas other MAbs are isolate specific, the MCA13 has been used to differentiate mild isolates and from those that cause decline and stem pitting in Florida (Permar *et al.,* 1990). No monoclonal antibody has been developed which reacts specifically to only decline or stem pitting CTV isolates (Yokomi, 2009a).

2.8.3. Molecular techniques

Molecular techniques are more sensitive than ELISA and are useful for detecting the virus from field samples even when the titer is not optimum for serological tests (Saponari *et al.,* 2007). After the complete nucleotide sequence of the CTV gRNA, a variety of diagnostic procedures based on specific detection of viral RNA were developed, including molecular hybridization with cDNA or cRNA probes (Barbarossa and Savino, 2006) and several RT-PCR based methods (Nolasco *et al.,* 1993; Hung *et al.,* 2000; Cambra *et al.,* 2000a; Roy *et al.,* 2005). Real-time RT-PCR protocols have greatly improved sensitivity of detection and allowed quantification of genomic RNA copies in infected citrus tissues or in viruliferous aphids (Bertolini *et al.,* 2008; Ruiz-Ruiz *et al.,* 2007; Saponari *et al.,* 2007). Bertolini *et al.* (2008), based on Olmos *et al.* 1996, developed a tissue print (TP) and squash real-time RT-PCR protocol using TaqMan chemistry to detect CTV in plant tissues and vectors without the use of extracts and nucleic acids purification. This procedure reduces the time and the cost of the analyses and is applicable in routine tests of a large number of samples; furthermore, Vidal *et al.* (2012) showed that TP real time RT-PCR technique was more sensitive than the standard reference technique of DTBIA using the validated monoclonal antibodies 3DF1 and 3CA5, whereas DTBIA showed the highest specificity.

Rapid procedures were devised to study CTV variation and to identify specific isolates or to try to associate pathogenicity characteristics with different molecular markers (Moreno and Guerri, 1997). Some of these procedures detected differences in the CP by analysis of peptide maps generated with endoproteases (Albiach-Martí *et al.,* 2000b). Other procedures detected variations in viral RNA, including: (1) differences in dsRNA pattern between isolates (Dodds *et al.,* 1987; Guerri *et al.,* 1991; Moreno *et al.,* 1990, 1993), later shown to be due to the presence of D-RNAs in CTV isolates; (2) hybridization pattern with cDNA or cRNA probes of several gRNA regions (Rosner and Bar-Joseph, 1984; Rosner *et al.,* 1986; Narváez *et al.,* 2000; Albiach-

Martí *et al.,* 2000b;); (3) restriction fragment length polymorphism (RFLP) analysis of the CP gene (Gillings *et al.,* 1993; Roy *et al.,* 2003); (4) RT-PCR amplification patterns with primer sets specific for several CTV genotypes (Hilf *et al.,* 1999, 2005), for 5'-UTR sequence types I, II and III (Ayllón *et al.,* 2001; Ruiz-Ruiz *et al.,* 2007), or for three groups of isolates differing by their p23 sequence (Sambade *et al.,* 2003); and (5) single-strand conformation polymorphism (SSCP) analysis of different gRNA regions (Rubio *et al.,* 1996; Ayllón *et al.,* 1999b; d'Urso *et al.,* 2000, 2003; Sambade *et al.,* 2002, 2007). This last technique has been used to characterize the population structure of CTV isolates and select specific variants for sequencing, thus allowing estimates of the genetic diversity within and between isolates (Sambade *et al.,* 2003; Rubio *et al.,* 2001; Ayllón *et al.,* 2006).

Cevik *et al*. (1996) categorized different CTV strains based on their biological activities; they found minor but consistent differences in their nucleotide sequences. Consequently, many probes have been designed including universal probes which hybridize with all known CTV strains, as well as specific probes enable to discriminate between decline, stem pitting and mild strains of CTV. This technique could be used to determine whether individual Citrus plants were coinfected with more than one strain of tristeza (Niblett *et al*., 2000). Multiple molecular markers (MMM) technique provides the assignment of a specific isolate genotype based on the similarity of its yielded marker amplifications profile with those of the designated standard isolates. MMM was successfully used for the characterization and the assessment of genetic relatedness of three hundred seventy two CTV isolates within an international collection of exotic citrus pathogens (Hilf *et al*., 2005).

Yokomi *et al.* (2010) developed a multiplex real time RT-PCR assay for rapid differentiation and identification of CTV potential severe strains, in which three strain specific probes were used to separate VT3 and T3, T36, and T-36 like genotypes.

2.9. CTV genetic diversity

Genetic variation is unevenly distributed along the CTV gRNA, the most conserved region being the 3'-UTR, with over 95% identity between isolates, and the most variable being the 5'-UTR, with identity values as low as 44.45% between sequence types I and III (López *et al.,* 1998). Uneven distribution of variation is also observed in coding regions and even within genes, probably reflecting different selective pressures along the gRNA (Moreno *et al.,* 2008).

Factors shaping CTV populations in the field include mutation, recombination events between diverged sequence variants, selection, genetic drift and gene flow due to repeated inoculation of field trees and movement of infected buds between regions. Mutation due the error-prone nature of RNA-dependent RNA polymerases is the basic mechanism generating diversity (Moreno *et al.,* 2008).

CTV populations are also affected by natural and cultural dispersal of the virus. Citrus are long-lived trees that can be repeatedly inoculated by aphids with divergent CTV variants, thus increasing within-isolate genetic diversity (Kong *et al.,* 2000; Rubio *et al.,* 2001, Ayllón *et al.,* 2006). Uneven distribution of sequence variants within infected plants and/or random selection of some of them during aphid acquisition or transmission may be additional factors contributing to CTV population changes in the field (d'Urso *et al.,* 2000, 2003; Sambade *et al.,* 2007). The presence of divergent sequence variants in a CTV isolate also increases the possibility of genetic variation via recombination (Vives *et al.,* 2005). By contrast, the movement of CTV-infected buds tends to reduce genetic diversity between regions creating a unique CTV population. For example, CTV isolates from California and Spain were shown to be genetically the same population (Rubio *et al.,* 2001).

Although CTV seems to be genetically stable in some hosts, human-driven interactions with different scion/rootstock combinations under different environmental conditions might have generated genetic variability, later modulated by other factors such as recombination, selection, genetic drift or gene flow between regions (Moreno *et al.,* 2008).

Phylogenetic analysis of the complete sequences reported for nine CTV isolates revealed three main clusters that included: (1) the severe SP isolates T318A from Spain (Ruiz-Ruiz *et al.,* 2007), SY568R from California (Z.N. Yang *et al.,* 1999; Vives *et al.,* 2005), NuagA from Japan (Suastika *et al.,* 2001) and VT from Israel (Mawassi *et al.,* 1996); (2) the mild isolates T30 from Florida (Albiach-Martí *et al.,* 2000b) and T385 from Spain (Vives *et al.,* 1999); and (3) isolates T36 from Florida (Karasev *et al.,* 1995; Pappu *et al.,* 1994), Qaha from Egypt (AY340974) and a Mexican isolate (DQ272579) (Fig. 7). Within-group nucleotide identities were over 97.5%, whereas the lowest identity (75.6%) was between VT and Qaha.

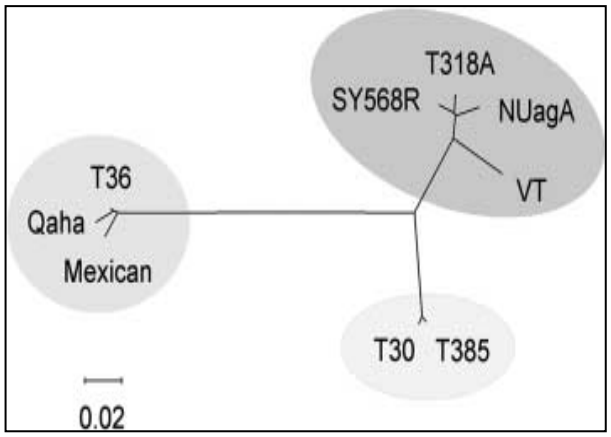


Figure 7. Neighbour-joining tree calculated with the complete nucleotide sequence of the genomic RNA from nine CTV isolates, using 1000 bootstrap replicates. Branch length indicates genetic distance. The three clusters obtained include severe isolates inducing seedling yellows (SY) and stem pitting in sweet orange and/or grapefruit (SP) (T318A, SY568R, NuagA and VT), mild non-SY, non-SP isolates (T30 and T385) and a group of isolates with intermediate characteristics (T36, Qaha and Mexican) (Moreno et al., 2008).

2.10. Transmission and epidemiology of CTV

CTV dispersal occurs by propagation of virus-infected buds and by vector transmission. The first is responsible for most CTV introductions into new areas, whereas the second is important for local spread (Moreno *et al.,* 2008). The virus has been also experimentally transmitted to healthy plants by dodder (*Cuscuta subinclusa*) (Weathers and Hartung, 1964) and by stem-slash inoculation with partially purified extracts (Garnsey *et al*., 1977), but these procedures are epidemiologically unimportant.

2.10.1. CTV transmission

Beside man, CTV is vector transmitted, in a semi-persistent manner (Bar-Joseph *et al.,* 1989) by several aphid species that vary in their efficiency of transmission (Garnsey and Lee, 1988). Members of Aphididae, *Toxoptera citricidus* (Kirkaldy), *Aphis gossypii* (Glover), and *A. spiraecola* (Patch) are the main vectors (Roistacher and Bar-Joseph, 1987). In a semi persistent mode, the previous aphid species may acquire and transmit the virus during short feeding probes with no latent period (Norman *et al*., 1972), but longer feeding times up to 24 h increase the efficiency of virus transmission (Roberts *et al*., 2001). CTV is reported to have no latent period with an acquisition and inoculation phases ranging from at least 30 min to 24 h (Costa and Grant, 1951; Bar-Joseph *et al*., 1989); but, acquisition and inoculation periods of

CTV by *T. citricidus* have been reported in seconds also (Retuerma and Price, 1972). Closteroviruses are not transmissible vertically, thus, aphids can remain viruliferous up to 24 - 48 hours after feeding in infected plants and normally lose their infectivity after two days feeding on a healthy plant and after molting (Roistacher, 2006). Previous investigations, using ELISA (Cambra *et al*., 1982), conventional RT-PCR (Mehta *et al*., 1997) and real-time RT-PCR diagnosis of purified TNA targets (Bertolini *et al*., 2008), reported that CTV can be successfully acquired by different vector and non-vector aphid species visiting citrus trees independently to their ability to transmit it.

2.10.1.1. The brown citrus aphid *Toxoptera citricidus* (Kirkaldy)

The brown citrus aphis (BrCA) *T. citricidus*, that means in Latin ''citrus killer'', is believed to be native to Asia where citrus originated. Since the first half of the twentieth century, the aphid has been known to be widely distributed on citrus in Asia, India, New Zealand, Australia, Pacific Islands (including Hawaii), Africa south of the Sahara, Madagascar, Indian Ocean Islands, and South America (Yokomi, 2009b). Recently, it has been detected in isolated citrus trees in northern Spain (Cambra, 2007) and Portugal (Ilharco *et al*., 2005), far from the important citrus-producing areas.

BrCA is the most important of the six reported aphid species that transmit CTV because of its high vector efficiency, prolific reproduction, and dispersal timed with citrus flush cycles to maximize chances of acquiring and transmitting the virus (Yokomi, 2009b). High populations of aphids during bloom periods can cause direct damage to citrus (Hall and Ford, 1933). The major damage associated with BrCA, however, is the transmission and spread of severe strains of CTV. Such strains cause rapid decline and death of citrus trees planted on sour orange rootstock regardless of tree age. The most virulent strains of CTV cause stem pitting in twigs, branches, and trunks of citrus trees regardless of rootstock. Stem pitting CTV weakens a tree and reduces fruit size, quality and quantity. This occurs over a period of 6 to 25 years depending on the virulence and challenge level of CTV (Yokomi, 2009b).

Virus dissemination by the BrCA has been particularly characterized as a bimodal transmission; whereby, varied aphid virus acquisition starvation can cluster around two periods: a short time period and a relatively long time probing (Chalfant and Chapman, 1962; Lim and Hagedorn, 1977). Powell *et al*. (2003) described the breakdown of "Ruby Red"

grapefruit trees cross-protected with three mild isolates of CTV (DD 102 bb, Guettler HS, and DPI 1-12-5-X-E), within 5 years after the BrCA get established in the experimental area. Consequently, infections with quick decline inducing CTV isolates reached 57, 81 and 71% for the protected trees by the three mild isolates, respectively, compared to 95% in unprotected ones.

The BrCA was vector responsible for the rapid spread of CTV decline that caused death of millions of citrus trees on sour orange in Brazil and Argentina in the 1930's and 1940's (Knorr and DuCharme, 1951) and in the 1970's in Colombia in 10-years period (Geraud, 1976; Lee *al.,* 1992). Currently, in South Africa, the BrCA is spreading CTV strains that are so virulent that economic longevity of grapefruit has been shortened to 6-8 years even though it contains a cross-protecting CTV isolate (Marais *et al.,* 1996). The BrCA was found to be 6 to 25 more efficient in transmission of various CTV isolates than was *A. gossypii*; it also transmits some strains of CTV that are not transmissible by other aphid species (Yokomi *et al.,* 1994). Currently, there are an estimated 200 million citrus trees on sour orange rootstock worldwide and are all at immediate risk to CTV decline (Garnsey *et al.,* 1996a).

The BrCA has been reported on other host species, such as *Rhododendron* sp., *Acerola* sp., *Malpighia punccifolia* and *Eugenia uniflora* in Puerto Rico (Yokomi, 1992; Yokomi *et al*., 1994), but also on *Passiflora* sp., *Mangifera* and *Anacardium* genera elsewhere (Roistacher *et al*., 2006).

The BrCA is anholocyclic and thelytokous throughout most of its range, preferring warm climates. It can, however, tolerate colder areas such as southern Japan by developing a holocyclic stage and overwintering as eggs (Komazaki, 1993).

The BrCA can be confused with *T. aurantii*, the black citrus aphid, because of its presence on citrus, dark brown-black coloration, size, and presence of stridulatory apparatus on the abdomen. However, alata of these aphids can be readily differentiated using a hand lens. *T. citricidus* has antennae III entirely black, forewing pterostigma light brown and media vein twice branched; *T. aurantii* has antennae III, IV, V, and VI banded at joints, forewing pterostigma conspicuously dark blackish-brown and media vein once-branched. Wingless adults and nymphs are more difficult to distinguish. The easiest character on apterae is the antennae. *T. aurantii* antennae have several banded joints, whereas *T. citricidus* have one prominent band near the middle. Setal length and patterns can be used to differentiate the aphids but require higher magnification. The cauda of *T. citricidus* is bushy with 25-40 setae, whereas that of *T. aurantii* is less bushy with 8-19 setae (Fig. 8). Another black aphid of citrus

is *A. carccivora* (Koch), cowpea aphid. It can be distinguished by its strikingly white legs (Knees of hind leg may be dark) and 7 caudal setae (Yokomi, 2009b).

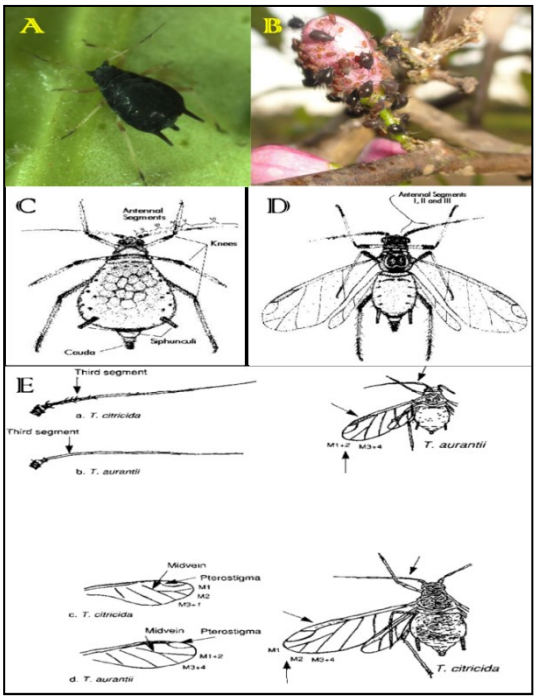


Figure 8. The brown citrus aphis *T. citricidus* (Kirkaldy). (A, C) Wingless adult; (B) colony of *T. citricidus*; (D) Winged adult; (E) Differential morphological characteristics of the front wings and antennae of ***T. citricida*** and *T. **aurantii*.**

2.10.1.2. The cotton or melon aphid *Aphis gossypii* (Glover)

Cotton aphid adults are soft-bodied insects, approximately 0.26 cm long, and vary from light yellow to dark green in color (Figs. 9A, B, C) (Blackman and Eastop, 1984; Slosser *et al.*, 1989; Ebert and Cartwright, 1997). Cotton aphids have both alatae and apterous (wingless) forms. Alatae forms occur in response to the deterioration of host plants, or overcrowding (Fry, 1982; Drees, 1993). Alatae aphids emigrate from deteriorating winter host plants and infest new host plants in the spring (Fry, 1982; Carter and Godfrey, 1999). Successive generations on the same host species during the spring and summer are usually apterous.

A. gossypii is a cosmopolitan and extremely polyphagous species. Ebert and Cartwright (1997) reported over 90 plant families in which at least one species was listed as a host. It is known to be the major pest of cultivated plants including *Cucurbitaceae*, *Rutaceae* and *Malvaceae*, as well as greenhouse plant species (Havelka, 1978). Damage occurs as a result of

direct feeding that reduces yield, production of honeydew and transmission of viruses causing more than 50 plant diseases, such as CTV, *Cucumber mosaic virus* (CMV) and *Water melon mosaic virus 2* (WMV 2) (Pitrat and Lecoq, 1980; Gray *et al.,* 1986).

A. gossypii is the second most efficient CTV vector, but is the main CTV vector in the Mediterranean basin and areas of North America (Dickson *et al.,* 1956; Raccah *et al.,* 1976; Hermoso de Mendoza *et al.,* 1984; Yokomi and Garnsey, 1987; Cambra *et al.,* 2000a; Marroquín *et al.,* 2004). This aphid is the most efficient vector of CTV in the absence of the BrCA. Comparative transmission experiments of several CTV isolates with these two vector species showed that *T. citricidus* can transmit CTV 6-25 times more efficiently than *A. gossypii* (Yokomi *et al.,* 1994). Dickson *et al.* (1956) reported that quick decline strains of tristeza were vectored by the cotton aphid in an extremely inefficient manner. However, Martinez and Wallace (1964), later reported that the cotton aphid transmitted three seedling yellows CTV isolates at rates from 25 to 78% using small replicate sizes.

2.10.1.3. The green citrus or spirea aphid *Aphis spiraecola* (Patch)

The green citrus or spirea aphid *A. spiraecola* is a small yellow or greenish-yellow aphid with black siphunculi and cauda, found in dense, moderately ant-attended colonies, curling and distorting leaves near the stem apices of a wide range of plants, particularly those of shrubby habit (Figs. 9D, E, F). Its numerous hosts are in more than 20 plant families, especially *Caprifoliaceae*, *Asteraceae*, *Rosaceae*, *Rubiaceae*, *Rutaceae*, and *Apiaceae*. Probably, its most important hosts are citrus species. *A. spiraecola* has a cosmopolitan distribution and appears to be mostly invasive and widespread around tropical and subtropical areas, while its presence in temperate climates is sometimes limited to sheltered or relatively warm habitats. In Japan, the aphid can also lay overwintering eggs on citrus (Komazaki, 1983, 1991, 1998), and its populations in the warm temperate areas (Mediterranean and comparable latitudes) show two main peaks of infestation, during spring and the early autumn months, synchronized with flushing of the shoots; lemon is not usually infested. Populations in most parts of the world are permanently parthenogenetic on secondary hosts, but in East Asia and North America, *A. spiraecola* has a sexual phase on Spiraea (Blackman and Eastop, 2007).

A. spiraecola has been reported as being less efficient CTV vector than *A. gossypii* under experimental conditions (Hermoso de Mendoza *et al.,* 1984; Yokomi and Garnsey 1987); however in some citrus areas *A. spiraecola* builds up larger populations than *A. gossypii* and

its role in CTV dispersal could be important (Hermoso de Mendoza *et al.*, 1984). Yokomi and Garnsey (1987), observed a 29% CTV transmission rate with *A. spiraecola* compared to 76% with *A. gossypii* using different Florida CTV isolates.

2.10.1.4. The black citrus aphid *Toxoptera aurantii* (Boyer de Fonscolombe)

T. aurantii affects all citrus species and cultivars (Figs. 9G, H, I). While polyphagous, mostly around the intertropical areas, citrus trees represent the main hosts of economic interest around the Mediterranean. It is considered to be strictly anholocyclic. Therefore, it overwinters as parthenogenetic morphs only, usually in sheltered environments (e.g. greenhouses) in those temperate zones that have a rather cold winter (Blackman and Eastop, 2007). It has been reported to be a minor vector of some CTV isolates, but not likely to be significant (Hermozo de Mendoza *et al*., 1984).

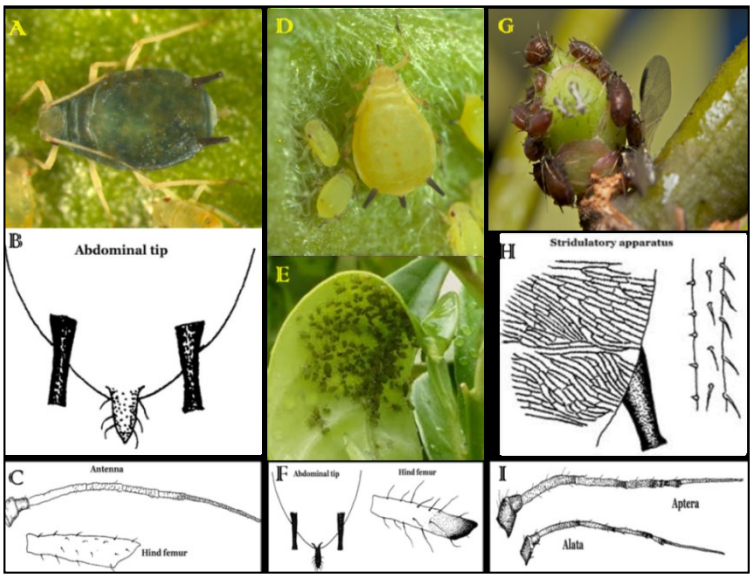


Figure 9. The cotton or melon aphid *A. gossypii* (Glover): (A) wingless adult of *A. gossypii*; (B, C) Differential morphological characteristics of the abdomen, antennae and hind femur of *A. gossypii*. The green citrus or spirea aphid *A. spiraecola* (Patch): (D) wingless adult of *A. spiraecola*; (E) colony *A. spiraecola* of and epinasty of citrus leaf; (F) Differential morphological characteristics of the abdomen and hind femur of *A. spiraecola*. The black citrus aphid *T. aurantii* (Boyer de Fonscolombe): (G) colony of *T. aurantii*; (H, I) Differential morphological characteristics of *T. aurantii*: stridulatory apparatus (H); (I) antennae.

2.10.2. CTV epidemiology

The CTV pathosystem is complex. Isolates of CTV vary greatly in symptom expression and a multitude of interactions can occur due to various combinations of the virus, the host tree, aphid vector species, and the environment. One of the most important characteristics of CTV epidemics is the complement of vectors that exist in the region. A number of factors influence the rate of CTV spread in a given area, including:

- ✓ composition of the aphid fauna (Gottwald *et al.,* 1996; Cambra *et al.,* 2000a);
- ✓ aphid population density (Dickson *et al.,* 1956);
- ✓ environmental conditions favoring new flush and aphid population build up (moderate temperatures, irrigation and fertilization) (Bar-Joseph and Loebenstein, 1973);
- ✓ susceptibility of the predominant citrus varieties (Roistacher and Bar-Joseph, 1984; Hermoso de Mendoza *et al.,* 1988b; Marroquín *et al.,* 2004);
- ✓ transmissibility of the predominant CTV isolates (Bar-Joseph and Loebenstein, 1973; Hermoso de Mendoza *et al.,* 1984, 1988a; Yokomi and Garnsey, 1987).

In many countries a lag period (sometimes more than 30 years) was observed between CTV introduction and noticeable field spread, suggesting a need for CTV isolates to become adapted to local aphid vector populations (Raccah *et al.,* 1980; Roistacher *et al.,* 1980; Bar-Joseph *et al.,* 1989). Failure to eradicate early CTV-infected trees allowed further epidemics, a situation that could be repeated with other exotic pathogens.

The BrCA and melon aphid have different patterns of spread reflecting differences in behavior (Gottwald *et al.,* 1996; Hughes and Gottwald, 1998). Citrus colonizers such as the BrCA, have the tendency to move virus from tree to tree with little distance travelled between infection centers and thus would spread disease more or less concentrically from initial points of entry and infection within a grove or region (Gottwald *et al.,* 1998). This species can reach such high populations during the flush period of the trees as to be a pest in its own right without including its ability to transmit CTV. The BrCA will not fly far when it moves between citrus trees, so adjacent trees are commonly colonized and infected with CTV in a radius of 4-8 trees (Gottwald *et al.,* 1998). Winter pruning produces excess flushing in the spring when aphids are active and tend to increase epidemics as it occurred in Israel (Bar-Joseph and Nitzan, 1991).

Conversely, much more diffuse, slow and random CTV spread pattern is generated by the cotton aphid compared to the BrCA (Gottwald *et al.,* 1998). However, when a mixture of

BrCA and cotton aphid coexists in the same area, the rapid spread of CTV over long distances may be due to *A. gossypii*, then, an aggregated efficient diffusion by *T. citricidus* results in a rapid increase of the CTV infections (Gottwald *et al.,* 1998).

2.10.3. Genomic alteration of viral CTV population by aphid transmission

CTV isolates may contain different sequence variants and it has been previously indicated that some of them can be altered by aphid or graft transmission upon a new host species (Moreno *et al*., 1993). Sub-isolates generated by these processes sometimes showed some divergence within the viral population of the source isolates. Separation of CTV variants from the field isolates has been reported in several studies through aphid passage (Gillings *et al*., 1993; Albiach-Martí *et al.,* 2000a; Brlansky *et al*., 2003). The BrCA has been commonly associated with effective transmission of severe SP isolates and sequence variation of the isolate after the transmission (Yokomi *et al*., 1994). As well, mild sub-isolates have been in some instances generated by *A. gossypii* transmission of more severe infections (Yokomi *et al.,* 1989). Conversely, the latter vector induced the emergence of severe sub-isolates that induced stem pitting on grapefruit and sweet orange from a designated mild isolate (B192) (Brlansky *et al.,* 2003).

Aphid transmission sometimes alters the expression of symptoms and the pathogenic characteristics of an isolate (Hermoso de Mendoza *et al*., 1988a; Moreno *et al*., 1993; Broadbent *et al*., 1996), as well as their serological reactivity against monoclonal antibodies (Kano and Koizumi, 1991; Cambra *et al*., 1993).

RFLP analysis of a cDNA of the *p*25 coat protein gene to differentiate CTV isolates showed disappearance of a marker after aphid transmission (Gillings *et al*., 1993). SSCP analysis, which has been efficiently applied for detecting single nucleotide mutation within the *p*25 gene of CTV isolates, provided its usefulness for analysis of genomic CTV populations and their variability after host passage (Rubio *et al*., 1996 and 2000).

In recent investigations, a preliminary screening of *A. gossypii* transmitted sub-isolates, deriving from the Californian SY568 isolate, has been performed based on RNase protection assays (RPAs) using the *p*65 heat-shock protein homolog probe; they showed that most of the segregants obtained were predicted to be severe in nature and caused some degree of stem pitting, stunting and vein corking, however, one of these new isolates (1B-a4) was predicted to be a mild strain (Velazquez-Monreal *et al*., 2009).

Individual aphid might sort from the same leaf some of the variants, even if these are not predominant, and transmit a subpopulation different from that of the source isolate; therefore, some minor variants of the former viral population could become predominant by a founder effect and give rise to new populations with divergent characteristics (D'Urso *et al*., 2000).

2.11. Control of CTV diseases

Control strategies for CTV differ according to the prevalence and severity of the CTV strains present (Garnsey *et al*., 1998; Lee and Bar-Joseph, 2000), the predominant scion/rootstock combinations and the population of the prevailing vector species in each area (Garnsey *et al*., 1998). Control of CTV is exceedingly difficult once established in a region if its natural vectors are also present. The best measures to avoid disease infection or dissemination in CTV-free areas include strict quarantine measures coupled with certification programs of plant propagation material, especially within countries of the Mediterranean Sea, where citrus are still exclusively grafted on sour orange rootstock (Navarro *et al*., 2002).

There is no single control strategy for CTV that will be applicable in all situations (Garnsey *et al*., 1998), therefore several approaches have been used to control the loss caused by CTV stains (Bar-Joseph *et al*., 1989; Rocha-Peña *et al*., 1995). Elimination of infected trees is the best means to avoid or delay an epidemic, if CTV is at low incidence and infected trees are grouped in a limited number of foci (Bar-Joseph *et al*., 1989; Kyriakou *et al*., 1996; Gottwald *et al*., 2002). If the incidence is less than 3%, eradication measures can provide various degrees of success (Bar-Joseph *et al*., 1989; Dodds *et al*., 1994). If the infected trees in the field can be destroyed in a greater number than the initiation of new infections, the incidence can be maintained at manageable levels (Dodds *et al*., 1994). Once the threshold of 5 % incidence has been reached, it becomes much more difficult to apply the eradication; therefore, others measures are needed (Bar-Joseph *et al*., 1989; Dodds *et al*., 1994).

2.11.1. Host tolerance

When the eradication of infected trees becomes unfeasible due to the efficient CTV dispersal through vectors, the propagation of citrus on tristeza-tolerant/resistant rootstocks is the only viable option to manage CTV without Tristeza decline (Moreno *et al*., 2008). However, a number of limiting factors makes more complicated the choice of suitable CTV-

tolerant/resistant species in an infected area regarding their response to the soil alkalinity, waterlogging in heavy soils, sensitivity to soil-borne and graft-transmissible pathogens, such as citrus blight and citrus sudden death (Román *et al*., 2004).

P. trifoliata and its hybrids Carrizo and Troyer citrange (sweet orange × *P. trifoliata*) and Swingle citrumelo (grapefruit × *P. trifoliata*) and Rangpur lime (*C. limonia* Osb.) are among the most widely used CTV tolerant rootstocks. The use of Rough lemon and mandarins as rootstocks instead of the sour orange enabled a good production of oranges in South Africa despite the coexistence of severe CTV strains and the BrCA vector (Bar-Joseph *et al*., 2010). Indeed, the presence of severe stem pitting (SP) inducing strains heavily damaged scions regardless to the tolerance of the used rootstocks.

2.11.2. Cross-protection

If severe SP isolates are widespread, the use of constitutive resistance to the virus or cross protecting sensitive varieties with appropriate mild isolates becomes necessary. This approach has prevented low yield and small-sized fruits of important varieties such as Pera sweet orange in Sao Paulo, Brazil (Costa and Müller, 1980), and Marsh grapefruit in South Africa (Van Vuuren *et al.,* 1993). Protected isolates were selected from field trees of the same cultivar that had grown for years displaying mild or no symptoms at all. It was assumed that these plants were protected against infection with the most aggressive variants and they were used as budwood sources to propagate new plants. However, the same protection strategy had limited success in other areas or with other varieties (Ieki and Yamaguchi, 1988; Müller *et al.,* 1988; Broadbent *et al.,* 1991), indicating that cross protection probably depends on the varieties, CTV strains and environmental conditions prevalent in each region.

The real mechanism behind the cross protection between related virus strains is still unclear. Whether cross protection is coat protein- or RNA-mediated has been a matter of discussion for years (Fraser, 1998). Although the role of the coat protein cannot be ruled out, post-transcriptional gene silencing (PTGS), a dsRNA-induced process, has recently arisen as a more likely mechanism to explain plant defense against viral infections and cross protection between closely related virus strains (Covey *et al.,* 1997; Ratcliff *et al.,* 1997, 1999; Hammond *et al.,* 2000). Indeed, Fagoaga *et al*. (2006) have observed high accumulation of viral-specific small-interfering (si)-RNAs in CTV-inoculated Mexican lime plants; this suggested that in cross protected host cells, the (si)-RNAs generated by the mild viral strain could prevent

subsequent infection by aggressive variants, able to depredate their genomic and subgenomic RNAs.

2.11.3. Breeding for resistance

Breeding to incorporate resistance genes in commercial cultivars is considered the best approach to avoid crop losses due to pathogens. However, different features of citrus biology, particularly their complex genetics and reproductive biology, together with their large plant size have greatly impaired genetic improvement through conventional breeding (Moreno *et al.,* 2008).

Although strain-specific resistance to CTV has been observed in some citrus species (Garnsey *et al.,* 1987a; Fang and Roose, 1999; Asíns *et al.,* 2004), resistance is more common in citrus relatives of other genera within the subfamily *Aurantioideae*, including some species of *Fortunella*, *P. trifoliata*, *Severinia buxifolia* and *Swinglea glutinosa* (Garnsey *et al.,* 1987a; Yoshida, 1996; Mestre *et al.,* 1997a, b). Particular attention has been devoted to *P. trifoliata*, which shows resistance to most CTV strains and is sexually compatible with *Citrus spp.*. This resistance is associated with a single dominant locus (*Ctv*) that has been thoroughly characterized and mapped (Yoshida, 1985, 1993; Gmitter *et al.,* 1996; Mestre *et al.,* 1997a; Fang *et al.,* 1998; Deng *et al.,* 2001;; Yang *et al.,* 2003). The *Ctv* locus has been restricted to a 121-kb segment in the Poncirus genome comprising ten genes (Rai, 2006).

2.11.4. Pathogen derived resistance

The first demonstration of pathogen-derived resistance to CTV came from the incorporation of the CP gene from a severe or a mild CTV strain in Mexican lime (Domínguez *et al.,* 2002). CTV inoculation of transgenic lines yielded two types of response: (1) most lines developed the same symptoms as the non-transgenic controls; and (2) some lines exhibited a resistance behavior that consisted of a fraction of plants (10–33%) being CTV immune, and the others showed a significant delay in virus accumulation and symptom onset.

Ghorbel *et al*. (2001) explored over-expression of the *p23* protein in transgenic plants as a potential method to interfere in the normal CTV infection process. Unexpectedly, constitutive expression of *p23* induced symptoms, resembling those incited by CTV in nontransgenic lime plants; whereas transgenic limes carrying a *p23*-truncated version were normal. Altogether, these results indicate that *p23* is an important CTV pathogenicity

determinant that interferes with plant development specifically in *Citrus* spp. and relatives (Fagoaga *et al.,* 2005).

Pathogen-derived resistance is feasible with CTV in its natural hosts, but factors other than the genetic background of the transgenic plant may affect the resistance phenotype displayed by transgenic plant propagations. Whether transgenic citrus plants expressing CTV-derived sequences could be an efficient alternative to cross protection for controlling SP damage in the field, or whether resistance to tristeza decline could be incorporated into the sour orange rootstock, remain to be tested (Moreno *et al.,* 2008).

2.11.5. Control of CTV vectors

Vector management should be one component of a disease management strategy, since CTV is transmitted only by aphid vectors that colonize citrus trees. Aphid populations on citrus are often too variable to provide sufficient natural enemies for effective vector control. Many of the natural enemies known to be effective against aphids include ladybeetles (Coleoptera: *Coccinellidae*), syrphid flies (Diptera: *Syrphidae*), lacewings (Neuroptera: *Chrysopidae*) and wasps (Hymenoptera: *Braconidae*) (John, 2000). Because new flushes of vegetation are available only in limited periods of the year, citrus aphids often occur together in time and space and tend to be attacked by the same range of antagonists (Michaud and Alvarez, 2000).

In Japan, *Lysiphlebus japonicus* Ashmead ((Hymenoptera: *Aphidiidae*) is the most important parasitoid of the BrCA (Takanashi, 1990). *L. testaceipes* (Cresson) was found attacking the BrCA in Puerto Rico (Yokomi and Tang, 1996) but parasitism rate was low as previously observed in Australia (Carver, 1984). Assuming that biological agents colonize new areas slower than their host, multiple augmentative releases of mass-reared parasitoids at various sites should be conducted (Wellings, 1994). *L. testaceipes* (Cresson) (Hymenoptera: *Braconidae*) is especially effective on *A. gossypii* and *T. aurantii,* causing up to 99% mortality (John, 2000), and readily *A. spiraecola* (Michaud and Alvazer, 2000).

Although insecticides may not act quickly enough to prevent primary infection by viruliferous aphids, reduction of aphid populations would decrease secondary spread. Insecticidal control effectiveness depends on longevity of suppression and extent of the treated area in relation to inoculums reservoir and migratory activity of the aphid (Knapp *et al.,* 1996). Insecticidal control of vector populations may have use in specific situations such in a citrus nursery or to protect budwood sources. A long residual systemic insecticide is

systemic insecticide with minimum impact on biological control agents is preferred. CTV titer is highest when trees are forming new shoots in spring and fall. Aphid flights also peak at this time and, hence, these periods should be targeted for control actions (Yokomi, 2009b).

Chapter III. OBJECTIVES

Following the report of the first CTV outbreak in the region of Loukkos in Northern Morocco (Afechtal *et al*., 2010), Tristeza is representing the major threat to the Moroccan citrus industry, especially that the CTV-sensitive sour orange is still the major rootstock used in the country and the most efficient virus vector, *T. citricidus*, is already established in neighboring countries Spain and Portugal. Therefore, the rapid identification of the CTV presence and its severity became critical for the management and control of the disease. As well, monitoring of *T. citricidus* and evaluation of the transmission efficiency of local CTV isolates by the present vectors are of utmost importance for predicting the disease dynamics, thus preventing further CTV outbreaks and/or maintaining the infection within acceptable limits.

In this context, the main objectives of this investigation are:

- ✓ Large scale monitoring of the presence, incidence and distribution of CTV in the main citrus growing areas of Morocco;
- ✓ Monitoring of CTV aphid vector species in the Loukkos and Gharb regions;
- ✓ Collection of CTV sources and study of their biological properties;
- ✓ Study of genetic variability of selected Moroccan CTV isolates by sequence analysis of the coat protein gene;
- ✓ Evaluation of aphid transmissibility of two Moroccan CTV isolates by the main CTV vector species landing on citrus;
- ✓ Study on the possible variations in CTV genetic structure after aphid transmission trials;
- ✓ Setting up of a Tissue Print Real Time RT-PCR assay for the rapid identification and differentiation of potential severe CTV strains and its use for further characterization of selected Moroccan CTV sources.

Chapter IV. MATERIAL AND METHODS

4.1. CTV survey

Sample collection was carried out in commercial orchards and nurseries in the main Moroccan citrus growing areas (Souss, Gharb, Moulouya, Tadla, Haouz and Loukkos) (Fig. 10), in addition to the varietal collections of INRA-El Menzeh in Kénitra and INRA-Tadla in Beni Mellal (Tables 2, 3, and 4). Samples were collected according to the hierarchical sampling (HS) scheme in which 25% of trees were monitored in the commercial orchards (Gottwald and Hughes, 2000) (Fig. 11). Tender shoots from 5192 trees were cut from different parts of the canopy and used for DTBIA test instead of the ELISA assay as applied by CIHEAM-MAIB. A detailed map was drawn up for each surveyed site, where information about position, rootstock, age, origin and location of each sampled tree were reported. In case of irregular citrus groves, sampled trees were marked directly in the field; any viral-like symptom and information potentially helpful for further sampling and investigation were registered, and representative photos were also taken. Some samples were collected from symptomatic trees which were not included in the HS scheme. Samples were kept in plastic bags, labeled and stored in a refrigerator and were transferred to the laboratory.

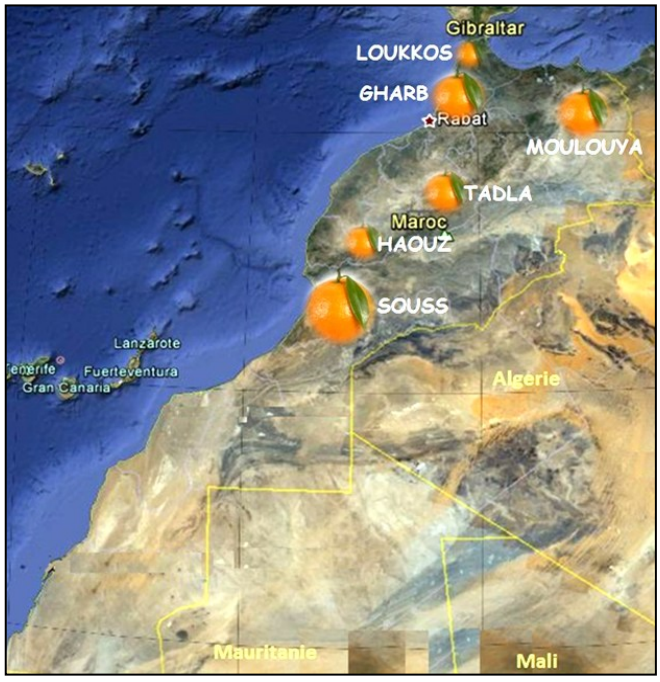


Figure 10. The main citrus growing areas in Morocco.

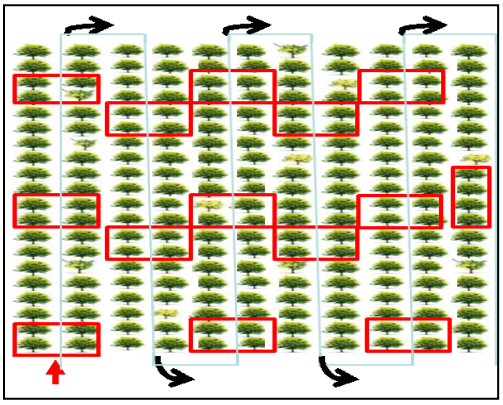


Figure 11. Hierarchical sampling scheme for CTV monitoring (Gottwald and Hughes, 2000).

Table 2. List of collected samples in commercial groves.

Region	Cultivar	Tested trees (No.)
Gharb	Maroc late orange	1147
	Washington navel	230
	Washington sanguine	142
	Thompson navel	15
	Common clementine	290
	Sidi Aissa clementine	74
Haouz	Maroc late orange	7
	Washington navel	7
	Washington sanguine	5
	Common clementine	28
	Nour clementine	8
Loukkos	Maroc late orange	762
	Common clementine	933
	Peau Fine clementine	15
	Sour orange	22
Moulouya	Berkane clementine	44
	Maroc late orange	3
	Navelina orange	8
	Nova mandarin	7
	Nour clementine	16
	Navel orange	4
	Ortanique mandarin	6
	Muska clementine	7
Souss	Isbal clementine	15
	Maroc late orange	20
	Ortanique mandarin	57
	Larache clementine	15
	Marisol clementine	7
	Nour clementine	38
	Nules clementine	37
	Common clementine	23
	Sidi Aissa clementine	8
	Navel orange	37
	Salustiana orange	15
Tadla	Maroc late orange	15
	Common clementine	23
	Navel orange	15
Total		**4105**

Table 3. Locations and number of trees sampled from citrus varietal collections during the survey.

Location	Varieties (No.)	Tested trees (No.)
INRA-Kénitra (Gharb)	52	940
INRA-Tadla	7	50
Total	**59**	**993**

Table 4. List of collected samples in the nurseries.

Region	Cultivar	Tested trees (No.)
Gharb (Mother trees)	Maroc late orange	2
	Washington navel	3
	Washington sanguine	3
	Navel lane late	4
	Larache clementine	2
	Sidi Aissa clementine	2
	Marisol clementine	2
	Nour clementine	3
	Nules clementine	2
	Nova mandarin	3
	Carte Noire clementine	1
	Lime	2
Moulouya (certified plants)	Berkane clementine	10
	Nour clementine	5
	Navel orange	5
	Nova mandarin	5
	Lime	10
Souss (certified plants)	Nules clementine	15
	Sidi Aissa clemetine	15
Total		**94**

4.2. Monitoring of citrus aphid species in the Gharb and Loukkos regions

The monitoring of aphid species was conducted in spring 2010 from 12 citrus orchards located in Gharb and Loukkos regions. The number of collected aphid samples was proportional to the citrus species present in the visited groves. In each grove, about 10 trees were randomly selected on the diagonals of the field and four infested shoots per tree (when present) were collected (Table 5). Aphids were collected from each infested shoot and stored in small vials in 70% ethanol.

Table 5. List of the surveyed orchards for the monitoring of citrus aphids.

Region	Orchard	Cultivars	Sample size (No. Trees)
Loukkos	1	Common clementine	10
	2	Common clementine	10
	3	Peau Fine clementine	10
	4	Common clementine and Maroc late orange	20
	5	Maroc late orange	10
	6	Maroc late orange	10
	7	Maroc late orange	10
Gharb	8	Common clementine	10
	9	Sidi Aissa clementine and Maroc late orange	20
	10	Maroc late orange	10
	11	Maroc late and W. navel oranges	20
	12	Thompson navel	10
Total			**150**

4.2.1. Aphids mounting

Clearing procedure and aphids mounting on slides were performed in order to improve the visualization of the insect morphology and to make easier and most effective the species identification when observed under a light compound microscope. About 15 aphids per grove were mounted singly on slides. According to Heikinheimo (1988), an appropriate mounting technique was used as below reported (Fig. 12A, B):

- ✓ ***Maceration:*** Aphids were transferred into staining vessels, where each individual was pricked with a sharp needle on the ventral side of the abdomen to intensify maceration. Aphids were then transferred in test tubes containing 10% KOH solution and moved to a water bath at 85-90°C for 5 min or longer prior to be transferred into cold KOH for 5-15 min and massaged till expelling most of their body content (embryos, etc.).
- ✓ ***Rinsing:*** Each rinsing phase lasted at least 10 min in distilled water containing 1-5% detergent fluid then in pure distilled water
- ✓ ***Clearing:*** Adult females were placed in 50% lactic acid in a waterbath at 85-90°C for 5-10 min.
- ✓ ***Slide preparation:*** Adult specimen (apterae and alatae) with whole legs and antennae were mounted on slides. Selected samples were transferred (dorsal side uppermost) on a drop of polyvinyl alcohol PVA (Danielsson, 1985), and placed on a microscopic slide (Annex 1). Antennae and legs were arranged under a stereozoom microscope (10X to 30X magnification). A clean coverslip was finally put in the middle of the slide to seal the sample.
- ✓ ***Labeling:*** Essential information was written on two standard labels 23x23 mm in size, glued onto slide at the side of the cover slip. The label on the right side included collection data about the origin, location, date, host plant, sample code number and collector's name, whereas the label on the left side included name of the genus, subgenus, and species, identifier's name, date of identification and mounting medium.
- ✓ ***Drying:*** Finally slides were transferred to a thermostatic stove at about 50°C for 10-14 days.

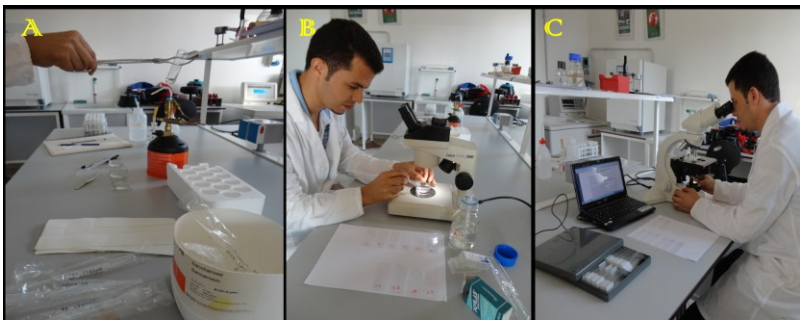


Figure 12. Aphids maceration and mounting (A, B); Taxonomic identification of aphid species under compound microscope based on the Blackman and Eastop (1984) Key (C).

4.2.2. Taxonomic identification of aphid species

Based on the key reported by Blackman and Eastop (1984), the taxonomic identification of the mounted specimens was made with a compound microscope at 100X to 400X magnification (Fig. 12C) (Annex 2). To evaluate the composition of aphid species in each surveyed field, only apterous adult aphids were observed directly under a stereozoom microscope for species discrimination and counting.

4.3. Serological detection and characterization

4.3.1. Direct Tissue Blot Immunoassay (DTBIA)

DTBIA is considered as sensitive as ELISA; it is a simple technique with little sample manipulation, which has been widely used for CTV detection (Garnsey *et al.*, 1993; Cambra *et al.*, 2000b; Djelouah *et al.*, 2001b). It can be performed using different plant tissues, but imprints obtained using flower sections give better results. DTBIA was employed to carry out a large-scale survey on the presence, incidence and spread of CTV in the main citrus growing areas in Morocco. DTBIA was performed using Plantprint kit (Spain); the plant stems and leaf petioles were printed into nitrocellulose membrane and processed as reported by Garnsey *et al*. (1993).

a. Preparation of the blotted membrane: Freshly made sections from tender shoots were pressed carefully on the nitrocellulose membrane, with four replicates, and left to dry for few minutes. The imprinted membranes were stored at 4°C and developed later at the CIHEAM/MAIB.

b. Membrane blocking: The printed membranes were blocked in 1% bovine serum albumin (BSA) solution, and incubated for 2 h at room temperature or overnight at 4°C with a slight agitation. Then, it was washed three times with PBS-Tween for 3 min each (Annex 3).

c. Addition of monoclonal antibodies (MAbs) linked to Alkaline Phosphatase (AP): The blocked membrane was incubated for 2-3 h with 3DF1+3CA5 mixture of monoclonal antibodies conjugated with alkaline phosphatase, and then washed.

d. Membrane development and reading: The membranes were incubated in the substrate buffer BCIP-NBT (Sigma fast tablets) till the appearance of the purple violet color in positive controls (3 to 7 min). The reaction was stopped by washing the membranes with tap water. Printings were observed under a low stereozoom microscope (10X to 30X) for the presence of the purple-violet precipitates in the vascular region of the imprints, indicating the presence of CTV.

4.3.2. DAS-ELISA

As recommended in the EPPO's protocols, the positive samples by DTBIA were confirmed by DAS-ELISA (Clark and Adams, 1977) using a commercial kit from Sediag (France).

Plates were coated with polyclonal antibodies diluted 1:100 in the coating buffer and incubated for 2 h at 37°C (Annex 3). After washing 3x3 min the plates with the washing buffer, fresh leaf samples were grinded in the extraction buffer and added to the plates; two wells were filled with 100µl each, in addition to negative and positive controls (Annex 3).

Plates were then incubated overnight at 4°C. After washing three times, 100µl of alkaline phosphatase linked antibodies diluted 1:100 in the conjugate buffer (Annex 3), were added to each well and the plates were incubated for 2 h at 37°C.

Plates were dried after the last wash, then 100µl of P-nitrophenyl phosphate prepared with 1mg/1ml in the substrate buffer (Annex 3), were added to each well. The plates were incubated at room temperature (RT) and an absorbance reading was done up to 2h in a conventional ELISA plate reader at 405nm. CTV sources were considered positive if the optical density (OD.405) values were more than 2.5 times above the values of healthy extracts.

4.3.3. Serotyping of selected CTV sources using the MCA13 monoclonal antibodies

Ten selected CTV sources from Loukkos, one from Gharb and one from Souss were collected and grafted for further characterization studies (Table 6). Aiming to evaluate the aggressiveness of these selected isolates, indirect antibody sandwich TAS-ELISA experiments were conducted using the MCA13 antibodies (MAbs) especially reactive with severe CTV strains from Florida (Permar *et al*., 1990). As reported by Cambra *et al*. (1991), ELISA plates were coated with polyclonal antibodies PAbs (Agritest, Italy) diluted (1:250) in the coating buffer and incubated for 2 h at 37°C (Annex 3). After washing the plates 3x3 min with the PBS-Tween washing buffer (Annex 3), fresh leaf samples were grinded in the extraction buffer (Annex 3) and added to the plates; two wells were filled with 100µl each, in addition to negative and positive controls. After an overnight incubation at 4°C and washing, 100µl of MAbs diluted in the conjugate buffer (1:2500) was added and incubated for 2 h at 37°C. After washing, 100 µl of antimouse diluted in the conjugate buffer (1:1000) were added and incubated again for 2 h at RT. Development of the plates was done by adding 100µl of P-nitrophenyl phosphate (1mg/1ml) in the substrate buffer (Annex 3). CTV sources were considered positive if the OD.405 values were more than 2.5 times above the values of healthy extracts.

Table 6. Selected CTV sources from Morocco for characterization studies.

Region	Cultivar	CTV source code
Gharb	Maroc late orange	G-Val-Mor
Loukkos	Common clementine	L-Clem1-Mor
	″	L-Clem2-Mor
	″	L-Clem3-Mor
	″	L-Clem4-Mor
	″	L-Clem5-Mor
	″	L-Clem6-Mor
	″	L-Clem7-Mor
	Maroc late orange	L-Val1-Mor
	″	L-Val2-Mor
	″	L-Val3-Mor
Souss	Ortanique mandarin	S-Ort-Mor

4.4. Biological characterization

The 12 previously selected CTV sources (see Table 6), were biologically characterized by inoculating Mexican lime seedlings, sour orange seedlings, Duncan grapefruit seedlings and

grafted Madame vinous sweet orange; Positive and negative controls were included (Roistacher, 1991). Budding and pruning equipments were disinfected in diluted sodium hypochloride solution.

Semi-hard wood cuttings from indicator plants were inoculated by chip budding using the bark tissue collected from the CTV sources, followed by grafting of these cuttings into citrange troyer. The inoculated plants were labeled and placed in greenhouse at cool temperatures (22-24°C). In order to verify the success of the graft inoculation into the specific indicators, DTBIA was carried out for all the indexed plants after 1 month.

The evaluation of the presence of CTV-syndromes was performed taking into account that vein-clearing and leaf-cupping can be induced by all CTV strains in Mexican lime (universal indicator); CTV-stem pitting strain (SP) can provoke pittings in grapefruit and rarely in sweet orange, whereas "yellows" reaction in sour orange and grapefruit seedlings are induced by CTV-seedling yellows strain (SY).

4.5. Molecular characterization

The 12 previously selected CTV sources (see Table 6), were used for molecular characterization using Single Strand Conformation Polymorphism analysis (SSCP) and sequence analysis of the coat protein gene.

4.5.1. Total nucleic acids extraction (TNAs)

Total nucleic acids (TNAs) were recovered from 0.2 g of leaf petioles ground in liquid nitrogen as described by Foissac *et al.* (2001). After grinding in 1 ml of the extraction buffer (Annex 4), a volume of 500µl of the extract was transferred to microcentrifuge tubes containing 100µl of 10% sodium lauryl sarcosyl and incubated at 70°C for 10 min with intermediate shaking, then briefly chilled in ice for 5 min. After centrifugation at 13.000 rpm for 10 min, 300µl of the collected supernatant were mixed with a solution containing a mix of 150µl of absolute ethanol, 35µl of re-suspended silica and 300µl of 6M sodium iodine solution (NaI) (Annex 4). The mixture was then incubated at room temperature for at least 10 min with intermittent shaking and then centrifuged at 6.000 rpm for 1 min. The pellet was recovered and washed 2-3 times by re-suspending in 500µl of the washing buffer (Annex 4). TNAs were then eluted in 150µl of RNase-free water after incubation for 4 min at 70°C and centrifugation at 13.000 rpm for 3 min. Finally, TNAs were stored at -20°C until use.

The concentration of TNAs was determined through spectrophotometer analysis at 260 nm and the product quality was monitored by the ratio A260/A280 and by agarose gel electrophoresis (Annex 5).

4.5.2. cDNA synthesis

For reverse transcription, first strand cDNA was synthesized from 0,5µg of total RNA in a final volume of 20µl of reaction mixture containing 4µl of 5X first strand buffer, 2µl of 0,1M dithiothreitol, 3µl of 10mM deoxy-nucleotidetriphosphate (dNTPs), 1µl random primer (0,5µg/µl concentration) and 0,8 µl of 200U Moloney-Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Invitrogen, USA), the obtained mix reaction was incubated at 37°C for 1h, prior to be heated at 70°C for 10 min in order to inactivate the reverse transcriptase enzyme, and stored at -20°C.

4.5.3. PCR

Five µl of CTV cDNA were used in a final volume of 25 µl of reaction vessel containing 1X GoTaq Flexi DNA polymerase buffer (Promega, USA), 1,5 mM $MgCl_2$, 0,2mM dNTPs, 0,2µM of each sense and anti-sense primers of the coat protein gene (Hilf *et al.,* 2005) and 0,2µl of GoTaq Flexi DNA polymerase (5U/µl) (Promega, USA). Amplification profiles comprised an initial denaturation at 95°C for 5 min, followed by 40 cycles of dsDNA denaturation at 95°C for 30s, primers annealing at 56°C for 1 min and extension at 72°C for 1 min. Amplicons were incubated for additional 7 min at 72°C for final DNA extension, prior to be maintained at 4°C until analysis. PCR products were electrophoresed in 1,2% agarose gel in 1X TAE buffer and stained with ethidium bromide (25ng/ml) and visualized on UV trans-illuminator (Annex 5). The expected molecular size of the PCR products was determined by comparison to the GeneRuler 100bp Plus DNA Ladder (Fermentas, UK).

4.5.4. Single Strand Conformation Polymorphism analysis (SSCP)

The test was performed for the CTV coat protein gene (CTV-CPg) amplified by RT-PCR using the P25f/r primers aiming a primary molecular differentiation between isolates prior to cloning and sequencing. For each sample, 2µl of RT-PCR products were mixed with 9 µl of the denaturing buffer (95% formamide, 20mM EDTA, 10 mM NaOH and trace of bromophenol-blue). Samples were then heated at 95°C for 5 min, and immediately chilled in

ice for 5 min. Denatured PCR products were separated by electrophoresis in a 8% non-denaturating polyacrylamide gel using 1X TBE buffer at 4°C and a constant voltage of 200V for 3-4 h (Annex 5). Afterward, classical colorimetric detection of the gel consisted on its fixation in 10% acetic acid solution for 20 min, incubation in 1% nitric acid solution for 3 min, then, its staining for 15-20 min in silver nitrate solution, separated by 3 min washing with distillated water (Annex 5). Finally, the developing solution was added and the gels were incubated till the appearance of the bands; this reaction was stopped by immersing the gel in 10% acetic acid solution for 10 min for better conservation (Beidler *et al*., 1982) (Annex 5).

4.5.5. Cloning and sequencing of the CTV-CPg

The 12 previously selected CTV sources (see Table 6) were submitted to further CPg cloning and sequencing.

Viral targets were amplified with the P25f/r universal primers yielding 672 bp and electrophoresed in 1.2% agorose gel, then eluted using the Wizard SV gel and PCR clean-up system kit (Promega, USA) according to the Manufacturer's instructions. Afterward, the purified amplicons were ligated the pGEM-T Easy Vector (50 ng/µl) (Promega, USA) and transformed to competent *Escherichia coli* DH5α bacterial cells (Invitrogen, USA) (Sambrook *et al*., 1989). As recommended by the Manufacturer's protocol, within each ligation reaction, a Control Insert DNA (4 ng/µl) was included for assessing whether the ligation proceeded efficiently, in addition to a background control to determine the number of blue colonies resulting from non-T-tailed pGEM-T Easy Vector. In order to optimize the number of recombinants, ligation reaction mixtures were incubated overnight at 4°C.

Preparation of *E. coli* competent cells

A single colony of *E. coli* DH5α (Invitrogen, USA) was inoculated in 2 ml of Luria-Bertani (LB) medium and incubated at 37°C for 16 h with shaking at 250 rpm (Annex 6). Additional growth of bacterial cells was performed by inoculating 100µl of bacterial culture in 10 ml of LB medium and incubating them at 37°C for 2 h with shaking at 250 rpm until the OD.600nm reaches 0.5-0.6. Then, the culture was transferred to a 50 ml falcon tube, chilled in ice for 15 min and the bacteria were collected centrifugation at 6.000 rpm for 5 min at 4°C. After supernatant discarding, the bacterial pellet was gently resuspended in 5 ml cold 0,1M $CaCl_2$ (Annex 6) then stored in ice for 30 min. After 5 min centrifuge at 6.000 rpm, the $CaCl_2$-treated

bacteria were resuspended again in 0.5 ml of 0.1M $CaCl_2$ and kept in ice for 2 h before the transformation step.

E.coli transformation

One hundred microliters of the $CaCl_2$-treated *E.coli* DH5α competent cells were mixed with 4µl of ligation mixture in a sterile tube and were kept in ice for 30 min. Incorporation of the plasmid into the competent cells suggested a heat shock treatment whereby the competent cells were incubated in a water bath at 42°C for 45s, and immediately transferred in ice for 5 min. Afterward, cells were supplied by 600µl of pre-warmed LB medium and incubated for 30 min at 37°C followed by additional 30 min at 37°C with agitation at 250 rpm (Annex 6). Once recovered, 150µl of bacterial suspension was plated in the LB-Agar medium supplied with 40µl of 20 mg/ml X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopiranoside) (Invitrogen, USA) and ampicillin (50 mg/L), using a sterile bent glass rod. Plates were incubated overnight at 37°C.

Screening of bacterial colonies

Among the transformed *E. coli* cells, a number of white colonies suspected to harbor the recombinant plasmid with the target insert were picked up, then, re-inoculated in new LB-Agar plates with a sterile toothpick, and simultaneously, eluted in 50µl of the triton buffer (1% triton X-100, 20mM Tris-HCl and 2mM EDTA, pH 8.0) in a new microcentrifuge tube. The latters were boiled for 10 min to release the suspected recDNA from the bacterial cells.

In order to confirm the presence of the target insert, PCR reactions were performed with the same universal primers P25f/r in a final volume of 25µl reaction using 2µl from the bacterial lysate.

Extraction of plasmid recombinant DNA (*rec*DNA)

E. coli selected colonies were picked up with sterile lance, inoculated in 2 ml of LB liquid containing ampicillin (50 mg/l) and incubated overnight at 37°C with shaking (250 rpm). Bacterial suspensions were poured into a 1,5 ml micro-centrifuge tube, and then centrifuged at 12.000 rpm for 30s and the supernatant was discarded. The pellet was resuspended in 350µl of STET buffer (Annex 6) and 20 µl of lysozyme (20 mg/ml) by rigorous vortex. Lysis of the bacterial cells was obtained by boiling for 40s, and immediate chilling in ice for 5 min.

After full speed centrifugation at 13.000 rpm for 20 min, the pellet of bacterial debris and genomic DNA were remove with sterile toothpicks. Plasmid DNA were extracted with 400µl of 1:1 (v/v) of phenol/chloroform by centrifugation at 13.000 rpm for 10 min. Supernatant were gently recovered, transferred to new tubes and precipitated for 20-30 min in ice, in the presence of 125µl of 5M ammonium acetate solution (pH 3,5) and 1 ml of cold absolute ethanol followed by centrifugation at 13.000 rpm for 10 min. Pellets were washed using 500µl of 70% cold ethanol and centrifuged again for 10 min at 13.000 rpm. After supernatant elimination and drying into vacuum pump for 1 h, it was resuspended in 50µl TE buffer containing 1µl of RNase A (10 µg/µl), vortexed and conserved in ice until analysis (Annex 6).

Plasmid enzymatic digestion and purification

The presence of the specific insert inside the extracted plasmids was confirmed by an enzymatic digestion with the restriction endonuclease EcoRI (Promega, USA) (Annex 7). Reactions were carried out in a total volume of 10µl, in which 10 to 15 ng of plasmid DNA were added to 1µl of 10X H buffer (Promega, USA), 2U EcoRI and incubated for 1 h at 37°C. The quality and the quantity of the digested products were confirmed by 1,2% agarose gel electrophoresis.

Further purification of the selected plasmids was performed by precipitation with 30 µl of the PEG-NaCl solution in ice for 1 h (Annex 6). Then, centrifuged at 13.000 rpm for 5 min; after supernatant removal, the pellet was washed by 100µl of 70% cold ethanol, centrifuged at 13.000 rpm for 5 min, dried for 1 h at RT and finally dissolved in 30µl of sterile distilled water.

PCR of recDNA and CPg sequence

White colonies were picked and added to a PCR mixture for recDNA amplification using the universal primers P25f/r then, SSCP analysis was performed in order to study the conformation complexity of the SSCP patterns obtained from each CTV source. Clones that presented different SSCP profiles originated from each CTV source were chosen for cDNA sequencing (Primm, Italy).

Computational-assisted sequence analysis

The obtained sequence from representative recDNAs were aligned using the BioEdit software version 7.0.9 (Hall, 1999) and the MEGA software version 5.05 (Tamura *et al.*, 2011). Obtained sequences were then confronted with other strain references available in the NCBI Data Base: T385, T30, 19-21, B301, VT, SY568, T36 and SP retrieved from the GenBank (Y18420.1, AF26065, AF184114, JF957196.1, U56902, AB046398, AY170468 and EU857538.1 accession numbers, respectively). Calculation of pair wise nucleotide distances between sequences and clustering were done using the phylogenetic and molecular evolutionary genetic analysis software MEGA version 5.05 for the estimation of the nucleotide homology (Tamura *et al.*, 2011).

4.6. CTV aphid transmission trials

4.6.1. CTV sources

Two CTV isolates from Morocco, L-Clem1-Mor and L-Clem2-Mor, were used in transmission trials. Infected CTV source plants from the field "donor plants (D.P)" were graft inoculated on Mexican lime seedlings and maintained under greenhouse conditions. Two replicates of donor plants were adopted during aphid transmission trials.

4.6.2. Aphid species as CTV vectors

A. gossypii and *A. spiraecola* species were used to conduct the transmission trials. Virginopare aphid species were picked up from infested citrus plantings (CTV-frees). The collected aphids were maintained in virus-free Carrizo citrange plants for 48h aiming to prevent eventual CTV infections. At the same time, some specimens of the collected aphids were subjected to rigorous mounting on slides as described by Heikinheimo (1988) and their identification was carried out using the Blackman and Eastop (1984).

4.6.3. Aphids rearing

A. gossypii rearing was performed on "celia" cotton (*Gossypium hirsutum*) in a growing chamber at 23/16°C, day/night, and a photoperiod of 16/8 h (Fig. 13A); while, populations of *A. spiraecola* were maintained on Viburum (*Viburnum suspensum*) and on healthy citrus plantlets.

Pure progeny was generated for each species starting from one single virginopare, in order to avoid the development of mixed aphid populations, and transfer of any parasitoids and/or predators from the rearing plants. The growing chamber was equipped with an air ventilation system, supplementary electric power, a thermohygrometer apparatus, cages with 200µm mesh diameter and abundant yellow aphid traps. Plants used as aphid hosts were renewed weekly.

4.6.4. CTV transmission

The methodology of aphid inoculation was partly based on the procedure described by Roistacher (2006). One leaf fully infested with aphids was divided into small pieces, which were deposited on new flushes of the selected donor plants in order that aphids acquire the virus during 24 to 48 h. For each transmission experiment, 20 aphids were transferred, using a fine brush, to the young tender leaves of each receptor Mexican lime seedlings (Fig. 13). After 48 hours of infection feeding, test plants were treated with an aphicide, transferred to a thermo-conditioned greenhouse (22°C to 28°C) and observed for typical tristeza symptoms over a minimum period of 60 days of post-inoculation as pointed out by Velazquez-Monreal *et al*. (2009).

Fifteen virus-free 2-6 months-old Mexican lime plants were used for transmission trials. Vector-exposed plants were given a code reflecting the isolate from which they were derived followed by the aphid species used for transmission (A.g and A.s corresponding respectively to *A. gossypii* and *A. spiraecola*) and the replication number, (e.g. L-Clem2-Mor Ag.1).

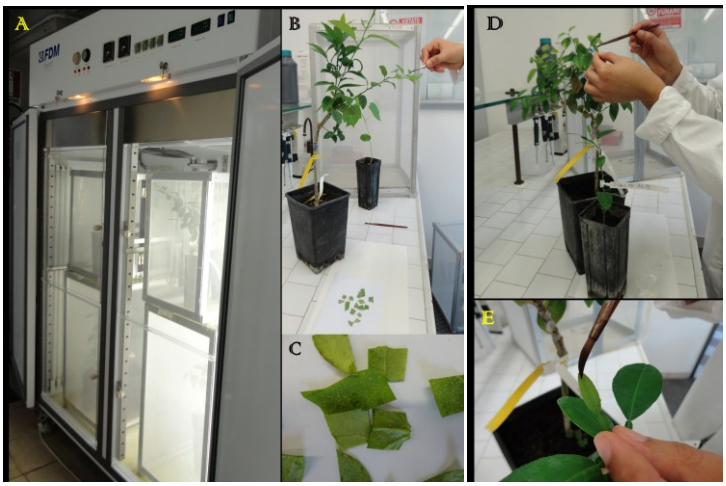


Figure 13. CTV aphids vector rearing and transmission trials. (A) incubator for rearing and transmission trials; (B) inoculation of a CTV infected donor plant using fully infested pieces of cotton with *A. gossypii*; (C) pieces of cotton infested with *A. gossypii*; (D and E) transfer of *A. spiraecola* using a brush from a donor plant to new flushes of a CTV free receptor plantlet.

4.6.5. CTV detection and characterization of aphid-inoculated sub-isolates

Aphid-infected plants were submitted to preliminary screening by DTBIA, prior to CTV detection by conventional RT-PCR analysis.

4.6.5.1. Serotyping of the CTV sub-isolates after aphid passage

TAS-ELISA was applied to test the aphid-inoculated sub-isolates using the MCA13 monoclonal antibody (see paragraph 4.3.3).

4.6.5.2. Molecular characterization of CTV sub-isolates after aphid passage

Successfully aphid transmitted CTV sub-isolates were subjected to further molecular analysis. Total nucleic acids were recovered from the parental CTV isolates (L-Clem1-Mor and L-Clem2-Mor) and from their correspondent aphid derived sub-isolates. In order to reveal eventual nucleotide mutations after the aphid passage, a serial of RT-PCR amplifications were performed, focusing on three genomic regions of CTV including p18, p23 and p25. cDNA templates (5 µl) were amplified in a final volume of 25 µl of reaction vessel containing 1X GoTaq Flexi DNA polymerase buffer (Promega, USA), 1.5mM $MgCl_2$, 0,2mM dNTPs, 0,2µl of GoTaq Flexi DNA polymerase (5U/µl) and 0,2µM of each sense and anti-sense primers (P18f/r, P20f/r and P25r/f) (Table 7). Amplifications conditions consisted in 5 min first denaturation at 95°C, followed by 40 cycles of 95°C for 30s, 56°C for 1 min and

72°C for 1 min. Amplicons were incubated for additional 7 min at 72°C for final DNA elongation. Resulted amplicons were checked by electrophoresis on 1,2% agarose gel.

Table 7. Oligonucleotide primers used for the amplification of the CTV genes encompassing p18, p23, and p25 proteins.

Primer	Oligonucleotide sequence (5′ to 3′)	Binding site*	Size bp
P18	(+)ATGTCAGGCAGCTTGGGAAATTT	16.754 -16.778	504
	(-)CTAAGTCGCGCTAAACAAAGTGA	17.234 -17.257	
P23	(+)ATGGATGATACTAGCGGACAAAC	18.357-18.382	630
	(-)TCAGATGAAGTGGTGTTCACGGA	18.962-18.986	
P25	(+) ATGGACGACGAAACAAAGAAATTG	16.117-16.141	672
	(-) TCAACGTGTGTTGAATTTCCCA	17.766-17.788	

*Binding site of the primers is given on the positive strand of gRNA of isolates T30 (EU937520).

4.6.5.2.1. Multiple SSCP analysis of the CTV vector inoculated sub-isolates

SSCP analysis was performed by denaturing RT-PCR products flanking three different amplified genomic fragments (p18, p23 and p25) in an 8% non-denaturing polyacrylamide gel. Small changes in the nucleotide sequence may alter conformation of ssDNA and, consequently, its electrophoretic mobility. Resulted patterns of the CTV sub-isolates derived from aphid transmission were then compared with those of their correspondent parental isolates (L-Clem1-Mor and L-Clem2-Mor).

4.6.5.2.2. Cloning and partial sequencing of the vector-inoculated sub-isolates

Once purified, PCR targets of p18, p23 and p25 genes were ligated into the PGEM-T Easy vector and used to transform the *E. coli* competent cells as previously described (paragraph 4.5.5). recDNA targets were subjected to additional PCR reactions with the correspondent primers used initially; then, a number of 20 clones were separated by SSCP analysis. Selected plasmids were purified prior to be sequenced in both directions using (Primm, Italy). For both isolates (L-Clem1-Mor and L-Clem2-Mor), the obtained sequences from the field were compared to their correspondent aphid derived sub-isolates using the BioEdit software version 7.0.9 (Hall, 1999).

4.7. Tissue Print Real Time RT-PCR

Tissue printing real time RT-PCR technique for a rapid differentiation and identification of potential severe strains of CTV was performed by combining two procedures developed by Bertolini *et al.* (2008) and Yokomi *et al.* (2010).

The Tissue Print (TP) and squash real-time RT-PCR procedure was developed as routine tests for a large number of samples using TaqMan chemistry to detect CTV in plant tissues and vectors without the use of extracts and nucleic acid purification Bertolini *et al.* (2008); this method showed that TP real time RT-PCR technique was more sensitive than the standard reference technique of DTBIA using the validated monoclonal antibodies 3DF1 and 3CA5 (Vidal *et al.*, 2012). Whereas, the second procedure, the multiplex real time RT-PCR assay was developed for rapid discrimination and identification of potential CTV severe strains (Yokomi *et al.*, 2010), in this method three strain specific probes were developed using intergene sequences between the major and minor coat protein genes (CPi); where the CPi-VT3 probe was designed for VT and T3 genotypes, the CP-T36 probe for T36 genotypes, the CPi-T36-NS probe to identify isolates in an outgroup clade of T36-like genotypes mild in California, and the CTV-G for broad spectrum detection of CTV (Table 8).

Table 8. Oligo nucleotide primers and TaqMan probes used in the TP real time RT-PCR assays (Yokomi *et al.*, 2010).

Primer/ Probe	Sequence (5′-3′)	Expected amplicon size[a]	Position
P27R	GACCCTTAAAGCAGTGCTCA	78	16.049-16.068[a]
P27F	TACGYGATTTGGGWAAGTAYTTDTA		15.990-16.014[a]
CPi-VT3 (TaqMan)	MGB-ACGGKGRTATTRCGC (6-FAM)		16.028-16.042
CPi-T36 (TaqMan)	MGB-ACGGTAACATTATACTATCCC (TET)		16.022-16.042
CPi-T36-NS (TaqMan)	MGB-ACGGTARTATYATRCCATCCT (6-FAM)		16.022-16.042
P25F	AGCRGTTAAGAGTTCATCATTRC	101	16.376–16.399[b]
P25R	TCRGTCCAAAGTTTGTCAGA		16.457–16.477[b]
CTV-G (TaqMan)	MGB-CRCCACGGGYATAACGTACACTCGG (TET)		16.412–16.437[b]

[a] Nucleotide positions and expected amplicon size referred to the GenBank accession no. AF001623.
[b] primer location based on GenBank accession number AF260651.

4.7.1. CTV isolates source population

Tissue print multiplex TaqMan-based real time RT-PCR assay was applied to identify potential severe strains of CTV, this assay was performed to differentiate a panel of 15 CTV sources belonging to the CIHEAM/MAIB Mediterranean collection (Table 9), each of these CTV sources was designated by the prefix (IAMB_Q) followed by a code number; in

addition, the 12 previously selected CTV sources collected in Morocco were also included (10 sources from Loukkos, 1 from Gharb and 1 from Souss).

Table 9. Mediterranean CTV sources used for the validation of the TP real time RT-PCR assay.

Origin	Isolate	Genotype
Albania	IAMB_Q3	T36
	IAMB_Q5	T36
	IAMB_Q91	T36
	IAMB_Q92	T36
	IAMB_Q95	T36
Egypt	IAMB_Q81	T3+VT
Iran	IAMB_Q98	T36+T30
Italy	IAMB_Q103	T30
	IAMB_Q113	T30
	IAMB_Q118	T30
Lebanon	IAMB_Q6	VT
Morocco	IAMB_Q74	T3+T30
	IAMB_Q75	VT+T30
Palestine	IAMB_Q40	VT
	IAMB_Q54	VT

4.7.2. Tissue print and extraction of RNA targets

Fresh sections of citrus leaf petioles belonging to the selected CTV sources were carefully printed; four partially overlapping imprints were made on a 1 cm^2 nitrocellulose membrane. The printed membranes were kept at room temperature for 30-60 min to dry and processed by DTBIA test. Small pieces of processed membranes harboring the printed samples were cut by using sterile blades and inserted into sterile eppendorf tubes; the nucleic acids were released by adding 100µl of the glycine buffer (0,1M glycine, 0,05M NaCl, 1mM EDTA, pH 8), incubated at 80°C for 10 min, followed by vortex and placed in ice for 5 min; the obtained extracts were used as template for real-time RT-PCR assays (Osman and Rowhani, 2006).

4.7.3. Multiplex real-time RT-PCR assays

Sequences analyses of P25 (CP) and P27 (CPm) coat protein genes of various isolates were used to design a selective 6'-carboxyl-fluorescein (6-FAM)-labeled minor groove binding (MGB) TaqMan probe CPi-VT3 for the identification of isolates containing VT and T3 genotypes (Fig. 14) (Saponari and Yokomi, 2010). Two additional strain specific TaqMan probes, tetrachloro-6-carboxylfluorescein (TET)-labeled MGB CPi-T36 probe and (6-FAM)-labeled MGB CPi-T36NS, were designed at the same nucleotide positions as the CPi-VT3

probe and were used with the primers P27F/P27R (Yokomi *et al.*, 2010; Saponari and Yokomi, 2010). For broad spectrum CTV detection, the generic primers CP25F/CP25R were used with TET-labeled MGB CTV-G probe.

For each tested sample, two treatments were performed in the duplex assays as follows: (A) CTV isolates were tested with a mixture of CTV-G and CPi-T36NS, and (B) CTV isolates were tested with a mixture of CPi-VT3 and CPi-T36. Assays were conducted with two replications for each sample, plus a CTV-negative control, a CTV-positive control, and a non-template control.

A one-step duplex real time RT-PCR protocol was used: two microliters of extracted RNA targets (≈ 50 ng of total RNA), from the immobilized samples on nitrocellulose membrane prepared as described above (paragraph 4.7.2), was used in a final volume of 25 µl containing 1× Path-ID™ Multiplex RT-PCR buffer (Applied biosystems, USA), 2.5 µl of Path-ID™ Multiplex RT-PCR enzyme mix and containing the following primers and probes concentrations: treatment (A): 80 nM for CTV-G probe, 160 nM for CPi-T36NS probe; 160 nM for P25R; and 320 nM for P25F, P27R, and P27F and (B): 160 nM for CPi-VT3 and CPi-T36 probes and 320 nM for each P27F/P27R primer (Yokomi *et al.*, 2010).

The amplification profile was one cycle at 48°C for 2 min and then 5 min at 95°C followed by 40 cycles at 95°C for 15 s and 56°C for 40 s.

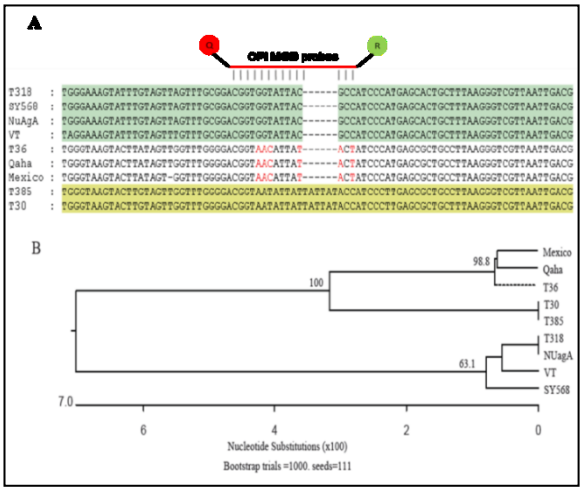


Figure 14. Minor coat protein (CPm) and CP intergene sequences. (A) Multiple alignment showing the location of the CPi-VT3, CPi-T36 and CPi-T36NS MGB-probes used for the selective detection of different CTV genotypes, and relative dendogram (B) constructed using MegAlign software (Lasergene 7, DNASTAR) (Saponari and Yokomi, 2010).

Chapter V. RESULTS AND DISCUSSION

5.1. CTV survey

A total of 1038 out of 5192 tested trees were proved to be CTV infected reaching an infection rate of 19,99%. CTV was detected in 3 out of 6 surveyed regions; prevailing in Loukkos orchards with an infection rate of 59.7% (Fig. 15); whereas, 3 CTV infected plants were detected in Souss region reaching an infection rate of 1.1%, and only a single plant out of 1898 was shown to be CTV infected in Gharb region. Interestingly, no positive samples were detected in Haouz, Moulouya and Tadla regions (Table 10).

Relatively to the distribution of CTV infection in varieties, the Clementine was the most infected one, reaching an infection rate of 66.95%, followed by Maroc late orange (30.92%); while only 3 Ortanique mandarins out of 4105 trees showed to be CTV infected (Fig. 16).

Furthermore, no CTV infected plant was detected in all the surveyed nurseries (94 samples) and varietal collections (993 samples). These results confirm the well established Moroccan certification program for the production of healthy propagating material. This may also support the fact that the CTV outbreaks findings in Morocco could be originated from illegal introduction of citrus plants from other countries. It is likely that the recent CTV outbreaks in Loukkos region could be originated by the illegal introduction of citrus plants from abroad. Actually, some European citrus producers are investing in this region and some may wonder if they introduced illegally citrus propagating material from abroad.

However, another possible source of contamination of the newly established commercial orchards can be associated to the ornamental sour orange trees which were planted along the roads in Larache (Loukkos region). In fact, 19 out of 22 tested plants were CTV infected.

Figure 15. CTV diseased trees in the Loukkos region.

Table 10. CTV incidence in commercial groves.

Region	Cultivar	Inspected trees		
		Tested (No.)	Infected (No.)	Infected (%)
Gharb	Maroc late orange	1147	1	0.08
	Washington navel	230	0	0
	Washington sanguine	142	0	0
	Thompson navel	15	0	0
	Common clementine	290	0	0
	Sidi Aissa clementine	74	0	0
Haouz	Maroc late orange	7	0	0
	Washington navel	7	0	0
	Washington sanguine	5	0	0
	Common clementine	28	0	0
	Nour clementine	8	0	0
Loukkos	Maroc late orange	762	320	42
	Common clementine	933	681	73
	Peau Fine clementine	15	14	93.33
	Sour orange	22	19	86.36
Moulouya	Berkane clementine	44	0	0
	Maroc late orange	3	0	0
	Navelina orange	8	0	0
	Nova mandarin	7	0	0
	Nour clementine	16	0	0
	Navel orange	4	0	0
	Ortanique mandarin	6	0	0
	Muska clementine	7	0	0
Souss	Isbal clementine	15	0	0
	Maroc late orange	20	0	0
	Ortanique mandarin	57	3	5.26
	Larache clementine	15	0	0
	Marisol clementine	7	0	0
	Nour clementine	38	0	0
	Nules clementine	37	0	0
	Common clementine	23	0	0
	Sidi Aissa clementine	8	0	0
	Navel orange	37	0	0
	Salustiana orange	15	0	0
Tadla	Maroc late orange	15	0	0
	Common clementine	23	0	0
	Navel orange	15	0	0
Total		**4105**	**1038**	**25.28**

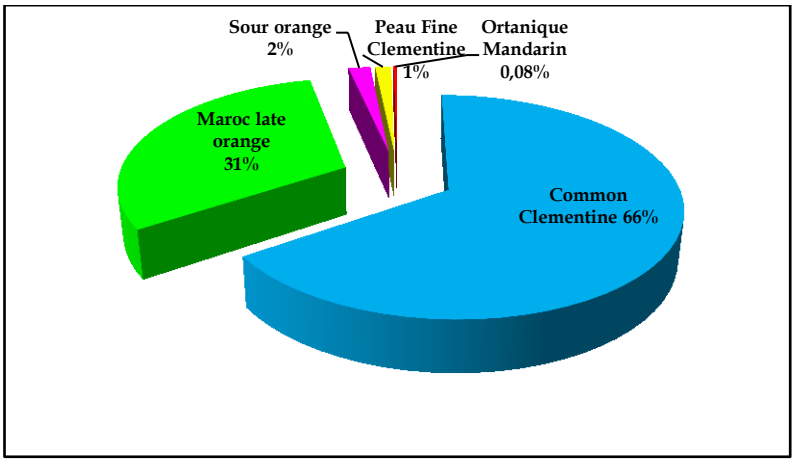


Figure 16. CTV incidence among citrus species.

It is interesting to indicate that this work represents the first large-scale survey of CTV conducted in Morocco. It has lead to the finding in the Loukkos region of the largest CTV outbreak ever reported so far from the country, in which the infected trees were belonging to an area of 720 Ha (ONSSA, personal communication).

The current situation of CTV and its distribution in Morocco is not at all reassuring. Consistent CTV infections on large scale were detected in the region of Loukkos (59.7%); this region represents the sixth citrus production area in the country with 1.280 Ha and may constitute a source of infection for the other citrus growing areas, especially for the Gharb region which is the closest one.

Several control measures have been taken by the ONSSA following the first CTV finding in Loukkos region in 2008 (Afechtal *et al.,* 2010): (i) organization of extension meetings on tristeza disease for producers and nurserymen; (ii) large scale surveys on the disease in the Loukkos region; (iii) eradication of CTV infected trees (Fig. 17); (iv) regulation of the movement of citrus planting material between different regions; (v) promoting the substitution of sour orange rootstock with tristeza-tolerant rootstocks.

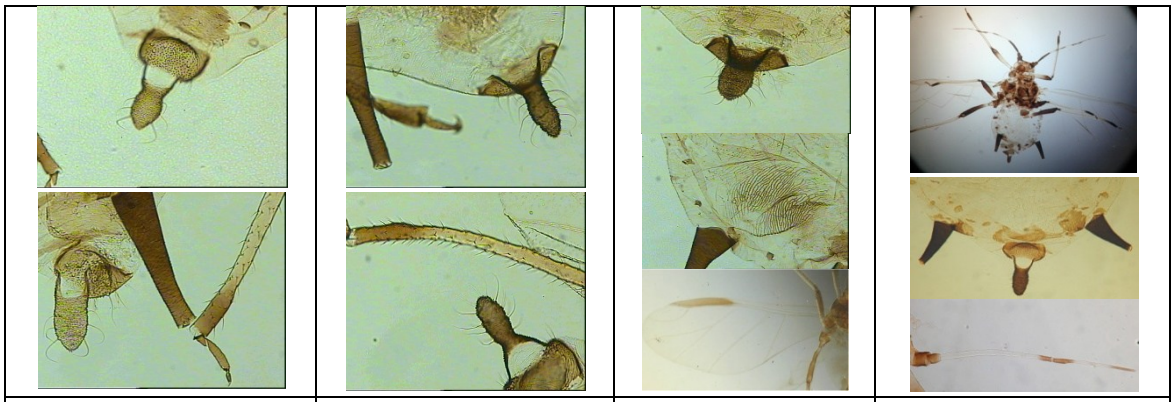
Beside the Loukkos region, CTV was also detected in a single Maroc late orange tree in the Gharb region and 3 Ortanique mandarin trees in the Souss region. Although all these latest infected trees were voluntary eradicated by the owners, further large scale surveys will be performed in order to understand the real CTV situation in these regions.

Figure 17. Eradication of a CTV infected orchard of cv. Clementine Peau Fine (93.33% infection rate) in the Loukkos region.

Considering (i) the current situation of CTV in Morocco; (ii) the predominant CTV-sensitive rootstock sour orange; (iii) the presence of *T. citricidus* "in the neighbouring countries" Portugal and Spain; (iv) the great importance of citrus in the Moroccan economy; preventive measures aiming at CTV monitoring and eradication should be sustained at national level; including vigorous controls in nurseries, enforcement and strengthening of quarantine measures, and encourage farmers and nurserymen to substitute the sour orange with other CTV tolerant rootstocks.

5.2. Monitoring of CTV vectors in the Gharb and Loukkos regions

To evaluate the status of the vectors species for better understanding the virus spread dynamics and the virus-vector relationship; the investigation was also extended to the potential vectors in the country. A monitoring of aphid species was conducted in the Gharb and Loukkos regions; the number of collected aphid was proportional to the citrus species present in the visited groves. Once collected and taken to the laboratory, the samples were examined under the stereozoom microscope and aphid specimens were divided into larvae stages, apterae and alatae morphs (Table 11). Aphid species identification was based on the Blackman and Eastop (1984) key (Fig 18).



Aphis gossypii	*Aphis spiraecola*	*Toxoptera aurantii*	*Toxoptera aurantii*
Cauda paler than siphunculi with 4-7 hairs. Femoral hairs all rather short, less than width of femur at base.	Cauda dark with 6-12 hairs. Some femoral hairs long and fine, exceeding width of femur at base.	Cauda with less than 20 hairs. Stridulatory apparatus present.	Cauda usually more than 20 hairs. Hairs on antennal segment III longer longer than the diameter of this segment at base.

Figure 18. Morphological discrimination between *A. gossypii*, *A. spiraecola*, *T. aurantii* and *T. citricidus* under the compound microscope.

The population of aphid was composed of 74.11% juvenile stages and 25.89% adults. The identification assays showed that *A. spiraecola* and *A. gossypii* were the most abundant aphid species in the visited citrus groves, representing respectively 56.29% and 32.28% of the total population in the two surveyed regions (Fig. 19), in agreement with previously reported data from other Mediterranean countries (Marroquin *et al.*, 2004; Yahiaoui *et al.*, 2009). Nevertheless, the percentage of *T. aurantii* considered as less efficient CTV vector, was noticeable and reached 8.13%, although, other species (mainly *Myzus persicae* and *A. fabae*) represented 3.30% of the total aphid population (Fig. 19). Fortunately, The BrCA was never found during this survey; however, considering the heavy CTV infections in the Loukkos region, which is located at the gates of Spain and Portugal, where *T. citricidus* is present, continuous surveys of this aphis need to be sustained.

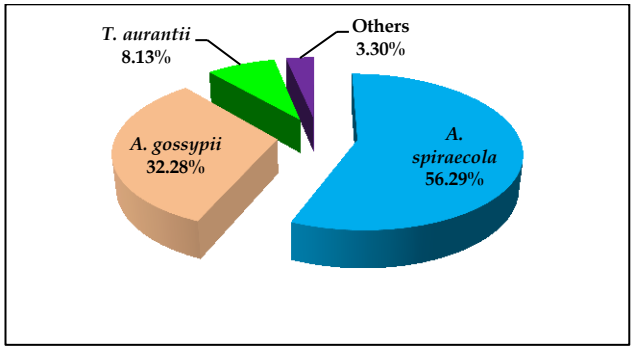


Figure 19. Relative percentage of aphid species in the surveyed citrus orchards.

Table 11. Results of the monitoring of aphid species in the visited citrus orchards from the Gharb and Loukkos regions.

Orchard	Region	Cultivar	Larvae*	Alatae*	Apterae*					Adults/shoot	Larvae/shoot
					A. gossypii	*A. spiraecola*	*T. aurantii*	*T. citricidus*	Others		
1	Loukkos	Common clementine	122.5	4.9	12.2	23.9	1.17	0	2.17	11.08	30.62
2		Common clementine	117.17	8.5	11.43	24.14	0	0	1	11.27	29.29
3		Peau Fine clementine	110.14	4.5	11.12	19	4.11	0	0.25	9.74	27.53
4		Common clementine	117.9	11.4	10.33	25.41	3.89	0	0	13	29.47
		Maroc late orange	85.34	2.4	4.23	13.1	1.34	0	0	5.27	21.33
5		Maroc late orange	100.5	4.83	11	12.22	5.33	0	2.75	9.03	25.12
6		Maroc late orange	58.12	2	9.11	9.34	2.57	0	1.93	6.24	14.53
7	Gharb	Common clementine	105.76	5.63	12	20.63	0.76	0	1	10	26.44
8		Commune clementine	129.65	7	8.45	16.75	0	0	3	8.8	32.41
9		Sidi Aissa clementine	119.27	7	11.12	21	5	0	0.45	11.14	29.82
		Maroc late orage	77.44	3.72	12.67	19.21	2.12	0	0.34	9.51	19.36
10		Maroc late orange	103.76	0	11.83	9.16	1.33	0	2	6.08	25.94
11		Maroc late orange	101.12	2.67	9.33	13.66	6.33	0	0	8	25.28
		Washington navel	97.51	1	8	19.52	1.53	0	0	7.51	24.38
12		Thompson navel	67.44	0.75	6.67	13.22	2.11	0	0.33	5.77	16.86

*Average per shoot (10 trees per cultivar were sampled from each surveyed orchard, 4 shoots were taken from each sampled tree)

Regarding the infestation of cultivars in the two surveyed regions, *A. spiraecola* prevailed on Clementine (59.93%) and Maroc late orange (47.65%) whereas *A.gossypii* and *T.aurantii* prevailed on Maroc late orange compared to the Clementine (Fig. 20).

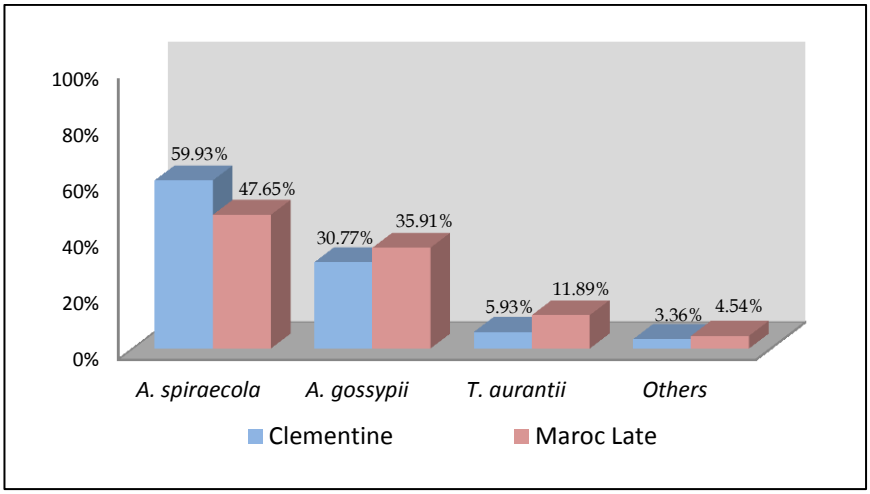


Figure 20. Relative percentage of Aphid species on different cultivars in the Gharb and Loukkos citrus orchards.

Relatively to the distribution of the aphid species in the surveyed areas, the highest population density of *A. spiraecola* was observed in the Gharb region (55.59%), followed by *A. gossypii* (33.42%) and *T. aurantii* (8.01%). In the Loukkos region, a comparable aphid population was scored, with a higher infestation of *A. spiraecola* compared to *A. gossypii* (57% and 31.12%, respectively) and *T. aurantii* (8.25%) (Fig. 21).

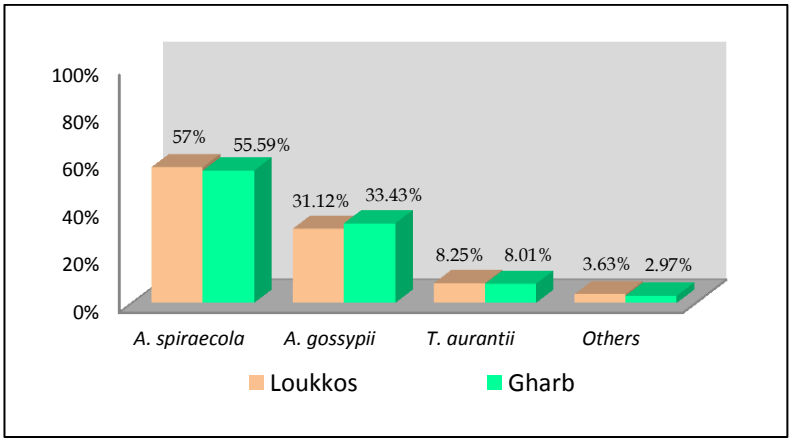


Figure 21. Relative abundance of aphid species in the Gharb and Loukkos citrus orchards.

5.3. Characterization of Moroccan CTV isolates

5.3.1. Serotyping using the MCA13 monoclonal antibodies

Among the twelve selected CTV sources from the Loukkos, Gharb and Souss regions (see Table 6); two collected isolates from the Loukkos region (L-Clem1-Mor and L-Clem3-Mor) reacted positively to the MCA13 MAb (Table 12). This antibody is known to differentiate between most of the severe and mild isolates (Permar *et al.*, 1990; Nikolaeva *et al.*, 1998), and constitutes a good indication of potential CTV aggressiveness. In order to more investigate the possible aggressiveness of these two isolates, further biological and molecular trials were carried out.

Table 12. MCA13 Serotyping of 12 selected Moroccan CTV sources.

Region	CTV source	MAb MCA13 (USA)
Gharb	G-Val-Mor	-
Loukkos	L-Clem1-Mor	+
	L-Clem2-Mor	-
	L-Clem3-Mor	+
	L-Clem4-Mor	-
	L-Clem5-Mor	-
	L-Clem6-Mor	-
	L-Clem7-Mor	-
	L-Val1-Mor	-
	L-Val2-Mor	-
	L-Val3-Mor	-
Souss	S-Ort-Mor	-

5.3.2. Biological properties of the CTV collected sources

All the 12 selected CTV sources induced typical leaf vein clearing in Mexican lime one month after inoculation (Fig. 22A). Two CTV isolates from the Loukkos region (L-Clem1-Mor and L-Clem3-Moro) in addition to the Souss isolate (S-Ort-Mor) induced leaf cupping on Mexican lime (Fig. 22B). Moreover, the two CTV isolates from the Loukkos region (L-Clem1-Mor and L-Clem3-Mor), which reacted positively to the MCA13, induced also mild stem pitting symptoms on Duncan grapefruit (Fig. 22C); thus confirming their aggressiveness. Furthermore, no seedlings yellows symptoms were observed on sour orange seedlings.

Figure 22. Biological indexing of the selected Moroccan CTV sources: (A) vein clearing symptoms in Mexican lime leaves; (B) leaf cupping in Mexican lime; and (C) mild stem pitting symptoms indicated by red arrows on Duncan grapefruit.

5.3.3. SSCP analysis

The preliminary assessment of the variability of the CP gene sequence of the 12 selected CTV isolates by SSCP analysis evidenced two different profiles, which could reflect the genetic diversity among these sources. Interestingly, the two CTV isolates L-Clem1-Mor and L-Clem3-Mor that reacted positively to the MCA13 MAb and induced mild stem pitting symptoms on Duncan grapefruit, showed similar SSCP profile between them but was different from the SSCP profile obtained with other CTV isolates (Fig. 23).

The difference in motility of the ssDNA fragments in SSCP analysis depends upon their spatial conformation, a simple self base pairing created by intra-molecular hydrogen bonding between the bases under the electrophoretic conditions (Rubio *et al.*, 1996; 2000). The different profiles which were obtained could indicate minor base mutations in the CPg nucleotidic sequence of the CTV population. In order to confirm this probability, coat protein (CPg) sequence analyses were performed.

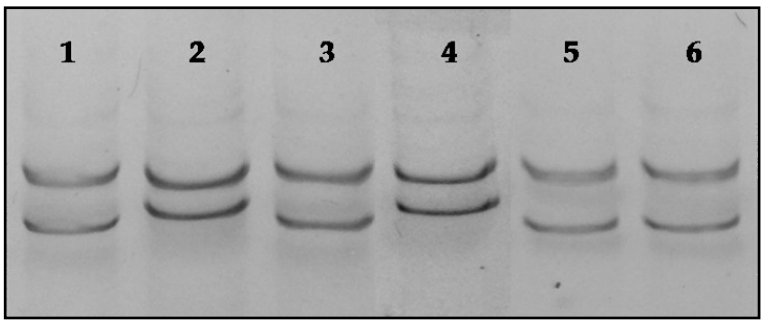


Figure 23. SSCP migration profiles of the CPg of six selected CTV sources from Morocco in an 8% non-denaturing polyacrylamide gel. (1) G-Val-Mor CTV source; (2) L-Clem1-Mor; (3) L-Clem2-Mor; (4) L-Clem3-Mor; (5) L-Val-Mor; and (6) S-Ort-Mor.

5.3.4. Phylogenetic analysis

Nucleotide sequence analyses of the CPg from the 12 selected Moroccan CTV isolates evidenced two different clusters and indicated high diversity among these isolates. The nucleotide homology among the selected CTV isolates varied from 91.6% to 99.7%.

Two CTV isolates (L-Clem1-Mor and L-Clem3-Mor) were clustered closely to the Portuguese 19-21 severe reference strain (AF184114) and to the *Poncirus trifoliata* resistance breaking (RB) severe isolate B301 from Puerto Rico (JF957196.1), showing a high sequence homology (98.5% to 99.2%); whereas, the other Moroccan CTV isolates were clustered close to the T30 (EU937520) mild reference strain (Florida) and T385 (Y18420.1) mild reference strain (Spain) with a sequence homology ranging from 98.6% to 99.4% (Table 13; Fig 24).

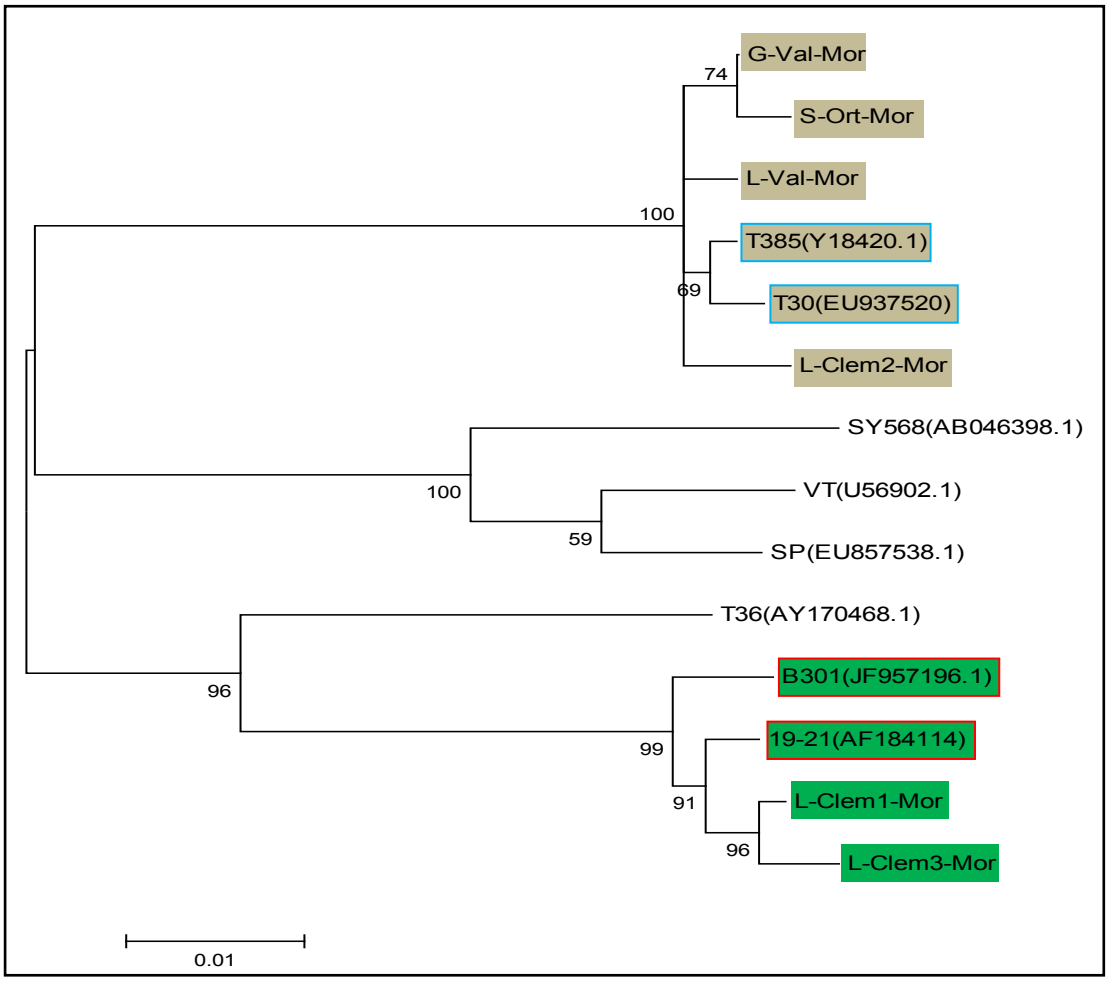


Figure 24. Neighbour-joining tree nucleotide sequences of the CP gene. Boostrap replicates values (percentage) are indicated at the nodes. Scale bar indicates changes per nucleotide.

Table 13. Pairwise nucleotide sequence identities (%) in the CP gene. In grey, identities with the reference isolates T385 and T30. In bleu, identities with the reference isolates 19-21 and B301.

Seq	G-Val-Mor	L-Clem1-Mor	L-Clem2-Mor	L-Clem3-Mor	L-Val-Mor	S-Ort-Mor	T385	T30	SY	SP	VT	T36	19-21	B301
G-Val-Mor	ID	91.9%	98.8%	91.6%	99.1%	99.7%	99.1%	98.9%	92.5%	93.0%	92.2%	93.0%	92.4%	92.2%
L-Clem1-Mor		ID	91.9%	99.4%	92.5%	92.2%	92.2%	92.2%	93.0%	93.1%	93.6%	94.6%	99.2%	98.8%
L-Clem2-Mor			ID	91.6%	99.1%	98.5%	99.1%	98.9%	92.5%	93.0%	92.2%	93.3%	92.4%	92.2%
L-Clem3-Mor				ID	92.2%	91.9%	91.9%	91.9%	92.7%	92.8%	93.3%	94.3%	98.9%	98.5%
L-Val-Mor					ID	99.1%	99.4%	99.2%	93.0%	93.4%	92.7%	93.3%	93.0%	92.8%
S-Ort-Mor						ID	98.8%	98.6%	92.4%	92.8%	92.4%	92.7%	92.7%	92.5%
T385							ID	99.5%	93.1%	93.8%	92.5%	93.3%	92.7%	92.8%
T30								ID	93.0%	93.4%	92.4%	93.1%	92.7%	92.8%
SY									ID	96.5%	96.5%	92.8%	93.4%	93.6%
SP										ID	98.0%	93.1%	93.6%	93.7%
VT											ID	93.0%	94.0%	93.8%
T36												ID	94.9%	94.6%
19-21													ID	98.9%
B301														ID

Considering that the MCA13 isolates reactivity is conferred to the presence of the amino acid phenylalanine (F) at the position 124 of the coat protein amino acids sequence (Pappu *et al.*, 1993) and this epitope is conserved among severe CTV isolates that cause either decline, stem pitting or seedling yellows.

A study was carried out on the presence of this epitope in the sequenced isolates. The phenylalanine was found at amino-acid position 124 for the clones obtained from the two Loukkos isolates (L-Clem1-Mor and L-Clem3-Mor) in agreement with the serological results as they were positive for MAb MCA13. Whereas for the remaining cloned isolates, which were MCA13 negative, the F epitope was replaced by tyrosine (Y), as a result of change of the base (T to A) at the position 371 in the nucleotide sequence of these isolates (Table 14).

Table 14. Partial multiple amino acids sequences alignment of the CP, from 6 Moroccan CTV isolates and other reference strains, confirming the presence or absence in the position 124 of MCA 13 epitope (F/Y) determined by the resulting codons TAT or TTT in the position 371 of the coat protein nucleotide sequence.

CTV isolates	CP amino acids sequence	CPg nucleotide sequence
	....|....| ....|....|	....|....| ....|....|
	125 135	365 375
L-Clem1-Mor	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAACTCCAA
L-Clem2-Mor	DIVYSSKGIG NRTNALRVWG	GACATCGTGT ATAGTTCTAA
L-Clem3-Mor	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAACTCCAA
L-Val-Mor	DIVYNSKGIG NRTNALRVWG	GACATCGTGT ATAATTCTAA
G-Val-Mor	DIVYNSKGIG NRTNALRVWG	GACATCGTGT ATAATTCTAA
S-Ort-Mor	DIVYNSKGIG NRTNALRVWG	GACATCGTGT ATAATTCTAA
T385 (Y18420.1)	DIVYNSKGIG NRTNALRVWG	GACATCGTGT ATAATTCTAA
T30 (EU937520)	DIVYNSKGIG NRTNALRVWG	GACATCGTGT ATAATTCTAA
SY568 (AB046398.1)	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAATTCTAA
T36 (AY170468.1)	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAACTCTAA
VT (U56902.1)	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAATTCTAA
19-21 (AF184114)	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAACTCCAA
SP (EU857538.1)	DIVFNSKGIG NRANALRVWG	GACATCGTGT TTAATTCTAA
B301 (JF957196.1)	DVVFNSKGIG NRTNALRVWG	GACGTCGTGT TTAACTCCAA

The characterization of twelve CTV sources (10 from Loukkos, 1 from Gharb and 1 from Souss) showed a high genetic and phenotypic diversity. To overcome the diffusion of the Citrus tristeza disease, the Moroccan Ministry of Agriculture is highly encouraging farmers and nurserymen to substitute the sour orange by other tolerant or resistant rootstocks; nevertheless, the finding of *P. trifoliata* Resistance breaking strain in the country will also limit the use of *Poncirus trifoliate* rootstocks, which is normally resistant to CTV infection. The presence of stem pitting form in the country, which is supported by previous studies that highlighted the presence of severe strains in Morocco (Zemzami *et al.*, 2002; Lbida, 2004), represents a serious threat to the Moroccan citrus industry, mainly if *T. citricidus* is introduced in the country.

5.4. Experimental transmission of two Moroccan CTV isolates

For better understanding the spread dynamics and the virus-vector relationship of CTV in the Loukkos region, transmission experiments using the main CTV vectors landing on citrus in this region, *A. spiraecola* and *A. gossypii*, were carried out.

Based on their molecular features, the two assayed Moroccan CTV isolates, the severe L-Clem1-Mor and the mild L-Clem2-Mor showed, a high genetic polymorphism related to their CPg sequences and different pathogenic behaviors on indicator plants. Both isolates were used for aphid transmission experiments by *A. gossypi* and *A. spiraecola*. Two donor

plants representing each CTV isolate were forced for actively developing tender shoots and used for the transmission trials.

5.4.1. CTV vector transmission efficiencies

The obtained aphid-infected plants were submitted to preliminary screening by DTBIA, prior to CTV detection by conventional RT-PCR analysis. As reported in the Table 15, a wide range of virus transmission values were obtained; the results showed that *A. gossypii* was able to transmit efficiently the L-Clem2-Mor mild isolate (40%), while less was the transmission rate (13.33%) for the L-Clem1-Mor severe isolate whereas, the mild isolate was transmitted at a rate of 6.67% by *A. Spiraecola,* which was not able to transmit the severe one (L-Clem1-Mor isolate).

Table 15. Experimental vector transmission efficiency of two Moroccan CTV isolates by *A. gossypii* and *A. spiraecola*.

CTV isolate	*A. gossypii*		*A. spiraecola*	
	Infection	**Transmission**	**Infection**	**Transmission**
L-Clem1-Mor	2/15	13.33%	0/15	0
L-Clem2-Mor	6/15	40%	1/15	6.67%

The obtained results were in accordance with some transmission trials conducted on several CTV isolates from the Mediterranean area and Florida under experimental conditions, showing that *A. spiraecola* was highly involved in the rapid dissemination of the CTV mild strains (T30 and T385) (Yokomi and Garnsey, 1987; Yahiaoui, 2009).

The transmission trials showed that the mild CTV isolate L-Clem2-Mor was more transmissible than the severe one L-Clem1-Mor; this may explain its prevalence in the region of Loukkos. This can also be confirmed by the study of the genetic diversity on the collected samples from the Loukkos region; in fact, out of 12 characterized CTV sources from this region, 10 were clustered close to the mild T385 (Spanish) and T30 (Florida) strains and only two CTV isolates were clustered close to the Puerto Rican severe trifoliate breaking resistance isolate B301. During the survey, some farmers were not using pesticide treatments against aphids, especially in the IPM conducted orchards, because such treatments may eliminate the mites' predators. With the obtained results that showed that, *A. gossypii* is

highly involved in transmitting local CTV strains in the Loukkos region, this practice should be revised.

5.4.2. Serotyping of the aphid-inoculated CTV sub-isolates using MCA13 MAb

TAS-ELISA was applied to test the infected donor isolates and their correspondent aphid inoculated sub-isolates. The overall response against MCA13 MAb did not change after aphid transmission (Table 16), indicating that no variation occurred on the epitopic conformation of CTV by aphid passage.

As expected, the aphid inoculated sub-isolates L-Clem1-Mor_Ag.7 and L-Clem2-Mor_Ag.13 (after transmission with *A. gossypii*) reacted against the CTV strain-discriminating MAb MCA13; while, none of the aphid transmitted sub-isolates from the L-Clem2-Mor isolate reacted with the MCA13 MAb.

Table 16. Serotyping of aphid-inoculated CTV sub-isolates using MCA13 MAb.

Isolate	Sub-isolates	MAb MCA13
L-Clem1-Mor	L-Clem1-Mor_Ag.7	+
	L-Clem1-Mor_Ag.13	+
L-Clem2-Mor	L-Clem2-Mor_Ag.1	-
	L-Clem2-Mor_Ag.2	-
	L-Clem2-Mor_Ag.6	-
	L-Clem2-Mor_Ag.9	-
	L-Clem2-Mor_Ag.11	-
	L-Clem2-Mor_Ag.14	-
	L-Clem2-Mor_As.6	-

5.4.3. Multiple SSCP analysis of the aphid-inoculated CTV sub-isolates

At molecular level, at least three proteins encoded by the CTV genome are of utmost interest since they are designated as RNA silencing suppressors in *N. benthamiana* and *N. tabacum* plants. The CPg suppresses intercellular silencing, p23 inhibits intracellular silencing, and p20 acts as suppressor at both levels. This kind of plant antiviral defence system can partially explain the high pathogenicity of CTV isolates (Lu *et al*., 2004). In addition to the p18, the mentioned proteins are reported to be affected by the vector transmission.

The population of genomic RNA sequence variants of the selected CTV isolates was firstly characterized by SSCP analysis of cDNA of the genes p18, p23 and p25. The comparison of

the parental CTV isolates (L-Clem1-Mor_D.P and L-Clem2-Mor_D.P) and their correspondent aphid-transmitted sub-isolates showed that the aphid transmission did not alter the SSCP patterns for the three studied genes (p18, p23 and p25). For both isolates (L-Clem1-Mor and L-Clem2-Mor), the obtained SSCP profile of the p25 gene from donor CTV isolate was similar to those of their correspondent aphid-transmitted sub-isolates (Fig. 25). Similar results were obtained for the p18 and p23 genes (results not shown).

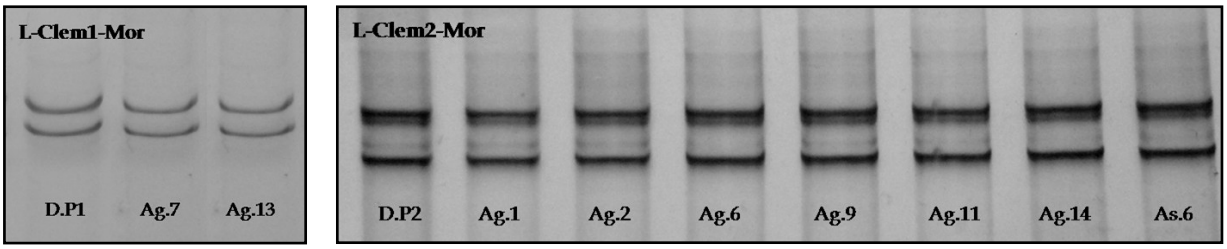


Figure 25. SSCP migration profiles of the p25 gene of two selected CTV isolates (L-Clem1-Mor and L-Clem2-Mor) and their correspondent aphid-transmitted sub-isolates in an 8% non-denaturing polyacrylamide gel. D.P1 and D.P2: donor plants of L-Clem1-Mor and L-Clem2-Mor CTV sources. Ag. and As. are *A. gossypi*i and *A. spiraecola* derived sub-isolates respectively.

5.4.4. Nucleotide and amino-acids sequences variation after aphid passage.

Since nucleotide sequencing provides precise information leading with specific gene mutations, nine sub-isolates and 2 donor CTV isolates belonging to the L-Clem1-Mor and L-Clem2-Mor isolates have been submitted to further sequence analysis.

5.4.4.1. The L-Clem1-Mor isolate

The L-Clem1-Mor CTV isolate has been successfully transmitted to two plants by *A. gossypii*; however *A. Spiraecola* was not able to transmit it. The p18, p23 and p25 genes recovered from donor plant (L-Clem1-Mor_D.P) and the two sub-isolates derived from aphid transmission by *A. gossypii* (L-Clem1-Mor_Ag.7 and L-Clem1-Mor_Ag.13) have been cloned and sequenced. The obtained sequences from donor plant were compared to their correspondent aphid derived sub-isolates.

5.4.4.1.1. The P25 gene

Multiple alignment of the p25 gene nucleotide sequences of the *A. gossypii* derived sub-isolates showed high sequence similarity with the p25 gene sequence obtained from the parental CTV isolate (L-Clem1-Mor_D.P) (Annex 8). However, a single new polymorphic position (NPP) was detected: C538⇒T in both aphid derived sub-isolates. This single NPP did not affect the predicted p25 coat protein amino acids sequences of these sub-isolates which remained identical to the CP sequence obtained from L-Clem1-Mor_D.P (Annex 9).

5.4.4.1.2. The P18 gene

In comparison with the p18 gene nucleotide sequence obtained from L-Clem1-Mor_D.P, multiple alignment (Annex 12) showed three NPPs in the *A. gossypii* derived sub-isolates: T113⇒A and A162⇒G within the L-Clem1-Mor_Ag.13 sub-isolate and A341⇒G in both aphid derived sub-isolates, resulting in minor variations in the p18 polypeptide sequences compared to the one from L-Clem1-Mor_D.P: F38⇒Y and 54I⇒M within the L-Clem1-Mor_Ag.13 sub-isolate and N114⇒S in both aphid derived sub-isolates (Annex 13).

5.4.4.1.3. The P23 gene

Multiple alignment of the p23 gene nucleotide sequences of the *A. gossypii* derived sub-isolates with the p23 gene sequence obtained from L-Clem1-Mor_D.P, disclosed three NPPs: C54⇒T and G136⇒A within the L-Clem1-Mor_Ag.13 sub-isolate and T498⇒C in both aphid derived sub-isolates (Annex 16), resulting in a single NPP in the predicted p23 amino-acids sequence V46⇒I within the L-Clem1-Mor_Ag.13 sub-isolate compared to the one from L-Clem1-Mor_D.P (Annex 17).

5.4.4.2. The L-Clem2-Mor isolate

During the transmission trials, the L-Clem2-Mor CTV isolate has been successfully transmitted to six plants by *A. gossypii* and to one plant by *A. Spiraecola*. From this isolate, the p18, p23 and p25 genes targets recovered from donor plant(L-Clem2-Mor_D.P) and the six sub-isolates derived from transmission by *A. gossypii* (L-Clem2-Mor_Ag.1, L-Clem2-Mor_Ag.2, L-Clem2-Mor_Ag.6, L-Clem2-Mor_A.g.9, L-Clem2-Mor_Ag.11 and L-Clem2-Mor_Ag.14), and the sub-isolate derived from *A. spiraecola* transmission (L-Clem2-Mor_As.6)

have been cloned and sequenced. The obtained sequences from donor plant were compared to their correspondent aphid derived sub-isolates.

5.4.4.2.1. The P25 gene

Multiple alignment of the p25 gene nucleotide sequences of the aphid (*A. gossypii* and *A. spiraecola*) derived sub-isolates showed high sequence similarity with the p25 gene sequence from parental CTV isolate (L-Clem2-Mor_D.P). However, six NPPs were revealed within the *A. gossypii* transmitted sub-isolates at positions: 56, 176, 298, 387, 597 and 608. Interestingly, the NPP at position 298 (G⇒A) was shared by all the *A. gossypii* transmitted sub-isolates. Single NPP was showed within the *A. spiraecola* transmitted sub-isolate (L-Clem2-Mor_As.6) at position 302 (Annex 10).

Compared to the p25 polypeptide sequence of L-Clem2-Mor_D.P, four NPPs were detected within the *A. gossypii* transmitted sub-isolates at positions: 19, 59, 100 and 203. As for the *A. spiraecola* transmitted sub-isolate (L-Clem2-Mor_As.6), a single NPP was revealed at position 101 (Annex 11).

5.4.4.2.2. The P18 gene

Multiple alignment of the p18 gene nucleotide sequences of the aphid derived sub-isolates with the p23 gene sequence obtained from L-Clem2-Mor_D.P revealed 4 NPPs within the *A. gossypii* transmitted sub-isolates at positions: 113, 145, 225 and 374. The NPP at position 145 (G⇒A) was shared by all the *A. gossypii* transmitted sub-isolates in addition to the *A. spiraecola* transmitted sub-isolate (L-Clem2-Mor_As.6) (Annex 14).

Three NPPs were detected within the p18 amino-acids sequences of the *A. gossypii* transmitted sub-isolates at positions: 38, 49 and 125 compared to the p18 amino-acids sequence from L-Clem2-Mor_D.P. Single NPP was detected within the *A. spireacola* transmitted isolate (L-Clem2-Mor_As.6) at position 49 (Annex 15).

5.4.4.2.3. The P23 gene

Comparing to the p23 gene nucleotide sequence obtained from L-Clem2-Mor_D.P, multiple alignment disclosed three NPPs in the *A. gossypii* derived sub-isolates at positions: 159, 229 and 520. The NPP at position 520 (C⇒T) was shared by all the *A. gossypii* transmitted sub-

isolates. Three NPPs were revealed within the *A. spiraecola* transmitted sub-isolate (L-Clem2-Mor_As.6) at positions: 159, 273, and 418 (Annex 18). One NPP was revealed in the p23 polypeptide sequence of a single *A. gossypii* transmitted sub-isolate (L-Clem2-Mor_Ag.1) at position 77 (K⇒E), in addition to another one in the *A. spiraecola* transmitted sub-isolate (L-Clem2-Mor_As.6) at position 140 (A⇒S) (Annex 19).

Comparison of field and aphid-transmitted isolates showed that aphid transmission induced minor changes in the nucleotide sequences of the genes p18, p23, p25, which was translated to minor changes in the predicted amino-acids sequences of the studied genes. Nevertheless, the slight variations induced by vector passage did not affect the phylogenetic grouping of the parental variant, but could influence the biological activity of the predominant CTV complex. Uneven distribution of the genomic RNA variants within the infected plant and sorting of some of these variants by individual aphids probably contribute to changes observed in the CTV population following aphid transmission. CTV isolates may contain different sequence variants and it has been previously indicated that some of them can be altered by aphid or graft transmission upon a new host species (Moreno *et al*., 1993). Sub-isolates generated by these processes sometimes showed some divergence within the viral population of the parental isolates. Furthermore, separation of CTV variants from the field isolates has been reported in several studies through aphid transmission (Gillings *et al*., 1993; Albiach-Martí *et al*., 2000a; Brlansky *et al*., 2003).

5.5. Tissue Print (TP) Real-time RT-PCR

5.5.1. Tissue print and extraction of RNA targets

Following a DTBIA test as reported by Garnsey *et al.* 1993, nucleic acids were extracted from the processed membranes by using the glycine buffer the obtained extracts were directly used as template for cDNA synthesis followed by a conventional PCR for the detection of the CTV-CPg. Specific amplification bands of the expected size (672 bp) were obtained from the positive control extracted either from processed membranes or with silica (paragraph 4.5.1), which didn't show any difference between the CTV extraction methods (Fig. 26).

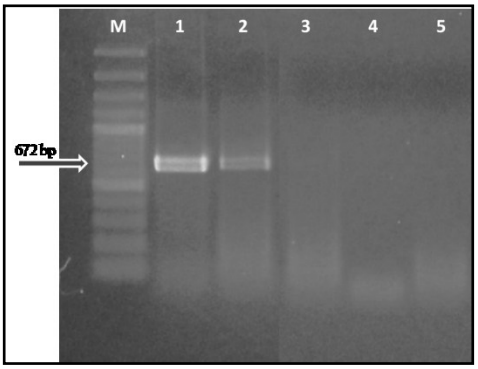


Figure 26. Agarose gel (2%) electrophoresis, showing the CTV-CPg PCR products. Lane M: GenRuler 100bp plus ladder DNA. Lane 1: CTV positive sample (IAMB_Q3) extracted with silica. Lane 2: CTV positive sample (IAMB_Q3) extracted from nitrocellulose membrane with glycine buffer after DTBIA test. Lane 3: CTV negative sample extracted with silica. Lane 4: CTV negative sample extracted from nitrocellulose membrane with glycine buffer after DTBIA test. Lane 5: no template water control.

5.5.2. Tissue print real time RT-PCR for the CTV detection

Following the DTBIA test and RNAs extraction from the processed membranes using the glycine buffer, the obtained extracts were directly used as template for real time RT-PCR test. A one-step real time RT-PCR assay was performed for broad CTV detection using the CTV-TET probe with the CP25f/r generic primers.

As reported by Yokomi *et al.* (2010) and Saponari and Yokomi, (2010), The broad spectrum CTV-G MGB-TaqMan probe and the generic P25f/r primers detected perfectly the RNA target; amplification of the target was obtained with the CTV positive control (IAMB_Q3) either extracted with silica or by DTBIA processed membranes (Cycle threshold Ct values of 16.36 and 21.53, respectively) (Fig. 27). These results confirm that CTV-RNA targets can be extracted from DTBIA processed membranes as previously reported by Bertolini *et al.* (2008) and later by Vidal *et al.* (2012).

Moreover, standard curve generated using four 10-fold dilutions with the CTV-G probe had a high correlation coefficient (R^2) of 0.994 indicating high reproducibility, and an amplification efficiency (E^2= 104.2%) in the optimal range of 90 to 120% (Figs. 28 and 29).

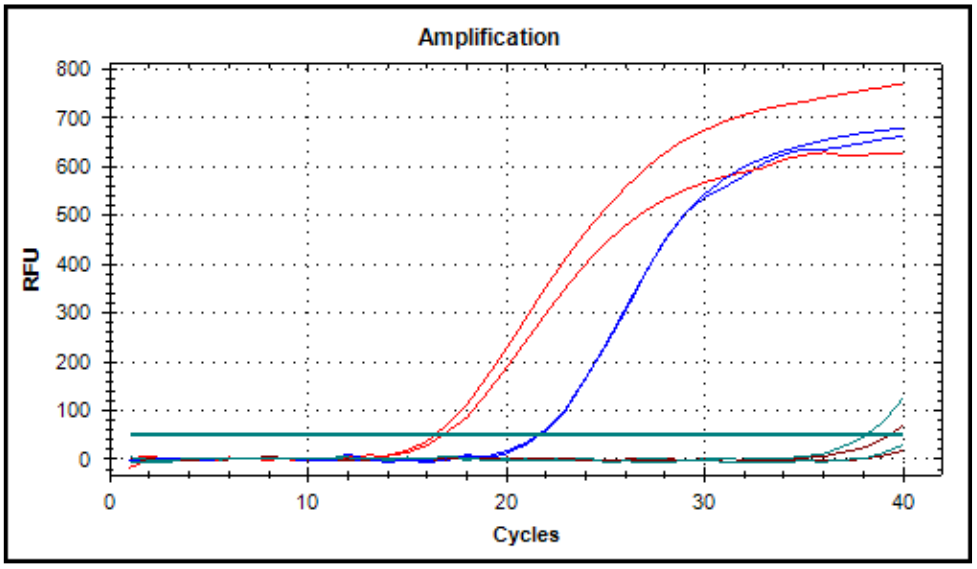


Figure 27. Tissue print real-time RT-PCR detection of CTV-RNA positive control (IAMB_Q3) using the broad spectrum CTV-G probe and the generic P25f/r primers. Red curve: extraction with silica; bleu curve: extraction from DTBIA processed membranes; clear bleu curve: negative control (extracted from DTBIA processed membrane); brown curve: non template water control.

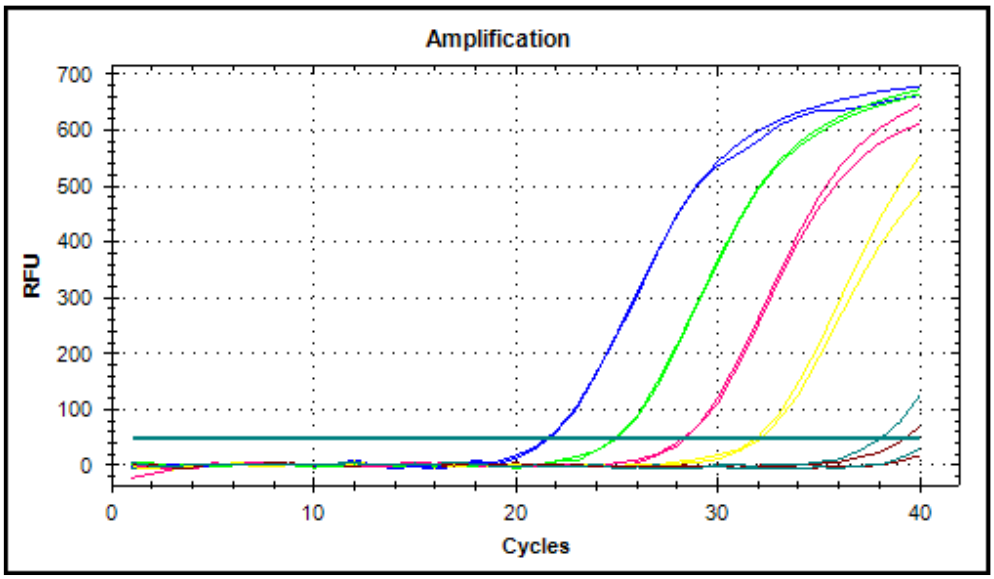


Figure 28. Tissue print real-time RT-PCR detection of CTV-RNA positive control (IAMB_Q3) extracted from DTBIA processed membranes, using the broad spectrum CTV-G probe and the generic P25f/r primers. Blue curve: amplification profile obtained from 50 ng of total RNA. Green, pink and yellow curves represent the amplification profiles obtained with 10-fold serial dilutions from 1 to 3 respectively. Clear bleu curve: negative control (extracted from DTBIA processed membrane). Brown curve: non template water control.

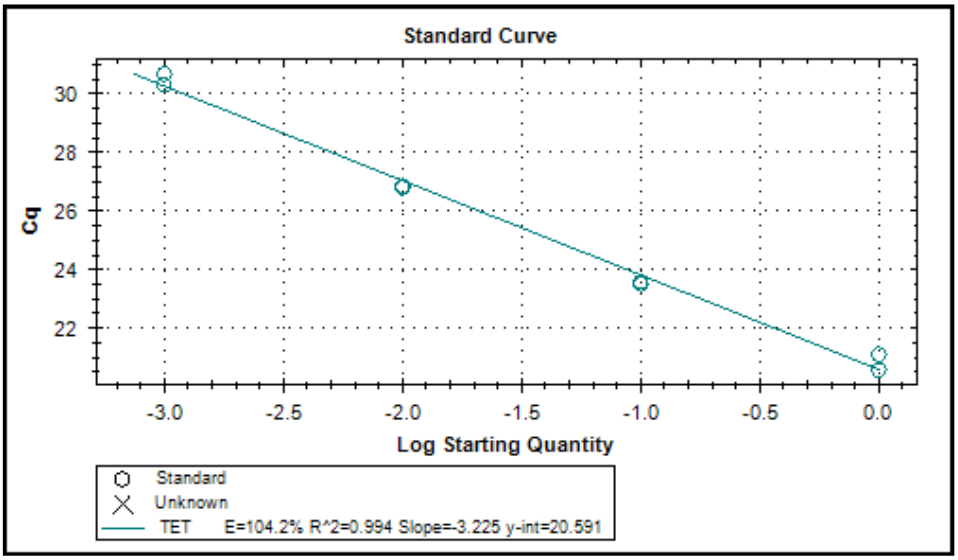


Figure 29. Standard curve generated using two replicates of a 4 series of 10-fold dilutions of the CTV-RNA positive control (IAMB_Q3) extracted from DTBIA processed membranes. The CTV-G probe curve was obtained using the generic primers P25f/r. The curve was generated by linear regression analysis plotting the threshold cycle values (Ct) in Y axis versus the logarithm of the starting dilutions in the X axis.

5.5.3. Multiplex tissue print real time RT-PCR for the identification and differentiation of potential severe CTV strains

Since CTV-RNA targets can be detected in extracts from DTBIA processed membranes, two duplex TaqMan-based real time RT-PCR assays were used in order to identify potential severe strains.

All the tested CTV isolates were detected by the broad spectrum CTV-G probe. Concomitantly, the selective CPi-T36NS MGB-TaqMan probe reacted perfectly with 2 Moroccan isolates (L-Clem1-Mor and L-Clem3-Mor), the ones that reacted positively with the MCA13 MAb and induced mild stem pitting on Duncan grapefruit (Table 17, Fig. 30). No significant changes in Ct values were obtained when the CPi-T36NS probe was used in single or duplex real time RT-PCR, demonstrating that the amplification of the two amplicons was balanced and only a minimal competition occurred. Broad spectrum detection by the CTV-G probe was comparable to that previously obtained when used in single real time RT-PCR. Yokomi *et al.* (2010) reported a high reproducibility and no reduction in the amplification efficiency when these two probes were used in duplex real time RT-PCR reactions.

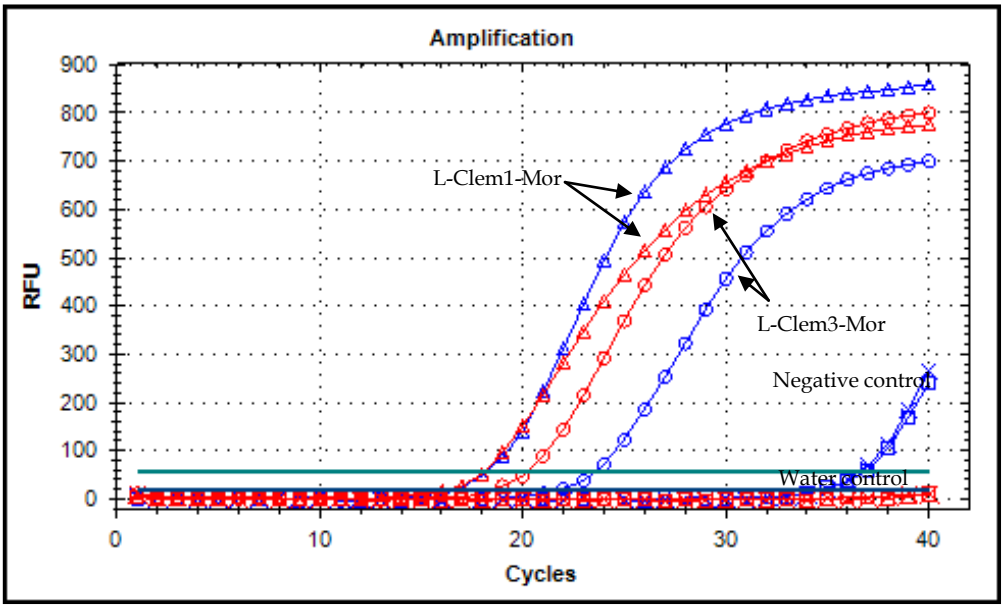


Figure 30. Tissue print one-step duplex real-time RT-PCR for broad detection of CTV, using the CTV-G probe and generic P25f/r primers, and identification of T36-like genotype isolates using the CPi-T36-NS probe with the P27r/f primers. Blue curves: CTV-G (TET) fluorophore and red color is for CPi-T36-NS (6-FAM) fluorphore. (The same samples are indicated by the same motif (circle, cross, triangle and squares). Negative and non template water controls were also included in the assay.

As expected and as reported by Yokomi *et al.* (2010), no significant changes in Ct values when the CPi-VT3 and CPi-T36 probes were used in single or duplex real time RT-CPR reactions, thus indicating high reproducibility and no reduction in the amplification efficiency. The CPi-VT3 and CPi-T36 effectively detected VT, T3 and T36 genotypes in single and natural mixed infections from the tested Mediterranean CTV collection (Table 17, Fig 31).

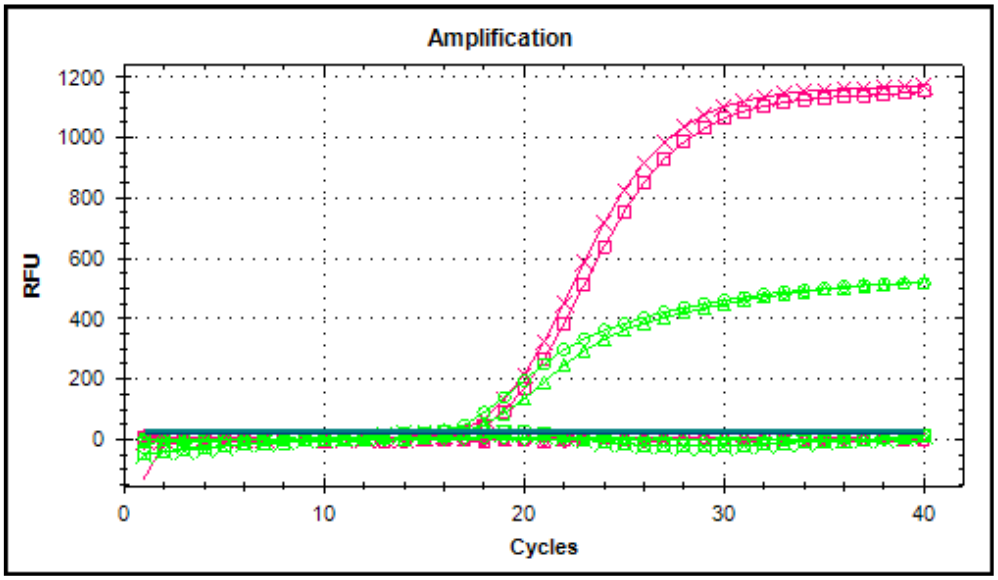


Figure 31. Tissue print one-step duplex real-time RT-PCR for the identification and differentiation of VT and T3 genotype isolates, using the CPi-VT3 probe, and T36 genotype isolates using the CPi-T36 probe with the P27r/f primers. Green curves: CPi-T36(TET) fluorophore and violet color is for CPi-VT3 (6-FAM) fluorphore. The same samples are indicated by the same motif (circle, cross, triangle and squares). Negative and non template water controls were also included in the assay.

Regarding the tested Mediterranean CTV isolates, panel tests using various CPi probes were in agreement with previous work conducted by Yahiaoui (2010) describing the biocharacterization and sequence grouping using Multiple Molecular Markers of each Mediterranean isolate and genotype of this panel (Table 17) (Hilf *et al.*, 2005), Moreover, all isolates that reacted with the CPi probes were also found to be positive with the MCA13 MAb (Table 17), this confirm the previous results obtained by Daden (2006) and Yahiaoui (2007),

The newly collected Moroccan CTV sources L-Clem1-Mor and L-Clem3-Mor, reacted perfectly with the CPi-T36NS probe suggesting their assignment to the T36-like genotype, furthermore, these 2 isolates reacted with the MCA13 MAb and based on nucleotide sequence, they were clustered close to the B301 Poncirus trifoliate-resistance breaking (RB) isolate from Puerto Ricco, these results are in accordance with those of Yokomi *et al.* (2010), which reported that NZRB-TH30, an RB isolate from New Zealand, could be detected by the CPi-T36NS probe.

Table 17. Differentiation and identification of potential severe strains of CTV by TP real time RT-PCR assays with Ct values for the correspondent reactions.

Origin	Isolate	Results of the real-time RT-PCR (Cycle threshold)				Obtained genotype	MCA13 reactivity	Genotype[b]
		CTV-G	CPi-T36NS	CPi-T36	CPi-VT3			
Albania	IAMB_Q3	21.71	0	19.45	0	T36	+[a]	T36
	IAMB_Q5	20.83	0	19.78	0	T36	+[a]	T36
	IAMB_Q91	19.17	0	19.05	0	T36	+[a]	T36
	IAMB_Q92	20.38	0	20.67	0	T36	+[a]	T36
	IAMB_Q95	19.38	0	18.45	0	T36	+[a]	T36
Egypt	IAMB_Q81	19.81	0	0	23.41	VT and/or T3	+[a]	T3+VT
Iran	IAMB_Q98	22.15	0	21.09	0	T36	+[a]	T36+T30
Italy	IAMB_Q103	21.61	0	0	0		-[a]	T30
	IAMB_Q113	19.10	0	0	0		-[a]	T30
	IAMB_Q118	20.08	35.76	0	0		-[a]	T30
Lebanon	IAMB_Q6	19.65	0	o	22.17	VT and/or T3	+[a]	VT
Morocco	IAMB_Q74	23.61	18.64	0	22.22	VT and/or T3 + T36-like	+[a]	T3+T30
	IAMB_Q75	18.25	16.45	0	21.98	VT and/or T3 + T36-like	+[a]	VT+T30
Palestine	IAMB_Q40	20.22	>36	0	19.56	VT and/or T3	+[a]	VT
	IAMB_Q54	21.66	>36	0	20.72	VT and/or T3	+[a]	VT
Morocco	G-Val-Mor	19.57	0	0	0	Unknown	-	nt
	L-Clem1-Mor	23.45	18.41	0	0	T36-like	+	nt
	L-Clem2-Mor	19.72	0	0	0	Unknown	-	nt
	L-Clem3-Mor	18.32	17.03	0	0	T36-like	+	nt
	L-Clem4-Mor	21.13	0	0	0	unknown	-	nt
	L-Clem5-Mor	22.15	0	0	0	unknown	-	nt
	L-Clem6-Mor	19.89	0	0	0	unknown	-	nt
	L-Clem7-Mor	20.71	0	0	0	unknown	-	nt
	L-Val1-Mor	20.33	0	0	0	unknown	-	nt
	L-Val2-Mor	23.01	0	0	0	unknown	-	nt
	L-Val3-Mor	19.56	0	0	0	unknown	-	nt
	S-Ort-Mor	21.44	0	0	0	unknown	-	nt

[a]MCA13 reactivity data were from previous work done by Daden (2006) and Yahyaoui (2007).
[b]Genotype classification data from previous work done by Yahyaoui (2010).
nt: not tested

Seiburth *et al.* (2005) tested some selective primers (VT-1, Type II and T3-2) and oligoprobes (ONP III, ONP IV, ONP V) on a large panel of CTV isolates to discriminate stem-pitting isolates and evaluated the procedure(s) for use as diagnostic tools for field trees. They found that no single marker could be associated with all CTV isolates which induce stem-pitting and concluded that biological, serological and molecular assays needs to be combined to best define the profile of a CTV isolate.

Real time RT-PCR protocols have been developed to detect CTV in citrus and in the aphid vector (Bertolini *et al.,* 2008; Ruiz-Ruiz *et al.,* 2007; Saponari *et al.,* 2007; Vidal *et al.,* 2012). Bertolini *et al.* (2008) developed, based on Olmos *et al.* 1996, a Tissue Print (TP) and squash real-time RT-PCR procedure to detect CTV in plant tissues and vectors without the use of extracts and nucleic acid purification, this procedure reduces the time and the cost of the analyses and is applicable in routine tests of a large number of samples; whereas, Vidal *et al.* 2012 showed that TP real time RT-PCR technique was more sensitive than the standard reference technique of DTBIA, showing also high specificity. More recently, real time RT-PCR assays were developed to detect CTV and simultaneously identify their association to the VT, T3, T36 or T36-like genotype (Saponari and Yokomi, 2010; Yokomi *et al.,* 2010).

In this work, a TP real time RT-PCR assay was developed by combining the two protocols developed by Bertolini *et al.* in 2008 and Yokomi *et al.* in (2010) and use TP for a rapid differentiation and identification of potential severe strains of CTV.

The TP TaqMan RT-PCR assays allowed detection of CTV in samples extracted from DTBIA processed membranes, while the positive reaction with one or more of the CPi probes identifies the presence of potential virulent virus strains. The CPi probes were able to differentiate MCA13-reactive isolates into at least three groups.

Differentiation of virulent versus mild CTV isolates is a major concern for the management of CTV. Introduction and spread of the brown citrus aphid, the most efficient CTV vector, and an anticipated increase in the incidence of virulent CTV strains once this vector is established have heightened the need for rapid differentiate of CTV strains, especially those that are associated with sweet orange and/or grapefruit stem pitting. The TP real time RT-PCR protocol is more simple, it is a great tool for large scale molecular characterization and differentiation of mild versus potentially virulent CTV isolates.

Chapter VI. CONCLUSIONS

The report of the first *Citrus tristeza virus* (CTV) outbreak in Loukkos region, in Northern Morocco, showed the great threat that Tristeza disease represents to the Moroccan citrus industry, not only for the susceptible graft combination on the sour orange rootstock of the majority of citrus groves, but mainly for the potential entrance and spread of the brown citrus aphid (BrCA), *T. citricidus*, from the neighboring Spain and Portugal, which could efficiently spread the severe virus strains (Afechtal *et al*., 2010). The CTV-quick decline strain can be controlled by replacing the sour orange with tolerant/resistant rootstocks. Differently is the case of the CTV-stem pitting strain which can be quickly transmitted by the BrCA regardless of the rootstock; therefore, large scale detection methods of the severe virus strains play a fundamental role for avoiding the rapid dissemination of these strains through the BrCA.

Within this context, the identification of the presence of CTV and BrCA in the Moroccan citrus areas, the characterization of the severity of the virus isolates, the evaluation of their transmission efficiency by the present aphid vectors and the development of a diagnostic tool for mass detection of the severe virus strains become a critical and urgent matter for predicting the disease dynamic, thus preventing further CTV outbreaks and/or maintaining the infection within acceptable limits.

The survey conducted in this study on the presence, incidence and distribution of CTV in the main citrus growing areas provided an updated situation on the occurrence of the infection in the country. Unfortunately the situation is not at all reassuring due to the 20% of infection rate, which is very high if we consider that it is referred to 1038 CTV infected out of the 5192 tested trees by DTBIA. Moreover, CTV was detected in three out of the six surveyed regions, prevailing in Loukkos with an infection rate of 59.7%. Fortunately, this region represents only the sixth citrus production area in the country with 1.280 Ha; however, the risk of CTV transmission from Loukkos to the neighbouring Gharb, where citrus is extensively cultivated and only one infected tree of Maroc late orange was found, is very high. Furthermore, the clementine was the most infected species in the region of Loukkos, reaching an infection rate of 66.95%, followed by the Maroc late orange (30.92%). Interestingly, in Souss region, the most important for citrus production, CTV was also detected in the Ortanique mandarin, which is not a local variety. Moreover, the absence of infected plants in the surveyed

nurseries and varietal collections, showed the positive impact of the Moroccan certification program for the production of healthy citrus propagating material. It is likely that the CTV outbreaks in Loukkos region could be originated by the illegal introduction of citrus plants from abroad with the consequent natural virus dissemination to the local varieties by the present aphid vectors. Actually, several investments from abroad in citrus plantings are occurring in Loukkos region. A similar situation is Souss region with the infected Ortanique trees. However, it is important to highlight that all CTV-infected trees have been voluntary eradicated by the owners thanks to the national phytosanitary campaign.

The characterization of twelve CTV sources from these regions (10 from Loukkos, 1 from Gharb and 1 from Souss) showed a high genetic and phenotypic diversity. The phylogenetic analysis of the CPg clustered ten sources in the same group of the T30 (Florida) and T385 (Spain) mild reference CTV strains, while two isolates from Loukkos region (L-Clem1-Mor and L-Clem3-Mor) were included in the same cluster of the Portuguese severe reference strain 19-21 and the *Poncirus trifoliata* resistance breaking (RB) severe isolate B301 (Puerto Rico). Interestingly, these two Moroccan isolates induced mild stem pitting symptoms on Duncan grapefruit indicator plants and reacted positively to the MCA13 monoclonal antibodies, which has been used in Florida to distinguish between mild and severe CTV strains (Permar *et al.,* 1990; Pappu *et al.,* 1993). Zemzami *et al.* (2002) and Lbida *et al*. (2004) highlighted the presence of stem pitting strains in Morocco. The mild CTV strains are likely to have been introduced from Spain (the closest country, from where most of the new citrus varieties were developed); as for the severe stem pitting strains, how could they have been introduced, considering the origin of the group type in which they are clustered? Moreover, how large is the spread of these strains in the citrus groves if we consider that 16% of the characterized CTV sources from Loukkos (two out of twelve) are CTV-stem pitting isolates? Whatever could be the answer, which will necessitate further and deeper studies; these findings confirm the problem of the great illegal introductions of citrus plants from abroad, which should be soon avoided. Moreover, the *P. trifolata* Resistance breaking strain of CTV could affect the replacement of the sour orange with resistant rootstocks, as the trifoliate and its hybrids, which is supported by the national programme for virus control.

To evaluate the status of vectors species for better understanding virus spread dynamics and virus-vector relationship, the investigation was also extended to the potential CTV vectors in the Loukkos and Gharb regions and to their efficiency in the transmission of the detected virus isolates.

In accordance with previously reported data from other Mediterranean countries (Marroquín *et al.,* 2004; Yahiaoui *et al.,* 2009), *A. spiraecola* and *A. gossypii* were the most abundant species on citrus trees in the surveyed regions, representing 56.29% and 32.28% of the total population, respectively. *T. aurantii* population, which is considered a minor CTV vector, was noticeable and reached 8.13%. Fortunately, *T. citricidus* was not found. Considering the heavy CTV infection and the presence of severe virus strains in the Loukkos region, which is located at the gates of Spain and Portugal where *T. citricidus* is present, measures for vector monitoring and control should be strengthened in this region in order to delay the entrance and spread of the BrCA thus protecting the most important citrus cropping areas of Morocco.

Aphid transmission of the two CTV isolates (one mild L-Clem2-Mor and the other severe L-Clem1-Mor) showed that *A. gossypii* was the most efficient vector. However, its transmission efficiency was less in the case of the CTV *P. trifoliata* RB isolate (13.33%) compared to the mild isolate (40%). Differently, *A. spiraecola* could transmit only the mild isolate at very low rate (6.67%). This finding, which confirms previous results on Mediterranean CTV isolates reported by Yahiaoui *et al.* (2009), provides supplementary data on the high involvement of *A. gossypii* in the rapid dissemination of the CTV mild strains (T30 and T385) in the Loukkos region.

The same CTV isolates were also evaluated for aphid/host co-infection processes in citrus species through a study on the genomic variability during aphid passage. Both parental isolates were then compared to their correspondent aphid derived sub-isolates using SSCP and sequencing analysis of the p18, p23 and p25 genes, which are known to be involved on the symptoms expression and to act as powerful RNA silencing suppressors in the host cells. The sequencing analysis of the p18, p23 and p25 genes revealed minor changes in the sequences obtained from aphid derived sub-isolates compared to their parental field isolates. Consequently, very limited changes were registered in the corresponding predicted amino acids sequences of the studied genes. Nevertheless, the slight variations induced by vector passage did not affect the phylogenetic grouping of the parental variant, but could influence the biological activity of the predominant CTV complex.

Considering the epidemiological results of this study and the necessity to investigate the presence of the CTV severe isolates on a large extent, it was necessary to develop an innovative and user friendly virus detection method, based on Tissue Print (TP) real time RT-PCR. To this aim, DTBIA processed membranes of 12 Moroccan isolates and 15

Mediterranean CTV isolates (from the MAIB collection) were used for RNA extraction template for real time RT-PCR with 3 CPi probes (CPi-VT3 for VT and T3 genotypes, the CP-T36 probe for T36 genotypes and the CPi-T36-NS probe for T36-like genotypes). Interestingly, the tested Mediterranean CTV isolates confirmed their classification as well as the two severe Moroccan isolates, which reacted positively with the CPi-T36-NS probe; the latter isolates confirmed their assignment to the severe T36-like genotype. The new developed Tissue Print real time RT-PCR method proved to be a great diagnostic tool of CT strains which could be applied for large scale molecular characterization and differentiation of mild versus potentially virulent CTV isolates.

Results of this present work show an alarming scenario of the Moroccan citrus industry, which will soon face the entrance and establishment of the BrCA, as it occurred in other countries worldwide. The massive destruction of citrus groves irrespective of the rootstocks can be predicted in Morocco due to the presence of severe stem pitting strains in the country. Therefore the innovative TP real time RT-PCR technique, which was set up in this work for performing large scale molecular characterization, is highly recommended in order to soon target severe strains for immediate eradication before the arrival of *T. citricidus*. An efficient vector control programme should then be applied to limit virus dissemination by the local vectors and to delay the entrance and spread of the BrCA in the country.

The Moroccan Ministry of Agriculture intends, in the framework of the Moroccan Green Plan 2010-2020 (Plan Maroc Vert), to extend the citrus production area by 18% and to increase the citrus production by 125%, reaching 3,8 million Tons in 2020. It is then desirable that this ambitious plan will consider the great contribution from the outstanding results of this present work which provides an updated scenario on CTV and relative vectors as well as innovative tools for their control.

REFERENCES

Afechtal M., Djelouah K. and D'Onghia A.M. (2010). Detection of an outbreak of *Citrus tristeza virus* in Morocco. *Integrated Control in Citrus Fruit Crops IOBC/wprs Bulletin* Vol. 62, 2011, p. 12.

Agranovsky A.A., Koonin E.V., Boyko V.P., Maiss E., Frotschl R., Lunina N.A. and Atabekov J.G. (1994). *Beet yellows Closterovirus*: Complete genome structure and identification of a leader papain-like thiol protease. *Virology*, 198: 311-324.

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

Albiach-Martí M.R., Mawassi M., Gowda S., Satyanayanana T., Hilf M.E., Shanker S., Almira E.C., Vives M.C., Lopez C., Guerri J., Flores R., Moreno P., Garnsey S.M. and Dawson W.O. (2000b). Sequences of *Citrus tristeza virus* separated in time and space are essentially identical. *Journal of Virology*, 74: 6856-6865.

Alzhanova D.V., Prokhnevsky A.I., Peremyslov V.V. and Dolja V.V. (2007). Virion tails of *Beet yellows virus*: coordinated assembly by three structural proteins. *Virology*, 359: 220-226.

Anonymous. (1984). Arrêté du Ministère de l'Agriculture et de la Réforme Agraire réf. 1478 du 21 Décembre 1983. *Bulletin officiel du Maroc* n° 3718.

Anonymous. (2011). Ministère de l'Agriculture et de la Pêche Maritime. *Situation de l'Agriculture Marocaine* n° 9, 204pp.

Asíns M.J., Bernet G.P., Ruiz C., Cambra M., Guerri J. and Carbonell E. (2004). QTL analysis of *Citrus tristeza virus*–citradia interaction. *Theoretical and Applied Genetics*, 97: 1145-1154.

Ayllón M.A., Lopez C., Navas-Castillo J., Mawassi M., Dawson W.O., Guerri J., Flores R. and Moreno P. (1999a). 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.

Ayllón M. A., Rubio L., Moya A., Guerri J., and Moreno P. (1999b). The haplotype distribution of two genes of *Citrus tristeza virus* is altered after host change or aphid transmission. *Virology*, 255(1): 32-39.

Ayllón 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(1): 27-40.

Ayllón M.A., Gowda S., Satyanayanana T., Karasev A.V., Adkins S., Mawassi M., Guerri J., Moreno P. and Dawson W.O. (2003). Effects of modification of the transcription initiation site context on *Citrus tristeza virus* subgenomic RNA synthesis. *J. Virology*, 77: 9232-9243.

Ayllón M.A., Gowda S., Satyanarayana T. and Dawson W.O. (2004). Cis-acting elements at opposite ends of the *Citrus tristeza virus* genome differ in initiation and termination of subgenomic RNAs. *Virology*, 322: 41-50.

Ayllón A.M., Rubio L., Sentandreu V., Moya A., Guerri J. and Moreno P. (2006). Variations in two gene sequences of *Citrus tristeza virus* after host passage. *Virus Genes,* 32: 119-128.

Ballester-Olmos J.F., Pina J.A., Carbonell E., Moreno P., Hermoso de Mendoza A., Cambra M. and Navarro L. (1993). Biological diversity of *Citrus tristeza virus* (CTV) isolates in Spain. *Plant Pathology*, 42: 219-229.

Bar-Joseph M. and Loebenstein G. (1973). Effect of strain, source plant, and temperature on transmissibility of *Citrus tristeza virus* by the melon aphid. *Phytopathology*, 63: 716-720.

Bar-Joseph M. and Lee R.F. (1990). *Citrus tristeza virus*. *Description of Plant Viruses n° 353. Commenwealth Mycological Institute / Association of Applied Biologist*. *Kew Surrey, England*. 7p.

Bar-Joseph M. and Nitzan Y. (1991). The spread and distribution of *Citrus tristeza virus* isolates in sour orange seedlings. *Proceedigns of the Eleventh Conference of the International Organization of Citrus Virologists. IOCV, Florida*, pp: 162-165.

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

Bar-Joseph M., Marcus R. and Lee R.F. (1989). The continuous challenge of *Citrus tristeza virus* control. *Annual Review of Phytopathology*, 27: 292-316.

Bar-Joseph M., Batuman O. and Roistacher C.N. (2010). The history of *Citrus tristeza virus* - Revisted. In: Karasev A.V. and Hilf M.E. (eds). *Citrus tristeza virus* Complex and Tristeza Diseases. *The American Phytopatological Society*, *APS Press*, pp: 3-26.

Barbarossa L. and Savino V. (2006). Sensitive and specific digoxigenin labelled RNA probes for routine detection of *Citrus tristeza virus* by dot-blot hybridization. *Journal of Phytopathology*, 154: 329-335.

Bederski K., Roistacher C.N. and Müller G.W. (2005). Cross protection against severe *Citrus tristeza virus* stem pitting in Peru. *Proc. 16th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 117-127.

Beidler L.L., Hilliard P.R. and Rill R.L. (1982). Ultrasensitive staining of nucleic acids with silver. *Analytical Biochemistry*, 126: 374-380.

Bertolini E., Moreno A., Capote N., Olmos A., Eduardo Vidal A., Pérez-Panadés J. and Cambra M. (2008). Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time RT-PCR. *European Journal of Plant Pathololgy*, 120:177-188.

Bitancourt A.A. (1940). A podridão das radicelas dos citrus na província de Corrientes, Argentina. *O Biològico*, 6: 285-288, 356-364.

Blackman R.L. and Eastop V.F. (1984). Aphids on the world's crops. An identification and information guide. *2nd edition. Wiley (eds). Chichester*. 466pp.

Blackman R.L. and Eastop V.F. (2007). Aphids as crop pests: Taxonomic issues. In: Van Emden H.F. and Harrington R. (eds). *Aphids as Crop pests*. *CAB International 2007, Cromwell Press, Trowbridge (UK)*: 1-30.

Bové C., Vogel R., Albertini D. and Bové J.M. (1988). Discovery of a strain of tristeza virus (k) inducing no symptoms in Mexicain lime. In: Timmer L.W., Garnsey S.M. and Navarro L. (eds). *Proc. 10th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 14-21.

Bové J.M. (1967). Maladies à virus des citrus dans le Bassin Méditerranéen. *Fruits*, 22: 125-140.

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

Broadbent P., Bevington K.B. and Coote B.G. (1991). Control of stem pitting of grapefruit in Australia by mild strain protection. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 64-70.

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.

Cambra M. 2007. An unexpected visit: *Toxoptera citricidus* in northern part Iberian peninsula. The current situation. *IOCV Newsletter*, May 2007: 7-8.

Cambra M., Hermoso de Mendoza A., Moreno P., and Navarro L. (1982). Use of enzyme-linked immunosorbent assay (ELISA) for detection of *Citrus tristeza virus* (CTV) in different aphid species. *Proceedings of the International Society of Citriculture*, 1: 444-448.

Cambra M., Camarasa E., Gorris M.T., Garnsey S.M. and Carbonell E. (1991). Comparison of different immunosorbent assays for *Citrus tristeza virus* (CTV) using CTV-specific monoclonal and polyclonal antibodies. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 38-45.

Cambra M., Camarasa E., Gorris M.T., Garnsey S.M., Gumpf D.J., Tsai M.C. (1993). Epitope diversity of *Citrus tristeza virus (*CTV) isolates in Spain. In: Moreno P., da Graça J.V. and Timmer L.W. (eds). *Proc. 12th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 33-38.

Cambra M., Gorris M.T., Marroquín C., Román M.P., Olmos A., Martínez M.C., Hermozo de Mendoza A., López A. and Navarro L. (2000a). Incidence and epidemiology of *Citrus tristeza virus* in the Valencian Community of Spain. *Virus Research*, 71: 75-85.

Cambra M., Gorris M.T., Roman M.P., Terrada E., Garnsey S.M., Camarasa E., Olmos A. and Colomer M. (2000b). Routine detection of *Citrus tristeza virus* by direct immunoprinting ELISA method using specific monoclonal and recombinant antibodies. *Proc. 14th Conf. Int. Org. Citrus Virol. IOCV, Brazil, 1998*, pp. 34-41.

Carter L. M. and Godfrey K. (1999). Evaluation of habitats for introduced parasites of the cotton aphid in the San Joaquin Valley. *Agricultural Research Projects-Summary Reports 1999. Cotton Incorporated. Cary, NC*, pp. 46-47

Carver M. (1984). The potential host ranges in Australia of some imported aphid parasites (Hym.: Ichneumonoidea: Aphidiidae). *Entomophaga*, 29: 351-359.

Cassin J. (1963). Découverte de 8 cas de tristeza parmi un lot de plants âgés de citrus introduit au Maroc. *Al-Awamia*, 9: 53-77.

Cevik B., Pappu S.S., Lee R.F. and Niblett C.L. (1996). Detection and differentiation of *Citrus tristeza closterovirus* using a point mutation and minor sequence differences in their coat protein genes. *Phytopathology*, 86: 101.

Chalfant A.S. and Chapman R.K. (1962). Transmission of cabbage viruses A and B by the cabbage aphid and the green peach aphid. *Journal of Economic Entomology*, 55: 584-590.

Chapot H. and Delucchi V.L. (1964). Maladies, troubles et ravageurs des agrumes au Maroc. *Publ. INRA-Rabat, les maladies à virus*, pp. 5-13.

Che X., Piestun D., Mawassi M., 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.

Che X., Mawassi M. and Bar-Joseph M. (2002). A novel class of large and infectious defective RNAs of *Citrus tristeza virus*. *Virology*, 298: 133-145.

Che X., Dawson W.O. and Bar-Joseph M. (2003). Defective RNAs of *Citrus tristeza virus* analogous to *Crinivirus* genomic RNAs. *Virology*, 310: 298-309.

Clark M.F. and Adams A.N. (1977). Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virology*, 34: 475-483.

CLAM (*Comité de Liaison de l'Agrimiculture Méditerranéene*). (2007). Les exportations d'agrumes du basin Méditerranéen. Statistiques, evaluations, répartitions, situation 2006-2007, 121pp.

Costa A.S. and Grant T.J. (1951). Studies on transmission of the tristeza virus by the vector, *Aphis citricidus*. *Phytopathology*, 41: 105-113.

Costa A.S. and Müller G.W. (1980). Tristeza control by cross-protection: a US-Brazil cooperation success. *Plant Disease*, 73: 692-597.

Covey S.N., Alkaff N.S., Langara A. and Turner D.S. (1997). Plants combat infection by gene silencing. *Nature*, 385: 781-782.

D'Urso F., Ayllón M.A., Rubio L., Sambade A., Hermoso de Mendoza A., Guerri J. 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.

D'Urso F., Sambade A., Moya A., Guerri J. and Moreno P. (2003). Variation of haplotype distributions of two genomic regions of *Citrus tristeza virus* populations from eastern Spain. *Molecular Ecology*, 12: 517-526.

Daden M. (2006). *Characterization of Mediterranean Citrus tristeza virus (CTV) isolates*. Mediterranean Agronomic Institute of Bari, IT. MSc thesis. 78pp.

Davino M, Catara A. and Terranova G. (1998). La tristezza degli agrumi, una grave minaccia per l'agrumicoltura italiana. *Informatore Fitopatologico*, 12: 12-20.

Davis R.A. (1924). Citrus growing in South Africa. *The Speciality Press of South Africa, Capetown, South Africa*.

Deng Z., Huang S., Ling P., Yu C., Tao Q., Chen C., Wendell M.K., Zhang H.-B. and Gmitter F.G.Jr. (2001). 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.

Dickson R.C., Johnson M.M., Flock R.A. and Laird E.F. (1956). Flying aphid populations in Southern California citrus groves and their relation to the transmission of the tristeza virus. *Phytopathology*, 46: 204-210.

Djelouah K. and D'Onghia A.M. (2001a). Occurrence and spread of *Citrus tristeza virus* (CTV) in the Mediterranean area. In: *Proceedings on production and exchange of virus-free plant propagating material in the Mediterranean region*. Options Méditerranéennes B 35, CIHEAM publications, pp. 43-50.

Djelouah K. and D'Onghia A.M. (2001b). Detection of *Citrus Psorosis virus* (CPsV) and *Citrus tristeza virus* (CTV) by direct tissue blot immunoassay. In: *Proceeding for the Mediterranean Network on Certification of Citrus*, Options Méditerraéennes B 33, CIHEAM publications, pp. 108-113.

Dodds J.A., Jarupat T., Lee J.G., and Roistacher C.N. (1987). Effects of strain, host, time of harvest and virus concentration on double-stranded RNA analysis of *Citrus tristeza virus*. *Phytopathology*, 77: 442-447.

Dodds J.A., Morris T.J. and Jordan R.L. (1994). Plant viral double-stranded RNA. *Ann. Rev. Phytopathology*, 22: 151-168.

Dolja V.V., Karasev A.V. and Koonin E.V. (1994). Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. *Ann. Rev. Phytopathology*, 32: 261-285.

Dolja V.V., Kreuze J.F. and Valkonen J.P.T. (2006). Comparative and functional genomics of closteroviruses. *Virus Research*, 117: 38-51.

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

Drees B.A. (1993). Aphid management. *Texas Agricultural Extension Service*. http://entowww.tamu.edu/extension/bulletins/uc/uc-031.html.

Du Charme E.P., Knorr L.C. and Banfi A. (1951). La presencia de 'stem pitting' en la Argentina. *IDIA*, 37: 15-20.

Ebert T.A. and Cartwright B. (1997). Biology and ecology of *Aphis gossypii* Glover (Homoptera: Aphididae). *Southwestern Entomologist*, 22: 116-153.

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

Fagoaga C., López C., Hermoso de Mendoza A.H., Moreno P., Navarro L., Flores R. and Peña L. (2006). Post-transcriptional gene silencing of the p23 silencing suppressor of *Citrus tristeza virus* confers resistance to the virus in transgenic Mexican lime. *Plant Molecular Biolology*, 66: 153-165.

Fang D.Q. and Roose M.L. (1999). A novel gene conferring *Citrus tristeza virus* resistance in *Citrus maxima* (Burm.) Merrill. *HortScience*, 34: 334-335.

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.

Fawcett H.S. (1936). Citrus diseases and their control. *2nd Eds. McGraw-Hill Book. New York & London*. 656pp. 440-455.

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

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.

Foissac X., Svanella-Dumas M.J., Candresse T. and Gentit P. (2001). Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested-PCR using degenerated and inosine containing primers. *Acta Horticolturae*, 550: 37-44.

Folimonov A.S., Folimonova S.Y., Bar-Joseph M. and Dawson W.O. (2007). A stable RNA virus-based vector for citrus trees. *Virology*, 368: 205-216.

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

Fraser R.S.S. (1998). Introduction to classical cross protection. In: Foster D. and Taylor S.J. (eds). *Methods in Molecular Biology vol. 81, Plant Virus Protocols. Totowa, NJ: Humana Press*, pp. 13-24.

Fry W.E. (1982). Principles of Plant Disease Management. *Academic Press Inc., San Diego, CA*.

Gandía M., Conesa A., Ancillo G., Gadea J., Forment J., Pallás V., Flores R., Duran-Vila N., Moreno P. and Guerri J. (2007). Transcriptional response of *Citrus aurantifolia* to infection by *Citrus tristeza virus*. *Virology*, 367: 298-306.

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

Garnsey S.M., Gonsalves D. and Purcifull D.E. (1977). Mechanical transmission of *Citrus tristeza virus*. *Phytopathology*, 67: 965-968.

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

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

Garnsey S.M., Civerolo E.L., Gumpf D.J., Yokomi R.Y. and Lee R.F. (1991). Development of a worldwide collection if *Citrus tristeza virus* isolates. In: *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 113-120.

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: *Proc. 12th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 39-50.

Garnsey S.M., Gottwald T.R. and Yokomi R.K. (1996a). Control strategies for citrus tristeza. In: Hadidi A., Khetarpal R.K. and Koganezawa H. (eds). *Plant Viral Disease Control: Principles and Practices. APS Press. St. Paul, MN*, pp. 639-658.

Garnsey S.M., Su H.J. and Tsai M. (1996b). Differential susceptibility of pummelo and *Swingle citrumelo* to different isolates of *Citrus tristeza virus*, In: da Graça J.V., Morno P. and Yokomi R.K. (eds). *Proc. 13th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 138-146.

Garnsey S.M., Gottwald T.R. and Yokomi R.K. (1998). Control strategies for *citrus tristeza virus*. In: Hadidi A., Khetarpal R. and Koganezawa H. (eds). *Plant Virus Disease Control*. *APS Press. St. Paul, MN*, pp. 639-658.

Geraud F. (1976). El Afido negro de los citricos, *Toxoptera citricida* Kirkaldy en Venezuela (Resumen) 1. *Encveatro Venezolano de Entomogia. Univ. Central de Venezuela, Facultad de Agronomia. Inst. Zoll. Agric. Maracay*.

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.

Gillings M., Broadbent P., Indsto J. and Lee R. (1993). Characterization 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.

Gmitter F.G.Jr., Xiao S.Y. Huang S. and Hu X.L. (1996). A localized linkage map of the *Citrus tristeza virus* resistance gene region. *Theor. Appl. Genetics*, 92: 688-695.

Gottwald T.R. and Hughes G. (2000). A new survey method for *Citrus tristeza virus* disease assessment. In: *Proc. 14th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp: 77-87.

Gottwald T.R., Garnsey S.M., Cambra M., Moreno P., Irey M. and Barbón J.C. (1996). Differential effects of *Toxoptera citricida* vs *Aphis gossypii* on temporal and spatial patterns of spread of citrus tristeza. In: da Graça V.J., Moreno P. and Yokomi R.K. (eds). *Proc. 13th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp: 120-129.

Gottwald T.R., Garnsey S.M., and Barbón J.C. (1998). Increase and patterns of spread of *Citrus tristeza virus* infections in Costa Rica and the Dominican Republic in the presence of the brown Citrus aphid, *Toxoptera citricida*. *Pyhotopathology*, 88(7): 621-636.

Gottwald T.R., Polek M. and Riley K. (2002). History, present incidence, and spatial distribution of *Citrus tristeza virus* in the California Central Valley. In: Duran-Vila N., Milne R.G. and da Graça J.V. (eds). *Proc. 15th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 83–94.

Gowda S., Satyanayanana T., Davis C.L., Navas-Castillo J., Albiach- Martí, M.R., 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.

Gowda S., Satyanayanana T., Ayllón M.A., Albiach-Martí M.R., Mawassi M., Rabindran S. and Dawson W.O. (2001). Characterization of the cis-acting elements controlling subgenomic mRNAs of *Citrus tristeza virus*: production of positive-and negative-stranded 3′-terminal and positive-stranded 5′ terminal RNAs. *Virology*, 286: 134-151.

Gowda S., Ayllón M.A., Satyanayanana T., Bar-Joseph M. and Dawson W.O. (2003a**)**.Transcription strategy in a Closterovirus: a novel 5′-proximal controler element of *Citrus tristeza virus* produces 5′- and 3′-terminal subgenomic RNAs and differs from 3′ open reading frame controler elements. *J. Virology*, 77: 340-352.

Gowda S., Satyanarayana T., Ayllón M.A., Moreno P., Flores R. and Dawson W.O. (2003b**)**. The conserved structures of the 5′ nontranslated region of *Citrus tristeza virus* are involved in replication and virion assembly. *Virology*, 317: 50-64.

Gray S.M., Moyer J.W., Kennedy G.G. and Campbell C.L. (1986). Virus-suppression and aphid resistance effects on spatial and temporal spread of *Watermelon mosaic virus 2*. *Phytopathology*, 76: 1254-1259.

Guerri J., Moreno P., Muñoz N. and Martínez M.E. (1991). Variability among Spanish *Citrus tristeza virus* isolates revealed by double-stranded RNA analysis. *Plant Pathology*, 40: 38-44.

Hall T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.

Hall W.J. and Ford W.K. (1933). Notes on some citrus insects of Southern Rhodesia. *British South Africa Co., Mazce Citrus Expt. Station Publ. 2a*.

Hammond S.M., Bernstein E., Beach D. and Hannon G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, 404: 293-296.

Havelka J. (1978). *Carnivorous gall midge Aphidoletes aphidimyza (Rond.) (Diptera, Cecidomyiidae): The bionomy, mass laboratory rearing and use against aphids on greenhouses crops*. Leningrad. PhD Thesis, 259pp.

Heikinheimo O. (1988). Mounting techniques, Aphid collection. In: Minks A.K. and Harrewijn P. (eds). *Aphids, their biology, natural enemies and control*. Amsterdam, *Elsevier*, pp. 31-44.

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: *Proc. 9th Conf. Int. Organ. Citrus Virologists, Riverside, CA*, pp. 68-70.

Hermoso de Mendoza A., Ballester-Olmos J.F. and Pina J.A. (1988a). Comparative aphid transmission of a common *Citrus tristeza virus* isolate and a seedling yellows isolate recently introduced into Spain. In: Timmer L.W., Garnsey S.M. and Navarro L. (eds). *Proc. 10th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 68-70.

Hermozo de Mendoza A., Ballester-Olmos J.F., Pina J.A., Serra J. and Fuertes C. (1988b). Differences in transmission of efficiency of *Citrus tristeza virus* by *Aphis gossypii* using sweet orange,

mandarin and lemon trees as donor or receptor host plants. In: Timmer L.W., Garnsey S.M. and Navarro L. (eds). *Proc. 10th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 63-64.

Herron C.M. (2003). *Citrus Tristeza Virus: characterization of Texas isolates, studies on aphid transmission and pathogen-derived control strategies*. Cambridge University, UK. PhD thesis. 258pp.

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 sequence divergence in the *Citrus tristeza virus* complex. *Phytopathology*, 89: 336-342.

Hilf M.E., Mavrodieva V.A. and Garnsey S.M. (2005). Genetic marker analysis of a global collection of isolates of *Citrus tristeza virus*: characterization and distribution of CTV genotypes and association with symptoms. *Phytopathology*, 95: 909-917.

Hughes G. and Gottwald T.R. (1998). Survey methods for assessment of *Citrus tristeza virus* incidence. *Phytopathology*, 88: 715-723.

Hung T.H., Wu M.L. and Su H.J. (2000). A rapid method based on the one step reverse transcriptase-polymerase chain reaction (RT-PCR) technique for detection of different strains of *Citrus tristeza virus*. *J. Phytopathology*, 148: 469-475.

Ieki H. and Yamaguchi A. (1988). Protective interference of mild strains of *Citrus tristeza virus* against a severe strain in Morita Navel orange. In: Timmer L.W., Garnsey S.M. and Navarro L. (eds). *Proc. 10th Conf. Int. Org. Citrus Virol. IOCV, Riversde, CA*, pp. 86-90.

Ilharco F.A., Sousa-Silva C.R. and Alvarez A. (2005). First report of *Toxoptera citricidus* (Kirkaldy), (Homoptera: Aphidoidea) in Spain and continental Portugal. *Agronomia Lusitana*, 51: 19-21.

Ismail M. and Zhang J. (2004). Post-harvest citrus diseases and their control. *Outlooks on Pest Management*, 15: 29-35.

John L.C. (2000). Melon Aphid or Cotton Aphid, *Aphis gossypii* Glover (Insecta: Hemiptera: Aphididae). *IFAS extension, EENY- 173, Florida*. 5pp: http://edis.ifas.ufl.edu.

Kano T. and Koizumi M. (1991). Separation of *Citrus tristeza virus* (CTV) serotypes through aphid transmission. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 82-5.

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

Karasev A.V. and Bar-Joseph M. (2010). *Citrus Tristeza virus* and the taxonomy of *Closteroviridae*. In: Karasev A.V. and Hilf M.E. (eds). *Citrus Tristeza virus: Complex and Tristeza disease*. *APS, Minnesota, USA*, pp. 119-132.

Karasev A.V., Nikolaeva O.V., Koonin E.V., Gumpf D.J. and Garnsey S.M. (1994). Screening of the closterovirus genome by degenerate primer-mediated polymerase chain reaction. *J. Gen. Virology*, 75: 1415-1422.

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., Lee R.F., Garnsey S.M., Lewandowski D.J. and Dawson W.O. (1995). Complete sequence of the *Citrus Tristeza virus* RNA genome. *Virology*, 208: 511-520.

Karasev A.V., Hilf M.E., Garnsey S.M. and Dawson W.O. (1997). Transcriptional strategy of closteroviruses: maping the 5′ termini of the *Citrus tristeza virus* subgenomic RNAs. *J. Virology*, 71: 6233-6236.

Klaassen V.A., Boeshore M.L., Koonin E.V., Tian T. and Falk B.W. (1995). Genome structure and phylogenetic analysis of *Lettuce infectious yellows virus*, a whitefly-transmitted bipartite *Closterovirus*. *Virology*, 208: 99-110.

Knapp J.L., Browning H.W., Lee R.F. and Stansly P.A. (1996). The brown citrus aphid, *Citrus tristeza virus* relationship and management guidelines for Florida citrus. *Citrus Indus.*, 77: 12-14, 16.

Knorr L.C. and DuCharme E.P. (1951). This is tristeza-ravager of Argentina's citrus industry. *Citrus Magazine*, 13: 17-19.

Koizumi M. (1991). Citrus tristeza virus field isolates from declined or dwarfed citrus trees in Japan. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol.* IOCV, Riverside, CA, pp. 25-30.

Komazaki S. (1983). Overwintering of the spirea aphid, *Aphis citricola* van der Goot (Homoptera: Aphididae) on citrus and spirea plants. *Applied Entomology and Zoology*, 18: 301-307.

Komazaki S. (1991). Studies on the biology of the spiraea aphid *Aphis spiraecola* Patch with special reference to biotypic differences. *Bulletin of the Fruit Tree Research Station 1991 (Supplement 2),* 60pp.

Komazaki S. (1993). Biology and virus transmission of citrus aphids. *Food and Fertilizer Technology Center. Technical Bull*, 136. 9pp.

Komazaki S. (1998). Difference of egg diapause in two host races of the spirea aphid. *Entomologia Experimentalis et Applicata*, 89: 201-205.

Kong P., Rubio L. Polek M. and Falk B.W. (2000). Population structure and genetic diversity within California *Citrus tristeza virus* (CTV) field isolates. *Virus Genes*, 21: 139-145.

Kreuze J.F., Savenkov E.I. and Valkonen J.P.T. (2002). Complete genome sequence and analyses of the subgenomic RNAs of S*weet potato chlorotic stunt virus* reveal several new features for the genus *Crinivirus*. *J. Virology*, 76: 9260-9270.

Kyriakou A., Ioannou N., Gavriel J., Bar-Joseph M., Papayiannis C., Kapar-Isaia, T. And Savva G. (1996). Management of *Citrus tristeza virus* in Cyprus. In: da Graça J.V., Moreno P. and Yokomi R.K. (eds). *Proc. 13th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 172-178.

Lacirignola C. and D'Onghia A.M. (2009). The Mediterranean citriculture: productions and perspectives. In: D'Onghia A.M., Djelouah K. and Roistacher C.N. (eds). *Citrus Tristeza Virus and Toxoptera citricidus: a serious threat to the Mediterranean citrus industry*. Options Méditerranéennes B 65, 2009, pp. 13-17.

Lbida B., Fonseca F., Santos C., Zemzami M., Bennani A. and Nolasco G. (2004). Genomic variability of *Citrus tristeza virus* (CTV) isolates introduced in Morocco. *Phytopathologia Mediterranea*, 43: 205-210.

Lbida B., Bannani A., Serrhini M.N. and Zemzami M. (2005). Biological, serological and molecular characterization of three *Citrus tristeza virus* isolates introduced in Morocco. *EPPO bulletin*, 35: 511-517.

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

Lee R.F. and Bar-Joseph M. (2000). Tristeza. In: Timmer L.W., Garnsey S.M. and Graham H. (eds.). *Compendium of Citrus Diseases, 2nd Edition. APS Press, St. Paul, Minn*, pp. 61-63.

Lee R.F., Roistacher C.N., Niblett C.L., Lastra R., Rocha-Peña M.A., Garnsey S.M., Yokomi R.K., Gumpf D.J. and Dodds J.A. (1992). Presence of *Toxoptera citricidus* in Central America: A threat to citrus in Florida and the United States. *Citrus Ind.*, 73: 13-24, 62, 63.

Lee R.F., Pappu H.R., Pappu S.S., Rocha-Peña 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(2): 79-87.

Lim W.L. and Hugedorn D.J. (1977). Bimodal transmission of plant viruses. In: Harris K.F. and Maramorosch K.K. (eds). *Aphids as Virus Vectors. Academic Press, New York*, pp. 237-251.

Ling K.S., Zhu H.Y. and Gonsalves D. (2004). Complete nucleotide sequence and genome organization of *Grapevine leafroll-associated virus 3*, type member of the genus *Ampelovirus*. *J. Gen. Virology*, 85: 2099- 2102.

López C., Ayllón M.A., Navas-Castillo J., Guerri J., Moreno P. and Flores R. (1998). Molecular variability of the 5′- and 3′-terminal regions of *Citrus tristeza virus* RNA. *Phytopathology*, 88: 685-691.

López C., Navas-Castillo J., Gowda S., Moreno P. and Flores R. (2000). The 23-kDa protein coded by the 3′-terminal gene of *Citrus tristeza virus* is an RNA-binding protein. *Virology*, 269: 462-470.

Lu R., Folimonov A., Shintaku M., Li W.X., Falk B.W., Dawson W.O. and Ding S.W. (2004). Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proceedigns of the National Academy of Sciences of the USA*, 101: 15742-15747.

Lutting M., Van Vuuren S.P. and Van Der Vyver J.B. (2002). Differentiation of single aphid subisolates from two South African *Citrus Tristeza Virus* isolates from grapefruit by single strand conformation polymorphism. In: Duran-Vila N., Milne R.G. and da Graça J.V. (eds). *Proc. 15th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 186-196.

Maccheroni W., Alegria M.C., Greggio C.C., Piazza J.P., Kamla R.F., Zacharias P.R.A., Bar-Joseph M., Kitajima E., Assumpcao L.C., Camarotte G., Cardozo J., Casagrande E.C., Ferrari F., Franco S.F., Giachetto P.F., Girasol A., Jordao H., Silva V.H.A., Souza L.C.A., Aguilar-Vildoso C.I., Zanca A.S., Arruda P., Kitajima J.P., Reinach F.C., Ferro J.A. and da Silva A.C.R. (2005).

Identification and genomic characterization of a new virus (*Tymoviridae* family) associated with citrus sudden disease. *J. Virology*, 79: 3028-3037.

Marais L.J., Marais M.L. and Ra M. (1996). Effect of citrus disease stem pitting on fruit size and yield of Marsh grapefruit in Southern Africa. In: Moreno P., da Graça J.V. and Yokomi R.K. (eds). *Proc. 13th Conf. Int. Org. Citrus Virol. IOCV Riverside, CA*, pp. 163-167.

Marroquín C., Olmos A., Gorris M.T., Bertoloni E., Martínez M.C., Carbonell E.A., De Mendoza A.H. and Cambra M. (2004). Estimation of the number of aphids carrying *Citrus tristeza virus* that visit adult citrus trees. *Virus Research*, 100: 101-108.

Martelli G.P., Agranovsky A.A., Bar-Joseph M., Boscia D., Candresse T., Coutts R.A.H., Dolja V.V., Falk B.W., Gonsalves D., Jelkmann W., Karasev A.V., Minafra A., Namba S., Vetten H.J., Wisler G.C. and Yoshikawa N. (2002). The family *Closteroviridae* revised. Report of the International Committee on Taxonomy of Viruses (ICTV), study group on closteroviruses and allied viruses. Virology Division News. *Archives of Virology* 147(10): 2039-2044.

Martinez A.L. and Wallace J.M. (1964). Studies on transmission of the virus components of citrus seedling yellows by *Aphis gossypii*. *Plant Disease*, 48: 131-133.

Mawassi M., Karasev A.V., 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 A.V., Lee R.F., Garnsey S.M., Dawson W.O. and Bar-Joseph M. (1995b). Multiple species of defective RNAs in plants infected with *Citrus tristeza virus*. *Virology*, 214: 264-268.

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

Mawassi M., Satyanayanana T., Albiach-Martí M.R., Gowda S., Ayllón M.A, Robertson C. and Dawson W.O. (2000a). The fitness of *Citrus tristeza virus* defective RNA is affected by the length of their 5′ and 3′ termini and by coding capacity. *Virology*, 275: 42-56.

Mawassi M., Satyanayanana T., Gowda S., Albiach-Martí M.R., Robertson C. and Dawson W.O. (2000b). Replication of heterologous combinations of helper and defective RNA of *Citrus tristeza virus*. *Virology*, 267: 360-369.

McClean A.P.D. (1963). The tristeza complex: Its variability in field-grown citrus in South Africa. *S. Afr. J. Agric. Sci.*, 6: 303-332.

McClean, A.P.D. (1960). Seedling-yellows in South African citrus trees. *South African Journal of Agricultural Science*, 3: 359-279.

Mehta 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*, 8: 1066-1069.

Meneghini M. (1946). Sôbre a natureza e transmissiblidade da doeça ‘‘tristeza’’ dos citrus. *O. Biológico*, 12: 285-287.

Mestre P.F., Asíns M.J., Pina J.A., Carbonell E.A. and Navarro L. (1997a). Molecular markers flanking *citrus tristeza virus* resistance gene from *Poncirus trifoliata* (L.) Raf. *Theor. Appl. Genetics*, 94: 458-464.

Mestre P.F., Asíns M.J., Pina J.A. and Navarro L. (1997b). Efficient search for new resistant genotypes to the *Citrus tristeza closterovirus* in the orange subfamily Aurantioideae. *Theor. Appl. Genetics*, 95: 1282-1288.

Michaud J.P. and Alvarez R. (2000). First collection of brown citrus aphid from Quintana Roo, Mexico. *Florida Entomologist*, 83: 357-358.

Moreira S. (1942). Observaçõs sobre a ''tristeza '' dos Citrus: ou ''Podridão das radicelas''. *O. Biológico*, 8: 269-272.

Moreno P. and Guerri J. (1997). Variability of *Citrus tristeza closterovirus* (CTV): Methods for differentiating isolates. In: *Filamentous Viruses of Woody Isolates. Trivandrum, India: Research Signpost*, pp. 97-107.

Moreno P. and Garnsey S.M. (2010). Citrus tristeza diseases: A worldwide perspective. In: Karasev A.V. and Hilf M.E. (eds). *Citrus Tristeza virus: Complex and Tristeza disease. APS Minnesota, USA*, pp. 27-49.

Moreno P., Guerri J. and Munoz N. (1990). Identification of Spanish strains of *Citrus tristeza virus* by analysis of double-stranded RNA. *Phytopathology*, 80: 477-482.

Moreno P., Guerri J., Ballester-Olmos J.F., Albiach R. and Martínez 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., Ambrós S., Albiach-Martí M.R., Guerri J., and Peña L. (2008). *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. *Mol. Plant Pathology*, 9(2): 251-268.

Muharam A. and Whittle A.M. (1991). Stem pitting strains of *Citrus tristeza virus* in Indonesia. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 150-155.

Müller G.W. and Garnsey S.M. (1984). Susceptibility of citrus varieties, species, citrus relatives, and non-rutaceous plants to slash-cut mechanical inoculation with *Citrus tristeza virus* (CTV). In: *Proc. 9th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 33-40.

Müller G.W., Costa A.S., Castro J.L. and Guirado N. (1988). Results from preimmunization tests to control the Capao Bonito strain of tristeza. In: Timmer L.W., Garnsey S.M. and Navarro L. (eds). *Proc. 10th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 82-85.

Nadori E.B. and Zemzami M. (1987). Contrôle de la tristeza dans la collection de Souihla. *Rapport de la Station Expérimentale de Recherches sur les Agrumes. INRA-El Menzeh, Maroc*, 48pp.

Nadori E.B. and Zemzami M. (1992). Citrus germoplasm blocks control for *Citrus tristeza virus* by enzyme-linked immunosorbent assay in Morocco. In: *Proc. Int. Soc. Citriculture*, 7: 265-267.

Narváez G., Skander B.S., Ayllón M.A., Rubio L., Guerri J. and Moreno P. (2000). A new procedure to differentiate *Citrus tristeza virus* isolates by hybridisation with digoxigenin-labelled cDNA probes. *J. Virol. Methods*, 85: 83-92.

Navarro L., Pina J.A., Juárez J., Ballester-Olmos J.F., Arregui J.M., Ortega C., Navarro A., Duran-Vila N., Guerri J., Moreno P., Cambra M., Medina A. and Zaragoza S. (2002). The Citrus Variety

Improvement program in Spain in the period 1975-2001. In: Duran-Vila N., Milne R.G. and da Graça J.V. (eds). *Proc. 15th Conf. Int. Org. Citrus Virologists, Riverside, CA*, pp. 306-316.

Navas-Castillo J., Gowda S., Hilf M.E., Garnsey S.M. and Dawson W.O. (1995). Replication of *Citrus tristeza virus* in *Nicotiana benthamiana* protoplasts. *Phytopathology*, 85: 1211.

Navas-Castillo J., Albiach-Martí 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.

Nhami A. (1981). *Maladies à virus et à mycoplasmes des agrumes au Maroc*. Université de Bordeaux, Bordeaux, France. Mémoire de D.E.A.

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 in strain differentiation of *Citrus Tristeza virus* and its application to the epidemiology of citrus tristeza disease. *Virus Research*, 71: 97-106.

Nikolaeva O.V., Karasev A.V., Garnsey S.M. and Lee R.F. (1998). Serological differentiation of *Citrus tristeza virus* isolates causing stem pitting in sweet orange. *Plant Disease*, 82: 1276-1280.

Nolasco G., Deblas C., Torres V. and Ponz F. (1993). A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant-viruses and subviral pathogens. *J. Virol. Methods*, 45: 201-218.

Norman P.A., Sutton R.A. and Selhime A.G. (1972). Further evidence that tristeza virus is transmitted semi-persistently by the melon aphid. *J. Econ. Entomology*, 65: 593-594.

Olmos A., Dasí M.A., Candresse T. and Cambra M. (1996). Print capture PCR: a simple and highly sensitive method for the detection of *Plum pox virus* (PPV) in plant tissues. *Nucleic Acids Research*, 24: 2192-2193.

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). *J. Virol. Methods*, 133: 130-136.

Pappu H.R., Pappu S.S., Niblett C.L., Lee R.F. and Civerolo E. (1993). Comparative analysis of the coat proteins of biologically distinct *Citrus tristeza closterovirus* isolates. *Virus Genes*, 73: 255-264.

Pappu H.R., Karasev A.V., Anderson E.J., Pappu S.S., Hilf M.E., Febres V.J., Eckloff R.M.G., McCaffery M., Boyko V., Gowda S., Dolja V.V., Koonin E.V., Gumpf D.J., Cline K.C., Garnsey S.M., Dawson W.O., Lee R.F. and Niblett C.L. (1994). Nucleotide sequence and organization of eight 3′ open reading frames of the *Citrus tristeza closterovirus* genome. *Virology*, 199: 35-46.

Peremyslov V.V., Pan Y.W. and Dolja V.V. (2004). Movement protein of a closterovirus is a type III integral transmembrane protein localized to the endoplasmic reticulum. *J. Virology*, 78: 3704-3709.

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

Pitrat M. And Lecoq H. (1980). Inheritance of resistance to *Cucumber mosaic virus* transmission by *Aphis gossypii* in *Cucumis melo*. *Phytopathology*, 70: 958-961.

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

Raccah B., Leobenstein G., Bar-Joseph M. and Oren Y. (1976). Transmission of tristeza by aphids prevalent on citrus and operation of the tristeza suppression program in Israel. In: Calavan E.C. (eds). *Proc. 7th Conf. Int. Ogr. Citrus Virol. IOCV, Riverside, CA*, pp. 47-49.

Raccah B., Loebenstein G. and Singer S. (1980). Aphid-transmissibility variants of *Citrus tristeza virus* in infected citrus trees. *Phytopathology*, 70: 89-93.

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 Mol. Biology*, 61: 399-414.

Ratcliff F., Harrison B.D. and Baulcombe D.C. (1997). A similarity between viral defense and gene silencing in plants. *Science*, 276: 1558-1560.

Ratcliff F., MacFarlane S. and Baulcombe D.C. (1999). Gene silencing without DNA. RNA-mediated cross-protection between viruses. *Plant Cell*, 11: 1207-1216.

Retuerma M.L. and Price W.C. (1972). Evidence that tristeza virus is stylet-borne. *FAO Plant Protection Bulletin*, 20: 111-114.

Roberts P.D, McGovern R.J, Lee R.F. and Niblett C.L. (2001). *Tristeza*. Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, 10pp. http://plantpath.ifas.ufl.edu

Rocha-Peña 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 *Toxopera citricida*: Threats to citrus production in the Caribbean and Central and North America. *Plant Disease*, 79(5): 437-444.

Roistacher C.N. (1988). Observation on the decline of sweet orange trees in coastal Peru caused by stem pitting tristeza. *FAO Plant Protection Bulletin*, 36: 19-26.

Roistacher C.N. (1991). Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. *FAO Eds*, Rome, 286pp.

Roistacher C.N. (2006). EcoPort slide shows on the internet related to citrus and citrus diseases. http://ecoport.org/ep?SearchType=domainContents&id=9&type=group

Roistacher C.N. and Bar-Joseph M. (1984). Transmission of tristeza and seedling yellows tristeza virus by *Aphis gossypii* from sweet orange, grapefruit and lemon to Mexican lime, grapefruit and lemon. In: *Proc. 9th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp: 9-18.

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

Roistacher C.N. and Moreno P. (1991). The worldwide threat from destructive isolates of *Citrus tristeza virus* - A review. In: Brlansky R.H., Lee R.F. and Timmer L.W. (eds). *Proc. 11th Conf. Int. Org. Citrus Virol. IOCV, Riverside, CA*, pp. 7-19.

Roistacher C.N., Nauer E.M., Kishaba A. and Calavan E.C. (1980). Transmission of *Citrus tristeza virus* by *Aphis gossypii* reflecting changes in virus transmissibility in California. In: Calavan E.C., Garnsey S.M. and Timmer L.W. (eds). *Proc. 8th Conf. Int. Organ. Citrus Virol. IOCV, Riverside, CA*, pp: 76-82

Román M.P., Cambra M., Juárez J., Moreno P., Duran-Vila N., Tanaka F.A.O., Alves E., Kitajima E.W., Yamamoto P.T., Bassanezi R.B., Teixeira D.C., Junior W.C.J., Ayres A.J., Gimenes-Fernandes N., Rabenstein F., Girotto L.F. and Bové J.M. (2004). Sudden death of citrus in Brazil: a graft-transmissible bud union disease. *Plant Disease*, 88: 453-467.

Rosner A. and Bar-Joseph M. (1984). Diversity of *Citrus tristeza virus* strains indicated by hybridization with cloned cDNA sequences. *Virology*, 139: 189-193.

Rosner A., Lee R.F. and Bar-Joseph M. (1986). Differential hybridization with cloned cDNA sequences for detecting a specific isolate of *Citrus tristeza virus*. *Phytopathology*, 76: 820-824.

Roy A., Ramachandran P. and Brlansky R.H. (2003). Grouping and comparison of Indian *Citrus tristeza virus* isolates based on coat protein gene sequences and restriction analysis patterns. *Arch. Virology*, 148: 707-722.

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. *J. Virol. Methods*, 129: 47-55.

Rubio L., Ayllón M.A., Guerri J., Pappu H.R., Niblett C.L. and Moreno P. (1996). Differentiation of *Citrus tristeza virus* (CTV) isolates by single-stranded conformation polymorphism analysis of the coat protein gene. *Ann. Appl. Biology*, 129: 479-489.

Rubio L., Guerri J., and Moreno P. (2000). Characterization of *Citrus tristeza virus* isolates by single-strand conformation polymorphism analysis of DNA complementary to their RNA population. In: *Proc. 14th Conf. Int. Org. Citrus Virologists, Riverside, CA*, pp. 12-17.

Rubio L., Ayllón 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. *J. Virology*, 75(17): 8054-8062.

Ruiz-Ruiz S., Moreno P., Guerri J. and Ambrós S. (2007). The complete nucleotide sequence of a severe stem pitting isolate of *Citrus tristeza virus* from Spain: Comparison with isolates from different origins. *Arch. Virology*, 151: 387-398.

Sambade A., Rubio L., Garnsey S.M., Costa N., Müller G.W., Peyrou M., Guerri J. and Moreno P. (2002). Comparison of the viral RNA populations of pathogenically distinct isolates of *Citrus tristeza virus*: application to monitoring cross protection. *Plant Pathology*, 51: 257-265.

Sambade A., Lopez C., Rubio L., Flores R., Guerri J. and Moreno P. (2003). Polymorphism of a specific region in gene p23 of *Citrus tristeza tirus* allows discrimination between mild and severe isolates. *Arch. Virology*, 148: 2325-2340.

Sambade A., Ambrós S., López C., Ruiz-Ruiz S., Hermoso de Mendoza A., Flores R., Guerri J. and Moreno P. (2007). Preferential accumulation of severe variants of *Citrus tristeza virus* in plants coinoculated with mild and severe variants. *Arch. Virology*, 152: 1115-1126.

Sambrook J., Fritsh E. and Maniatis T. (1989). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, *Cold Spring Harbor*, NY. 1885pp.

Saponari M. and Yokomi R.K. (2010). Use of the Coat Protein (CP) and Minor CP intergene sequence to discriminate severe strains of *Citrus tristeza virus* (CTV) in three U.S. CTV isolate collections. In: *Proc. 17th Conf. Int. Org. Citrus Virol. IOCV*, pp: 43-57.

Saponari M., Manjunath K. and Yokomi R.K. (2007). Quantitative detection of *Citrus tristeza virus* in citrus and aphids by real-time reverse transcription-PCR (TaqMan). *J. Virol. Methods*, 147: 43-53.

Satyanayanana T., Gowda S., Boyko V.P., Albiach-Martí M.R., Mawassi M., Navas-Castillo J., Karasev A.V., Dolja V., Hilf M.E., Lewandowsky 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. *Proc. Natl. Acad. Sci. USA*, 96: 7433-7438.

Satyanarayana T., Gowda S., Mawassi M., Albiach-Martí M.R., Ayllón M.A., Robertson C., Garnsey S.M. and Dawson W.O. (2000). Closterovirus encoded HSP70 homolog and p61 in addition to both coat proteins function in efficient virion assembly. *Virology*, 278: 253-265.

Satyanarayana T., Gowda S., Ayllón M.A., Albiach-Martí M.R. and Dawson W.O. (2002a). Mutational analysis of the replication signals in the 3′-non translated region of *Citrus tristeza virus*. *Virology*, 300: 140-152.

Satyanarayana T., Gowda S., Ayllón M.A., Albiach-Martí M.R., Rabindram R. and Dawson W.O. (2002b). The p23 protein of *Citrus tristeza virus* controls asymmetrical RNA accumulation. *J. Virology*, 76: 473-483.

Satyanayanana T., Gowda S., Ayllón M.A. and Dawson W.O. (2004). Closterovirus bipolar virion: evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5′ region. *Proc. Natl. Acad. Sci. USA*, 101: 799-804.

Schneider H. (1959). The anatomy of tristeza-virus-infected citrus. In: Wallace J.M. (eds). *Citrus Virus Diseases*. University of California, Divison of Agricultural Sciences. Berkeley, CA, pp. 73-84.

Sekiya M.E., Lawrence S.D., McCaffery M., and Cline K. (1991). Molecular cloning and nucleotide sequencing of the coat protein gene of *Citrus tristeza virus*. *J. Gen. Virology*, 72: 1013-1020.

Slosser J.E., Pinchak W.E. and Rummel D.R. (1989). A review of known and potential factors affecting the population dynamics of the cotton aphid. *Southwestern Entomologist*, 14: 302-313.

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

Takanashi M. (1990). Development and reproductive ability of *Lysiphlebus japonicus* Ashmead (Hymenoptera: Aphidiidae) parasitizing the citrus brown aphid, *Toxoptera citricidus* (Kirkaldy) (Homoptera: Aphididae). *Japanese Journal of Applied Entomology and Zoology*, 34: 237-243.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* (submitted). http://www.kumarlab.net/publications

Tian T., Rubio L., Yeh H.-H., Crawford B. and Falk B.W. (1999). *Lettuce infectious yellows virus*: *In vitro* acquisition analysis using partially purified virions and the whitefly, *Bemisia tabaci. J. Gen. Virology,* 80: 1111-1117.

Timmer L.W., Garnsey S.M. and Graham J.H. (2000). Compendium of citrus diseases. *APS press*. USA, 61pp.

Toxopeus H.J. (1937). Stock-scion incompatibility in citrus and its cause. *Journal of Pomology and Horticultural Science*, 14: 360-364.

Tzanetakis I.E., Postman J. and Martin R.R. (2005a). characterization of a novel member of the family closteroviridae from *Mentha spp*. *Phytopathology*, 95: 1043-1048.

Tzanetakis I.E., Reed J.C. and Martin R.R. (2005b). Nucleotide sequence, genome organization and phylogenetic analysis of *Strawberry pallidosis associated virus*, a new member of the genus *Crinivirus*. *Arch. Virology*, 150: 273-286.

Valiela M.F.V. (1948). Informe preliminar acerca de la etiologia de la "Podredumbre de las raicillas" del naranjo agrio injertado. *Revista de Investigaciones Agricolas*, 2: 139-146.

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

Vela C., Cambra M., Cortés E., Moreno P., Miguet S.G., Pérez de San Román C. and Sanz A. (1986). Production and characterisation of monoclonal antibodies specific for *Citrus tristeza virus* and their use for diagnosis. *J. Gen. Virology*, 67: 91-96.

Velazquez-Monreal J.J., Mathews D.M. and Dodds J.A. (2009). Segregation of distinct variants from *Citrus tristeza virus* isolate SY568 using aphid transmission. *Phytopathology*, 99: 1168-1176.

Vidal E., Yokomi R.K, Moreno A., Bertolini E. and Cambra M. (2012). Calculation of diagnostic darameters of advanced serological and molecular tissue-print methods for detection of *Citrus tristeza virus*: A model for other plant pathogens. *Phytopathology*, 102(1): 114-121.

Vives M.C., Rubio L., López C., Navas-Castillo J., Albiach-Martí 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. *J. Gen. Virology*, 80: 811-816.

Vives M.C., Rubio L., Sambade A., Mirkov T.E., Moreno P. and Guerri J. (2005). Evidence of multiple recombination events between two RNA sequence variants within a *Citrus tristeza virus* isolate. *Virology*, 331: 232-237.

Wallace J.M. (1978). Virus and virus like diseases. In: Reuther W., Calavan E.C. and Carman G.E. (eds). *The Citrus Industry*. University of California, Division of Agricultural Sciences, Berkeley, CA, pp. 67-184

Weathers L.G. and Hartung M.K. (1964). Transmission of citrus viruses by dodder, *Cuscuta subinclusa*. *Plant Disease Report,* 48: 102-103.

Webber, H.J. (1943). The ''Tristeza'' disease of sour orange rootstock. *Proceedings of the American Society for Horticultural Science*, 43: 160-168.

Webber H.J. (1948). History and development of the citrus industry. In: Webber, H.J., and Batchelor, L.D. (Eds.). *The Citrus Industry* - Volume I, *History, Botany and Breeding*. University of California Press, Riverside, pp: 1-25.

Wellings P.W. (1994). How variables rates of colonization?. *European Journal of Entomology*, 91: 121-125.

Yahiaoui D. (2007). *Monitoring of Citrus tristeza virus (CTV) vectors in Apulia region and experimentation trials on the transmissibility of Mediterranean CTV isolates by Aphids*. Mediterranean Agronomic Institute of Bari, IT. MSc thesis. 60pp.

Yahiaoui D. (2012). *Assessment of genetic diversity of Mediterranean Citrus tristeza virus (CTV) isolates and genomic RNA variability associated to their vecotor transmission*. University of Catania, IT. PhD thesis. 177pp.

Yahiaoui D., Adante R., Djelouah K. and D'Onghia A.M. (2009). Preliminary monitoring of Citrus tristeza (CTV) vectors in Apulia region. In: D'Onghia A.M., Djelouah K. and Roistacher C.N. (eds). *Citrus tristeza virus and Toxoptera citricida: a serious threat to the Mediterranean citrus industry*. Options Méditerranéennes, B 65, 2009, pp: 173-175.

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. *J. Virology*, 71: 9800-9802.

Yang G., Che X., Golfman 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., 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., Mathews D.M., Dodds J.A. and Mirkov T.E. (1999). Molecular characterization of an isolate of *Citrus tristeza virus* that causes severe symptoms in sweet orange. *Virus Genes*, 19: 11-142.

Yokomi R.K. (1992). Potential for biological control of *Toxoptera citricidus* (Kirkaldy). In: *Citrus tristeza virus and Toxoptera citricidus in Central America: Development of Management Strategies and the Use of Biotechnology for Control*. CATIE University of Florida-INIFAP/SAH-Universidad de Central de Venezuela-USDA, Maracay, Venezuela, pp. 194-198.

Yokomi R.K. (2009a). *Citrus tristeza virus*. In: D'Onghia A.M., Djelouah K. and Roistacher C.N. (eds). *Citrus tristeza virus and Toxoptera citricida: a serious threat to the Mediterranean citrus industry*. Options Méditerranéennes, B 65, 2009, pp: 19-33.

Yokomi R.K. (2009b). The brown citrus aphid, *Toxoptera citricida*. In: D'Onghia A.M., Djelouah K. and Roistacher C.N. (eds). *Citrus tristeza virus and Toxoptera citricida: a serious threat to the Mediterranean citrus industry*. Options Méditerranéennes, B 65, 2009, pp: 35-46.

Yokomi R.K. and Garnsey S.M. (1987). Transmision of *Citrus tristeza virus* by *A. gossypii* and *A. citricola* in Florida. *Phytophylactica*, 19: 169-172.

Yokomi R.Y. and Tang Y.Q. (1996). A survey of parasitoids of brown citrus aphid. (Homoptera: Aphididae) in Puerto Rico. *Biological Control*, 6: 222-225.

Yokomi R.K., Garnsey S.M., Civerolo E.L. and Gumpf D.J. (1989). Transmission of exotic *Citrus tristeza virus* isolates by a Florida colony of *Aphis gossypii*. *Plant Disease*, 73: 552-556.

Yokomi R.K., Lastra R., Stoetzel M.B., Damgstreet V.D., Lee R.F., Garnsey S.M., Rocha-Peña M.A. and Niblett C.L. (1994). Establishment of the brown citrus aphid *Toxoptera citricida* (Kirkaldy) (Homoptera: aphididae) in Central America and the Caribbean Basin, and its transmission of *Citrus tristeza virus*. *J. Econ. Entomology*, 87: 1078-1085.

Yokomi R.K., Saponari M. and Seiburth P.J. (2010). Rapid differentiation and identification of potential severe strains of *Citrus tristeza virus* by real-time reverse transcription-polymerase chain reaction assays. *Phytopathology*, 100(4): 319-327.

Yoshida T. (1985). Inheritance of susceptibility to *Citrus tristeza virus* in trifoliate orange (*Poncirus trifoliata* Raf.). *Bulletin of Fruit Tree Research Station*, 12: 17-25.

Yoshida T. (1993). Inheritance of immunity to *Citrus tristeza virus* of trifoliate orange in some citrus intergeneric hybrids. *Bull. Fruit Tree Res. Station*, 25: 33-43.

Yoshida T. (1996). Graft compatibility of Citrus with plants in the Aurantioideae and their susceptibility to *Citrus tristeza virus*. *Plant Disease*, 80: 414-417.

Zemzami M. and Nadori E.B. (1989). Contrôle de la tristeza dans les collections de Aïn Taoujdate, Melk Zhar et Aïn Chaïb. *Rapport de la Station Expérimentale de Recherches sur Agrumes. INRA-El Menzeh, Maroc*, 2pp.

Zemzami M., Soares C.M., Bailey A.M., Niblet C.L. and Nolasco G. (2002). Comparaison of molecular and biological characteristics of fourteen Moroccan isolates of *citrus tristeza virus*. In: *Proc. 15th Conf. Int. Org. Citrus Virologists, Riverside, CA*, pp. 8-12.

Zemzami M., Benali D., Moukhtari A. and Soulaymani A. (2009). Historical review of citrus tristeza in Morocco. In: D'Onghia A.M., Djelouah K. and Roistacher C.N. (eds). *Citrus Tristeza Virus and Toxoptera citricidus: a serious threat to the Mediterranean citrus industry*. Options Méditerranéennes, B 65, 2009, pp: 95-101.

ANNEXES

ANNEX 1. Mounting medium for aphids (Heikinheimo, 1988).

Polyvinyl alcohol chips "Mowiol N 4-98"	25 g
Polyvinyl alcohol chips "Mowiol N 56-98"	5 g
Distilled water	105 ml
96% ethanol	30 ml
Lactic acid (pure)	105 ml

ANNEX 2. Identification key for worldwide citrus aphid species (Blackman and Eastop, 1984).

1	Antennal tubercles weakly developed	2
	Antennal tubercles well developed	10
2	Terminal process a little shorter than base of last antennal segment. Siphunculi much shorter than cauda.	A
	Terminal process much longer than base of last antennal segment. Siphunculi shorter or longer than cauda.	3
3	Cauda helmet-shaped in dorsal view, not longer than its width at base.	B
	Cauda tongue-shaped or triangular in dorsal view, longer than its basal width.	4
4	Dorsal abdomen with an extensive solid black patch.	C
	Dorsal abdomen without a black central patch.	5
5	Siphunculi much shorter than cauda.	D
	Siphunculi longer or at least as long as cauda.	6
6	Terminal process more than 3.5 times longer than base of last antennal segment. Cauda with not less than 10 hairs.	7
	Terminal process less than 3.5 times longer than base of last antennal segment. Cauda usually with less than 10 hairs.	9
7	Cauda with usually more than 20 hairs. Hairs on antennal segment III longer than the diameter of this segment at base. Thoracic tergites often partly sclerotized.	E
	Cauda with usually less than 20 hairs. Hairs on antennal segment III often shorter than the diameter of this segment at base. Thoracic tergites usually unsclerotized.	8
8	Siphunculi less than 1.5 times longer than cauda. Stridulatory apparatus present.	F
	Siphunculi more than 1.5 times longer than cauda. Stridulatory apparatus absent.	G
9	Cauda paler than siphunculi, with 4-7 hairs. Femoral hairs all rather short, less than width of femur at base.	H
	Cauda dark, with 6-12 hairs. Some femoral hairs long and fine, exceeding width of femur at base.	I
10	Inner face of antennal tubercles convergent.	J
	Inner face of antennal tubercles paralled or divergent.	11
11	Siphunculi a little shorter than the long dark cauda	K
	Siphunculi much longer than cauda.	12
12	Head, legs, and antennae mainly dark; femora basally pale but with distal one-half to threequarters black. Siphunculi slightly swollen over distal two-thirds. Cauda with a constriction.	L
	Head, legs, and antennae mainly pale. Siphunculi tapering or parallel over most of length. Cauda without a constriction.	13
13	Inner faces of antennal tubercles parallel. Siphunculi without polygonal reticulation. Cauda only one-tenth to one-eight of length of body.	M
	Inner faces of antennal tubercles divergent. Siphunculi with a subapical zone of polygonal reticulation. Cauda longer, one-seventh to one-fifth of length of body.	N

** The letters in the right column refer to the aphid species, whereas the letters in the same column refer to each step number of the first column.*

A: *Brachyunguis harmalae*
B: *Brachycaudus helichrysi*
C: *Aphis craccivora*
D: *Toxoptera odinae*
E: *Toxoptera citricidus*
F: *Toxoptera aurantii*
G: *Aphis nerii*
H: *Aphis gossypii*
I: *Aphis spiraecola*
J: *Mysus persicae*
K: *Sinomegoura citricola*
L: *Aulacorthum magnoliae*
M: *Aulacorthum solani*
N: *Macrosiphum euphorbiae*

Casual aphid species: *Aphis arbuti, A. fabae, Brachycaudus cardui, Pterochloroides persicae and Rhapalosiphum maidis*.

ANNEX 3. Buffers used in serological assays: DTBIA and ELISA (Clark and Bar-Joseph, 1984).

Buffer	Components	Proportions	Notes
Phosphate buffer saline (PBS) 1X; pH 7.4	NaCl KH_2PO_4 Na_2HPO_4 NaN_3 KCl	8.0 g 0.20 g 1.15 g 0.20 g 0.20 g	Dissolution in 1l distillated water.
Washing buffer (W.B); pH 7.4	PBS (1X) Tween-20	1L 0.5 ml	
Extraction buffer (E.B); pH 7.4	Polyvinilpirolidine Tween-20	20.0 g 0.5 ml	Dissolution in 1l of PBS (1X).
Coating buffer (C.B); pH 9.6	Na_2CO_3 NaN_3 $NaHCO_3$	1.59 g 2.93 g 0.20 g	Dissolution in 1l distillated water.
Conjugate buffer (C.B); pH 7.4	Polyvinilpirolidine Tween-20 Bovine serum albumin (BSA)	20.0 g 05 ml 2.0 g	Dissolution in 1l of PBS (1X).
Substrate buffer (S.B); pH 9.8	Diethanolamine NaN_3	97 ml 0.20 g	Dissolution in 1l distillated water.

ANNEX 4. Total nucleic acid extraction buffers.

Buffer	Material	Quantity	Note
Grinding buffer (pH 5.6 - 5.8)	Guanidine thiosianate. NaOAc, pH 5.2 EDTA. KOAc PVP-40 Sodium bisulphate	4M 0.2 M 25 mM 1.0 M 2.5% 2%	Adjust pH using CH_3COOH Sterilize by autoclaving. Keep it at 4°C. Add Sodium bisulphate before using.
NaI (6M)	Na_2SO_3 NaI (Sigma S8379)	0.75 g 36 g	Dissolve in 40 ml distilled water. Sterilize by autoclaving. Keep it in dark at 4°C.
Silica particles solution (pH 2.0)	Silica particles (Sigma 12% S5631)	12%	Add 60 g silica to 500 ml H_2O. Mix and let settle for 24 hours. Discard the upper 470 ml supernatant (90% of the supernatant). Add H_2O up to 500 ml and mix well. Let settle 5 h. Discard 440 ml (85% of the supernatant). Adjust the remaining 60 ml slurry to a pH 2.0 with HCl. Autoclave and store in dark at room temperature.
Washing buffer (1x)	Tris-HCl, pH7.5 (1M) EDTA (5 M) NaCl (0.5 M) Ehanol	10.0 mM 0.5 mM 50 mM 50%	Sterilize by autoclaving before adding ethanol. Keep it at 4°C.

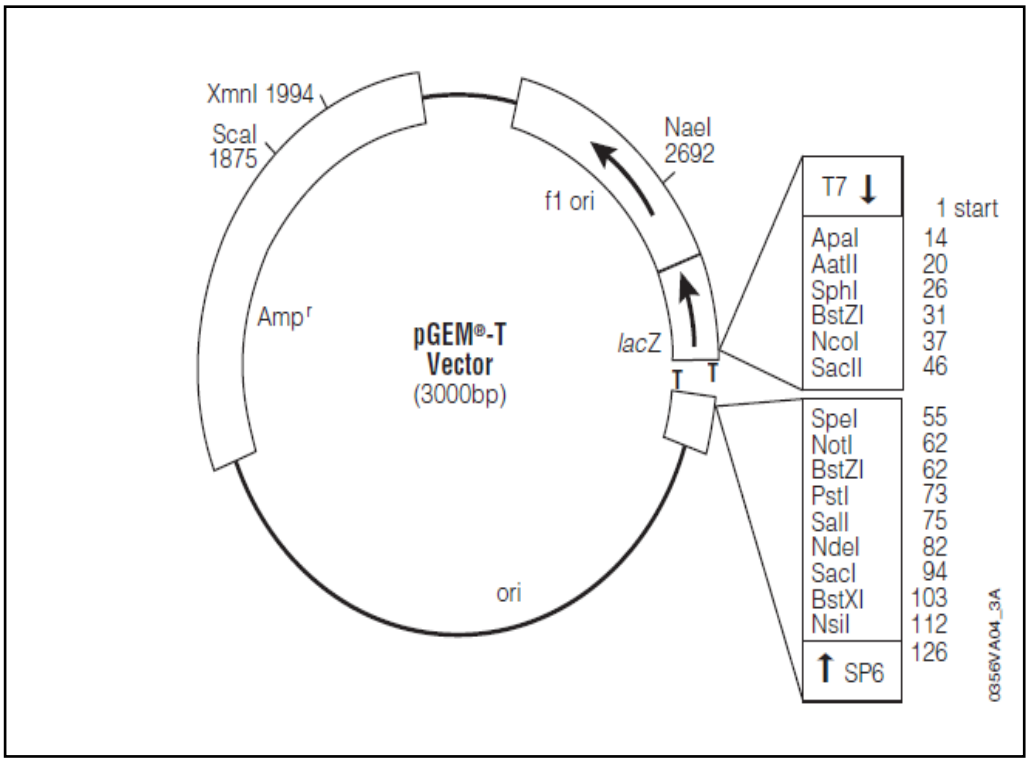
ANNEX 5. Buffers and gels used for electrophoresis.

Buffer	Material	Quantity	Note
TAE 20x (pH 7.2)	Tris Sodium acetate EDTA	0.4M 0.4M 0.02M	Adjust pH using acetic acid Sterilize by autoclaving Keep it at room temperature
TBE 10x (pH 8.3)	Tris Boric acid 99.5% EDTA	1M 61.3 g 0.01M	Dissolve in 1L distilled water Sterilize by autoclaving Keep it at room temperature
1%-Agarose-TBE gel	Distilled water TBE 10x Agarose	90 ml 10 ml 1 g	Boil the mixture for few minutes.
5%-Polyacrylamide-TAE gel	Distilled water Acryl-bis 40% TAE 5x APS 10% TEMED	3.86 ml 650 µl 500 ml 60 µl 6 µl	For 5ml volume
10%- Polyacrylamide-TBE gel	Distilled water Acryl-bis 40% TAE 5x APS 10% TEMED	3.86 ml 1.3 ml 1 ml 120 µl 12 µl	For 5ml volume
Fixing solution	Acetic acid glacial	10 ml	Bring volume to 100 ml using deionized water
Nitric acid solution	Nitric acid pure	1ml	Bring volume to 100 ml using deionized water
Silver equilibrium Solution	$AgNO_3$ Formaldehyde 37%	100 mg 150 µl	Bring volume to 100 ml using deionized water
Developing solution	$NaCO_3$ Formaldehyde 37% Sodium thiosulphate 200 mg/ml	3g 150 µl 4 µl	Bring volume to 100 ml using deionised water

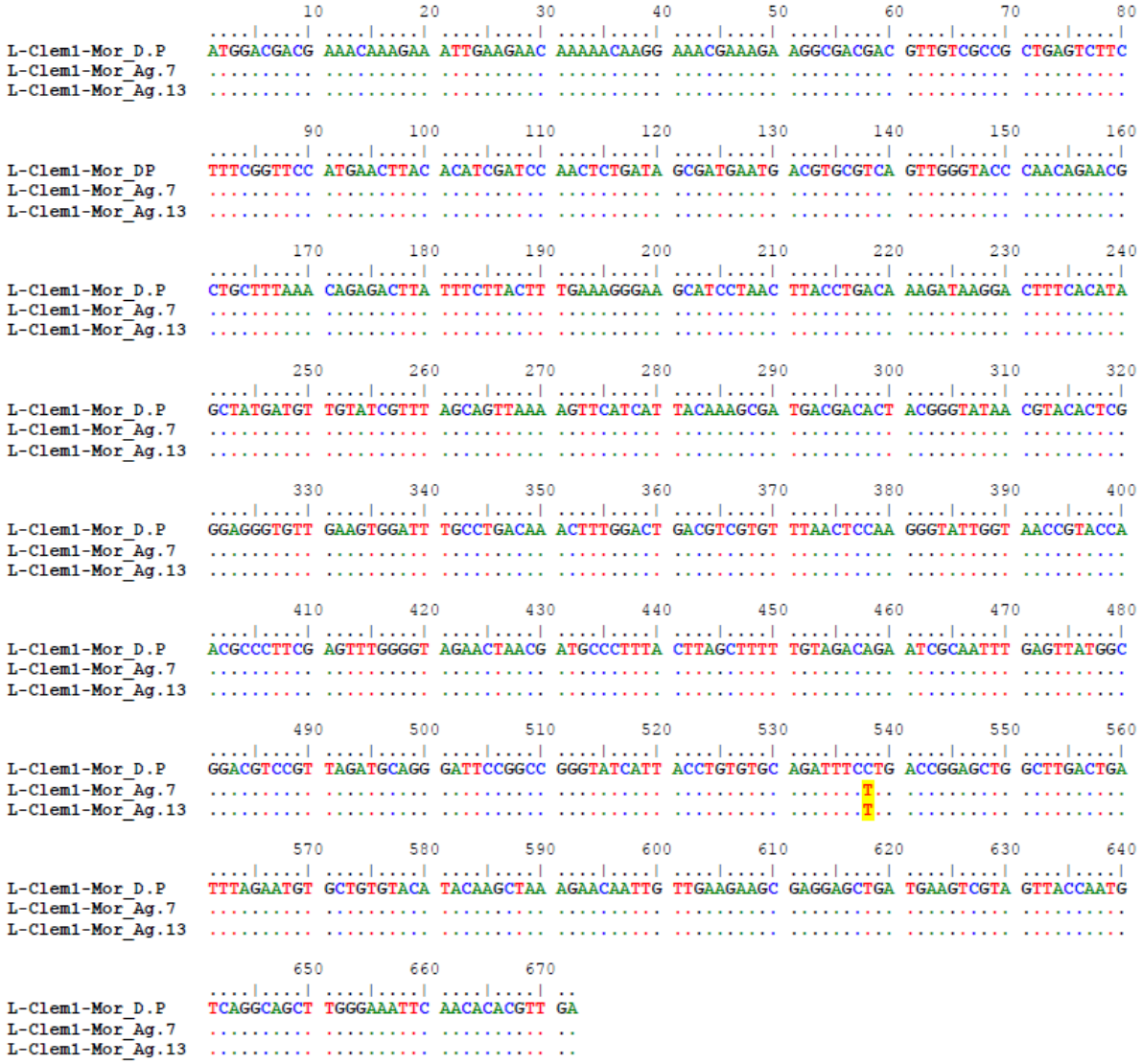
ANNEX 6. Materials and solutions used for cloning and plasmid extraction.

Solutions	Components	Quantities	Notes
STET (100 ml)	NaCl	0.1 M	
	Tris-HCL	2 mM	pH 8
	EDTA	50 mM	pH 8
	Saccarose	8 %	
	Trition X-100	0.5 %	
Lysozyme		20 mg/ml	Store at -20°C
Ammonium acetate pH 5.5	$CH_3CO_2NH_4$	5 M	Sterilize by autoclaving
PEG-NaCl	PEG 8000	20 %	Sterilize by autoclaving
	NaCl	2.5 %	
Luria-Bertani (LB) Liquid	Tryptone	1 %	Sterilize by autoclaving
	Yeast extract	0.5 %	
	NaCl	0.5 %	
Luria-Bertani (LB) Solid	Tryptone	1 %	Sterilize by autoclaving
	Yeast extract	0.5 %	
	NaCl	0.5 %	
	Agar	1.8 %	
TE Buffer	Tris	10 mM	Sterilize by autoclaving
	EDTA	1 mM	

ANNEX 7. Site map of the pGEM-T Easy vector cloning.



ANNEX 8. CTV p25 nucleotide sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.



D.P: donor plant, Ag.: *A. gossypii* subisolates.

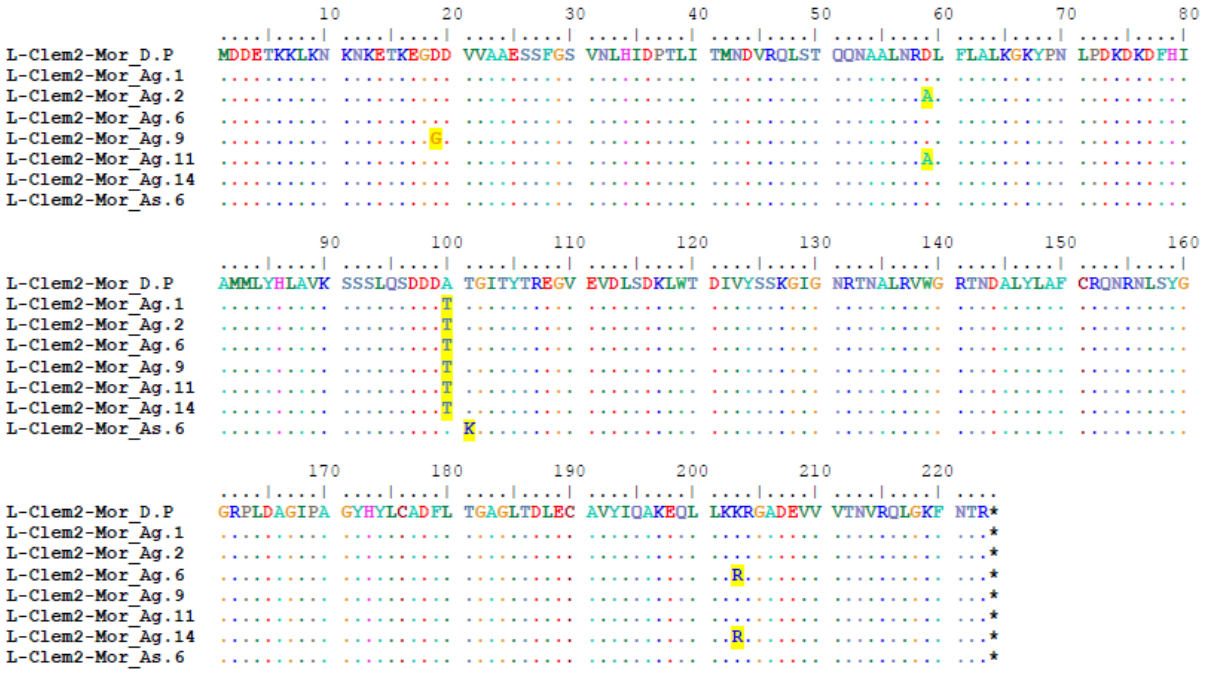
ANNEX 9. Predicted p25 amino-acid sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.

```
                        10         20         30         40         50         60         70         80
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P MDDETKKLKN KNKETKEGDD VVAAESSFGS MNLHIDPTLI AMNDVRQLGT QQNAALNRDL FLTLKGKHPN LPDKDKDFHI
L-Clem1-Mor_Ag.7 .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13 .......... .......... .......... .......... .......... .......... .......... ..........

                        90        100        110        120        130        140        150        160
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P AMMLYRLAVK SSSLQSDDDT TGITYTREGV EVDLPDKLWT DVVFNSKGIG NRTNALRVWG RTNDALYLAF CRQNRNLSYG
L-Clem1-Mor_Ag.7 .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13 .......... .......... .......... .......... .......... .......... .......... ..........

                       170        180        190        200        210        220
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....
L-Clem1-Mor_D.P GRPLDAGIPA GYHYLCADFL TGAGLTDLEC AVYIQAKEQL LKKRGADEVV VTNVRQLGKF NTR*
L-Clem1-Mor_Ag.7 .......... .......... .......... .......... .......... .......... ...*
L-Clem1-Mor_Ag.13 .......... .......... .......... .......... .......... .......... ...*
```

ANNEX 10. CTV p25 nucleotide sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.

D.P: donor plant; Ag.: *A. gossypii* sub-isolates; and As.: *A. spiraecola* sub-isolates.

ANNEX 11. Predicted p25 amino-acid sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.



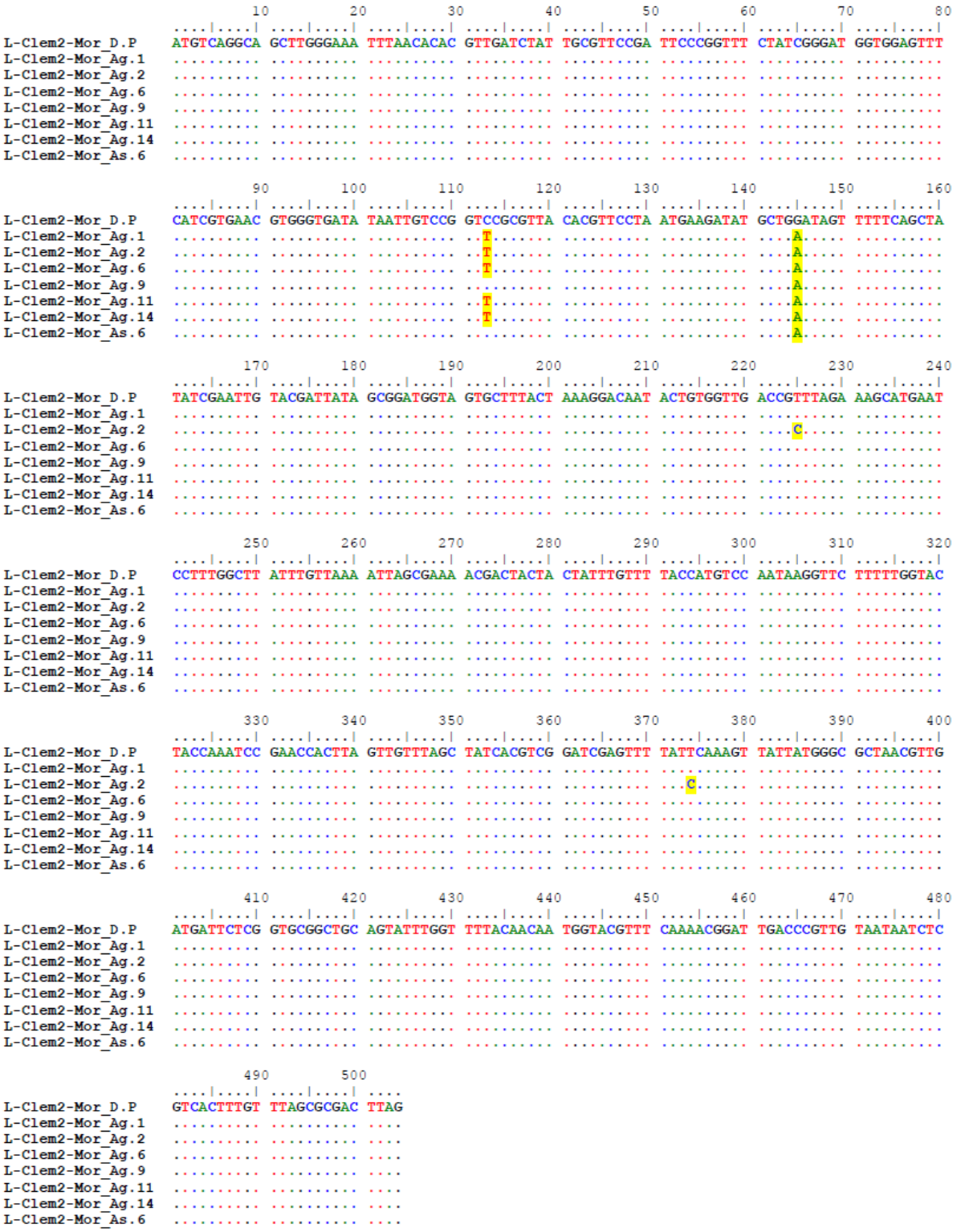
ANNEX 12. CTV p18 nucleotide sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.

```
                          10         20         30         40         50         60         70         80
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    ATGTCAGGCA GCTTGGGAAA TTTAGCACAC GTTGATCTGT TGCGTTCCGA TTCCCGGTTT CTATCGGGAT GGTGGAGTTT
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                          90        100        110        120        130        140        150        160
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    CATCGTGAAC GTGGGCGATA TAATAGTCCG GTTTGCGTTA CACGTTCCTA ATGAAGATAT GCTGAATAAT TTTTCAGCTA
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... ..A....... .......... .......... .......... ..........

                         170        180        190        200        210        220        230        240
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    TATCGAATTG TACGATCATA GCGGACGGTA GTGCTTTACT AAAAGACAAT ACCGTGGTTG ATCGTTTAGA AGGCATGAAT
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .G........ .......... .......... .......... .......... .......... .......... ..........

                         250        260        270        280        290        300        310        320
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    CCTTTGGCTT ATTTGTTAAA TTTAGCGAAA ACGACTACTA CTATTTGTTT TACTATGTCT AATAAGGTTC TTTTTGGTAC
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         330        340        350        360        370        380        390        400
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    TACTAAATCC GAACCTCTTA ATTGTTTAGC TATCACGTCA GATCGAGTTT TGTTCAAAGT TATTATGGGT ACTAACGTTG
L-Clem1-Mor_Ag.7   .......... .......... G......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... G......... .......... .......... .......... .......... ..........

                         410        420        430        440        450        460        470        480
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    ATGATTCTCG ATGCGGCTGC AGTATTTGGT TTTACAACAA TGGTACGTTT CAAAACGGAT TGATCCGTTG TAATAATCTC
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         490        500
                   ....|....| ....|....| ....
L-Clem1-Mor_D.P    GTCGCTTTGT TTAGCGCGAC TTAG
L-Clem1-Mor_Ag.7   .......... .......... ....
L-Clem1-Mor_Ag.13  .......... .......... ....
```

ANNEX 13. Predicted p18 amino-acid sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.

```
                    10        20        30        40        50        60        70        80
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P     MSGSLGNLAH VDLLRSDSRF LSGWWSFIVN VGDIIVRFAL HVPNEDMLNN FSAISNCTII ADGSALLKDN TVVDRLEGMN
L-Clem1-Mor_Ag.7    .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13   .......... .......... .......... .......Y.. .......... ...M...... .......... ..........

                    90        100       110       120       130       140       150       160
                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P     PLAYLLNLAK TTTTICFTMS NKVLFGTTKS EPLNCLAITS DRVLFKVIMG TNVDDSRCGC SIWFYNNGTF QNGLIRCNNL
L-Clem1-Mor_Ag.7    .......... .......... .......... ...S...... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13   .......... .......... .......... ...S...... .......... .......... .......... ..........

                    ....|...
L-Clem1-Mor_D.P     VALFSAT*
L-Clem1-Mor_Ag.7    .......*
L-Clem1-Mor_Ag.13   .......*
```

ANNEX 14. CTV p18 nucleotide sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.



ANNEX 15. Predicted p18 amino-acid sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.

```
                          10        20        30        40        50        60        70        80
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem2-Mor_D.P   MSGSLGNLTH VDLLRSDSRF LSGWWSFIVN VGDIIVRSAL HVPNEDMLDS FSAISNCTII ADGSALLKDN TVVDRLESMN
L-Clem2-Mor_Ag.1  .......... .......... .......... .......F.. ........N. .......... .......... ..........
L-Clem2-Mor_Ag.2  .......... .......... .......... .......F.. ........N. .......... .......... ..........
L-Clem2-Mor_Ag.6  .......... .......... .......... .......F.. ........N. .......... .......... ..........
L-Clem2-Mor_Ag.9  .......... .......... .......... .......... ........N. .......... .......... ..........
L-Clem2-Mor_Ag.11 .......... .......... .......... .......F.. ........N. .......... .......... ..........
L-Clem2-Mor_Ag.14 .......... .......... .......... .......F.. ........N. .......... .......... ..........
L-Clem2-Mor_As.6  .......... .......... .......... .......... ........N. .......... .......... ..........

                          90        100       110       120       130       140       150       160
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem2-Mor_D.P   PLAYLLKLAK TTTTICFTMS NKVLFGTTKS EPLSCLAITS DRVLFKVIMG ANVDDSRCGC SIWFYNNGTF QNGLTRCNNL
L-Clem2-Mor_Ag.1  .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem2-Mor_Ag.2  .......... .......... .......... .......... ....S..... .......... .......... ..........
L-Clem2-Mor_Ag.6  .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem2-Mor_Ag.9  .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem2-Mor_Ag.11 .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem2-Mor_Ag.14 .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem2-Mor_As.6  .......... .......... .......... .......... .......... .......... .......... ..........

                  ....|...
L-Clem2-Mor_D.P   VTLFSAT*
L-Clem2-Mor_Ag.1  .......*
L-Clem2-Mor_Ag.2  .......*
L-Clem2-Mor_Ag.6  .......*
L-Clem2-Mor_Ag.9  .......*
L-Clem2-Mor_Ag.11 .......*
L-Clem2-Mor_Ag.14 .......*
L-Clem2-Mor_As.6  .......*
```

ANNEX 16. CTV p23 nucleotide sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.

```
                          10         20         30         40         50         60         70         80
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    ATGGACGATA CTAGCGGACA AACTTTCGTT TCTGTGAACC TTTCTGACGA AAGCAACACA GCGAGTACTA GAGTTGAAAA
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... ...T...... .......... ..........

                          90        100        110        120        130        140        150        160
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    CGTAAAATCG GAAGCGGATC GCTTGGAGTT TTTACGTAAA ATGAATCCCT TCATTGTTGA CGCTCTGGTG CGGAAAACTA
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .....A.... .......... ..........

                         170        180        190        200        210        220        230        240
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    ATTATCAGGG CGCTCGCTTT CGTGCGAGAA TAATAGGAGT GTGCGTGGAT TGTGGTAGAA AACACGACAA GGCGCTCAAG
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         250        260        270        280        290        300        310        320
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    ACTGAACGTA AGTGTAAGGT CAACAATACG CAGTCTCAGA ACGAGGTGGC GCATATGTTG ATGCACGATC CCGTTAAGTA
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         330        340        350        360        370        380        390        400
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    TTTAAACAAA AGAAAGGCTA GAGCCTTTTC TAACGCAGAG ACGTTTGCGA TTGAATTGGT TTTGTACACC AAGGAAAAGC
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         410        420        430        440        450        460        470        480
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    AATTGGCGGT TGATTTGGCC GCCGAAAGGG AGAAGGCGAG GTTGGCTCGT AGACACCCAA TGCGTTCTCC AGAGGAAACT
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... .......... ..........

                         490        500        510        520        530        540        550        560
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    CCGGAACATT ATAAATTTGG TATGACTGCT AAAGCGATGT TACCGGAATT CAACGCTGTA GGCGTTGGTG ATAACGAAGA
L-Clem1-Mor_Ag.7   .......... .......C.. .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......C.. .......... .......... .......... .......... .......... ..........

                         570        580        590        600        610        620        630
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
L-Clem1-Mor_D.P    CACTTCGTCG GAATACCCAG TAAGTTTGAG TGTTTCCGGT GGAGTTCTCC GTGAACACCA CTTCATCTGA
L-Clem1-Mor_Ag.7   .......... .......... .......... .......... .......... .......... ..........
L-Clem1-Mor_Ag.13  .......... .......... .......... .......... .......... .......... ..........
```

ANNEX 17. Predicted p23 amino-acid sequence of the Moroccan CTV L-Clem1-Mor isolate and its aphid derived sub-isolates.

ANNEX 18. CTV p23 nucleotide sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.

ANNEX 19. Predicted p23 amino-acid sequence of the Moroccan CTV L-Clem2-Mor isolate and its aphid derived sub-isolates.