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Assessment of genetic diversity of Mediterranean Citrus Tristeza Virus (CTV) isolates and genomic RNA variability associated to their vector transmission

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Abstract

Citrus tristeza virus (CTV), is the causal agent of devastating epidemics that changed the course of citrus industry worldwide. To date, global CTV infection caused the loss of almost 100 million trees, of which 40 millions were from countries of the Mediterranean basin, where potential risks for further serious devastations are foreseen, since the CTV-sensitive sour orange is still the major rootstock adopted over large extends.

CTV is adapted to replicate in the phloem cells of *Citrus* spp., where latent infections are quiet common and may last symptomless for a long time. Meanwhile, dramatic losses have been readily induced by the interaction of severe CTV variants with different scion-rootstock combinations, enabling to discriminate between three distinct syndromes known as quick decline, stem pitting and seedling yellows. Furthermore, CTV is a stylet-borne virus adopted to be transmitted in nature by several aphid species in a semi-persistent manner. The most efficient vector *Toxoptera citricidus* (Kirkaldy) became recently established in northern Spain and Portugal, which raised the risk for the rapid spread of severe CTV isolates, representing a serious threat to the Mediterranean citrus industry.

Moreover, *Aphis gossypii* (Glover) is a pandemic aphids species that has been reported to be the origin of major CTV epidemics in the Mediterranean basin. Additionally, minor CTV vectors including *A. spiraecola* (Pagenstecher) and *Toxoptera. aurantii* (Boyer de Fonscolombe) are prevalent and their extensive populations may have a significant impact in the local virus spread.

CTV populations can occur as a mixture of genotypes, which have been reported to be separated by aphid transmission bottlenecks and/or graft propagation, but the mechanism involved during these changes is still largely unknown.

The main aim of present research was to conduct a molecular characterization and to evaluate the genetic diversity of the CTV populations within the Mediterranean basin. Furthermore, the experimental vector-transmissibility of some isolates by local aphid biotypes has been evaluated and studies on eventual genomic variations after aphid passage have also been investigated.

To reach the above mentioned goals, a total number of 75 CTV sources originated from 15 Mediterranean countries (Albania, Algeria, China, Croatia, Cyprus, Egypt, Italy, Iran, Lebanon, Montenegro, Morocco, Palestine, Portugal, Syria and Trinidad), maintained within CTV collection of the Mediterranean Agronomic Institute of Bari (MAI-B, Italy), have been assigned a genotype profile according to their specific signals using a set of linked molecular markers. Within each exhibited genotype profile, CTV-CP gene targets have been amplified from a representative isolate and subjected to partial genome sequence analysis.

In order to evaluate the vector transmission kinetics of CTV within the Mediterranean area, a number of CTV isolates showing diversified genomic traits and pathogenecity have been subjected to experimental transmission trials using the most prevailing local aphid biotypes, including *A. gossypii* (Glover), *A. spiraecola* and *T. aurantii*. Further serological and molecular assays flanking multiple genomic regions have been applied to the aphid-derived sub-isolates, in order to assess eventual alterations in the parental virus populations by aphid passage.

The 5'terminal ORF typing and the phyllogenetic relationship of CTV-CPg analysis of CTV isolates, highlightes the high genetic diversity of the Mediterranean CTV populations between and within countries, and evidences the predominant occurrence of CTV isolates as a mixture of genomic variants. The major outcome of this research is that T30 mild strain genotype (Florida) is a stable viral component on the studied area, while, severe VT (Israel) and T36 (Florida) genotypes appeared as relevant variants in the Middle East and East

Adriatic regions, respectively. Nevertheless, some p25 haplotype populations clustered close to standard isolates associated with high pathogenic potential, such as T3 (Florida) and SY568 (New Zealand).

Vector transmission experiments of the studied isolates showed diverse transmission efficiencies of and evidenced a great spread potential of either mild and severe seedling yellows -*A. gossypii* combinations. Conversely, the T36-like isolate (MAIB_Q3). was poorly transmissible.

In correlation with the consistent prevalence of *A. gossypii* species on citrus plantings, these findings reflect the high involvement of the citrus entomofauna in the rapid spread and the major outbreaks of tristeza in the Mediterranean area. Within the aphid-generated CTV sub-isolates, minor nucleotide mutations occurred on p18, p20 and p23 genomic regions, but also over the highly conserved CPg sequence, exhibiting new haplotypes. When translated to amino-acid sequences, specific variations on the p23 and p25 regions resulted in slight protein divergences, which in some instances may be accompanied by changes in the pathogenic behaviour of the parental isolate.

Riassunto

Il Virus della Tristeza (CTV) è agente causale di devastanti epidemie che hanno cambiato l'andamento della produzione agrumicola di tutto il mondo. Ad oggi le infezioni da CTV hanno causato la perdita di circa 100 milioni di alberi, di cui 40 milioni in Paesi nel Bacino del Mediterraneo, ancora a rischio di ulteriori gravi devastazioni, poiché il portainnesto maggiormente usato in queste aree rimane l'arancio amaro, molto sensibile a CTV.

CTV si è adattato a replicarsi nelle cellule floematiche delle specie di Citrus, dove le infezioni latenti sono molto comuni e possono restare asintomatiche per molto tempo. Sono state provocate grosse perdite indotte dall'interazione fra varianti di CTV molto aggressive su diverse combinazioni nesto-portainnesto, causando diverse sindromi note come quick decline (morte repentina), stem pitting (butteratura del fusto) e seedling yellows (ingiallimento dei semenzali). CTV è un virus dalla forma a stiletto che si è adattato ad essere trasmesso in natura da diverse specie di afidi in modo semi-persistente. Il vettore più efficiente è la *Toxoptera citricidus* (Kirkaldy) (recentemente stabilitasi nelle zone settentrionali di Spagna e Portogallo), che ha innalzato il rischio per la rapida diffusione di diversi isolati di CTV, che rappresentano una grave minaccia per la produzione agrumicola mediterranea.

Una specie di afide pandemico, ritenuto all'origine delle principali epidemie di CTV nel bacino del Mediterraneo è l'*Aphis gossypii* (Glover); altri vettori ritenuti minori ma comuni sono l'*A. spiraecola* (Pagenstecher) e la *T. aurantii* (Boyer de Fonscolombe), le cui ampie popolazioni possono avere un impatto significativo nella diffusione locale del virus. Gli isolati di CTV possono contenere un misto di genotipi, che, come riportato dalla letteratura, si sono separati a seguito della trasmissione tramite afidi e/o propagazione attraverso innesto, ma il meccanismo di questi cambiamenti è ancora sconosciuto.

Scopo principale della presente ricerca è stata la caratterizzazione molecolare e valutazione della diversità genetica della popolazione CTV

all'interno del Bacino del Mediterraneo. E' stata inoltre valutata la trasmissibilità sperimentale tramite vettori di alcuni isolati tramite biotipi locali di afidi e sono stati anche studiate eventuali variazioni genomiche dopo il passaggio degli afidi.
Per raggiungere gli obiettivi di cui sopra è stato assegnato un profilo genotipico (secondo l'analisi dei marcatori molecolari), ad un numero totale di 75 isolati di CTV provenienti da 15 Paesi Mediterranei (Albania, Algeria, Cina, Croazia, Cipro, Egitto, Italia, Iran, Libano, Montenegro, Marocco, Palestina, Portogallo, Siria e Trinidad conservati nella collezione CTV dell'Istituto Agronomico Mediterraneo di IAM-Bari, Italia). All'interno di ogni profilo genotipico il gene CP è stato amplificato da un isolato rappresentativo e sottoposto all'analisi parziale della sequenza del genoma.

Un certo numero di isolati di CTV rappresentativi di diversi tipi di genomi e grado di virulenza, è stato sottoposto a prove sperimentali di trasmissione, utilizzando i biotipi di afidi locali prevalenti, tra cui *A. gossypii* (Glover), *A. spiraecola* and *T. aurantii*, in modo da valutare la cinetica della trasmissione tramite vettori nell'area mediterranea. Ulteriori saggi sierologici e molecolari su altre regioni del genoma, sono stati effettuati sui sub-isolati derivati dagli afidi, in modo da valutare eventuali alterazioni nella popolazione parentale del virus col passaggio degli afidi.

Dallo studio della parte terminale del genoma e dall'analisi filogenetica del gene CPg degli isolati CTV, è stata evidenziata una elevata diversità genetica nella popolazione CTV mediterranea, tra e all'interno dei Paesi e mette in risalto il fatto predominante di avere isolati CTV composti da più varianti genomiche.

Il più importante risultato di questa ricerca è che il genotipo del ceppo blando T30, e i virulenti VT (Israele) e T36 (Florida) sono comparsi come varianti rilevanti rispettivamente in Medio Oriente e nelle regioni orientali dell'Adriatico. Tuttavia alcune popolazioni aplotipiche di *p*25 si sono raggruppate vicino agli isolati standard T3 (Florida) e SY568.

Gli esperimenti di trasmissione attraverso vettori hanno mostrato un diverso grado di efficienza nella trasmissione degli isolati e dimostrato che esiste

un grande potenziale di diffusione sia degli isolati blandi che di quelli aggressivi in combinazione con l' *A. gossypii*. Al contrario l'isolato T36 (MAIB_Q3) è stato scarsamente trasmesso. In relazione alla prevalenza di *A. gossypii* su impianti di agrumi, questi risultati riflettono l'alta importanza dell'entomofauna agrumicola nella diffusione rapida e nelle principali epidemie di Tristeza nell' Area mediterranea.

All'interno del sub-isolato CTV generato dall'afide si sono verificate mutazioni nucleotidiche minori sulle regioni genomiche di *p*18, *p*20 e *p*23 ma anche sul gene Cpg dando luogo a nuovi aplotipi.

Le putative sequenze aminoacidiche mostravano variazioni specifiche sulle regioni *p*23 and *p*25 dando luogo a cambiamenti nel comportamento patogenetico degli isolati parentali.

RESUME

Citrus tristeza virus (CTV), est l'agent causal d'une épidémie dévastatrice. Ce virus a changé le cours de l'industrie des agrumes dans le monde entier. Jusqu'ici, l'infection globale du CTV a causé la perte de presque 100 millions d'arbres, dont 40 millions étaient des pays du bassin Méditerranéen, où des risques potentiels sont prévus pour d'autres dévastations encore plus graves, étant donnè que le bigaradier, sensible au CTV est toujours le porte greffe principalement adopté dans la majeure partie des pays du bassin méditerranéen.

Le CTV est adapté pour se multiplier dans les cellules de phloème des espèces d'agrumes, où les infections latentes sont communes et peuvent durer asymptomatiques pendant longtemps. En attendant, des pertes dramatiques ont été aisément induites par l'interaction des variantes sèvères de CTV avec différentes combinaisons de greffon-porte/greffe, permettant de distinguer entre trois syndromes divers connus sous le nom de « Quick Decline », « Stem pitting » et « Seedling yellows ». En outre, le CTV est un virus transmis naturellement par plusieurs espèces de pucerons d'une façon semi-persistante. L'espèce *Toxoptera citricidus* (Kirkaldy) est le vecteur le plus efficace, il a ètè récemment repertoriè en Espagne nordique et au Portugal, où on a pu soulever le risque pour la diffusion rapide des isolats graves du CTV, représentant une menace grave à l'industrie méditerranéenne des agrumes.

D'ailleurs, l'*Aphis gossypii* (Glover) est une des espèces pandémiques de puceron. Il a ètè rapporté comme ètant l'origine des épidémies importantes de CTV dans le bassin méditerranéen. Parmi les vecteurs mineurs de CTV, l'*A. spiraecola* (Pagenstecher) et *Toxoptera aurantii* (Boyer de Fonscolombe) sont des populations répandues et ils peuvent avoir un impact significatif dans la diffusion locale de virus.

Les populations de CTV peuvent se produire comme mélange de génotypes, leur transmission peut se produire par des pucerons et/ou greffage,

mais le mécanisme impliqué pendant ces changements est toujours en grande partie inconnu.

Le but principal de la recherche actuelle était de conduire une caractérisation moléculaire et d'évaluer la diversité génétique des populations de CTV dans le bassin méditerranéen. En outre, l'expérimentation vecteur-transmissibilitè de quelques isolats par des biotypes locaux de pucerons a été évaluée et des études sur certaines variations genomiques après que le passage de pucerons, aient été également recherchèes.

Pour atteindre les buts mentionnés ci-dessus, un nombre total de 75 sources de CTV a ètè provenu de 15 pays méditerranéens (Albanie, Algérie, Chine, Croatie, Chypre, Egypte, Italie, Iran, Liban, Montenegro, Maroc, Palestine, Portugal, Syrie et Trinidad), maintenus dans la collection de CTV de l'Institut Agronomique Méditerranéen de Bari (IAM-B, Italie), Les sources de CTV ont été assignés en un profil de génotype selon leurs signaux spécifiques utilisant un ensemble de marqueurs moléculaires. Dans chaque profil de génotype exhibé, des cibles de gène de CTV-CP ont été amplifiées d'un isolat représentatif et soumises à l'analyse partielle d'ordre de génome.

Afin d'évaluer la cinétique de transmission du vecteur du CTV dans l'aire méditerranéenne, un certain nombre d'isolats de CTV montrant des traits genomiques et pathogenecitè divers ont été soumis aux épreuves expérimentales de transmission utilisant les biotypes de pucerons locaux les plus prèsents, y compris l' *A.gossypii* (gantier), l'*A. spiraecola* et le *T. aurantii.* Encore d'autres analyses sérologiques et moléculaires flanquant des régions genomiques multiples ont été appliquées aux sous-isolats générés par les puceron, afin d'évaluer certains changements des populations parentales de virus moyennant le passage de pucerons.

L'alignement selon le cadre ouvert de lecture de la terminaison 5' et le rapport phyllogènique de l'analyse PCg-CTV des isolats de CTV, ont montré la diversité génétique élevée des populations méditerranéennes de CTV au sein des pays et entre les different pays de la mèditèrrannèe, et ont mis en évidence

l'occurrence prédominante des isolats de CTV sous forme de mélange de variantes genomiques. Les résultats principaux de cette recherche sont les suivant: Le génotype T30 induisant des symptomes lègers (la Floride) est un composant viral stable dans l'aire étudié, tandis que, les génotype sèvères VT (l'Israël) et T36 (la Floride) est apparu en tant que variantes spècifiques du Moyen-Orient et des régions adriatiques. Néanmoins, les populations du haplotype *p*25 ont ètè groupé à proximitè des isolats standards liés au potentiel pathogènique élevé, tel que T3 (la Floride) et SY568 (Nouvelle Zélande).

L'expérimentation de transmission des isolats étudiés a montré des capacitès diverses de transmission et a démontré un grand potentiel de diffusion de la combinaison d'*A gossypii*-symptomes lègers et sèvères de jaunissement.. Réciproquement, l'isolat similaire au T36 (MAIB_Q3) était médiocrement transmissible. Dans la corrélation avec la prédominance conformée des espèces d'*A gossypii* sur des plantations d'agrumes, ces résultats reflètent la participation élevée de l'entomofaune infèodèe aux agrumes dans la diffusion rapide et les manifestations principales de tristeza dans la zone méditerranéenne.

Au niveau des sous-isolat du CTV générés par la transmission par les pucerons, des mutations mineures de nucléotide se sont produites sur les régions genomiques *p*18, *p*20 et *p*23, mais également au-dessus de l'ordre fortement conservé de la PCg, montrant de nouveaux haplotypes. Une fois traduite en acide aminé, les variations spécifiques sur les régions du *p*23 et les *p*25 ont eu comme conséquence de légères divergences de protéine, qui peuvent être accompagnées parfois de changements du comportement pathogène de l'isolat parental.

Table of contents

Acknowledgment…… i
Abstract……..……iii
Riassunto……vi
Résumé……ix
Chapter 1. Introduction..... 1
1.1. Early introduction of Citrus spp. into the Mediterranean basin 1
1.2. Worldwide Citrus industry 3
1.3. Citrus disorders and advance to Tristeza pandemics..... 5
Chapter 2. LITERATURE REVIEW: *Citrus tristeza disease*..... 8
2.1. Origins of Citrus tristeza disease: Historical perspective and economic impact 8
2.2. Causal agent: Citrus tristeza closterovirus taxonomy 10
2.3. Host range and field diagnosis 12
2.4. Evolutionary diagnosis 16
2.4.1. Biological indexing 16
2.4.2. Serological diagnosis..... 17
2.4.3. Molecular diagnosis 18
2.5. Viral aspect and molecular features 20
2.5.1. CTV genome organization 20
2.5.2. CTV replication..... 23
2.5.3. CTV population structure and genetic diversity..... 25
2.6. Epidemiology and vector transmission of Citrus tristeza virus..... 26
2.6.1. Concepts in CTV epidemiology 26
2.6.2. CTV aphid vectors..... 28
2.6.2.1. Taxonomic rank:..... 28
2.6.2.2. Morphology and biological behavior of CTV aphid vector species. 29
2.6.2.3. Semi-persistent CTV vector transmission 31
2.6.2.4. Major CTV aphid vector species..... 32
(ii) The cotton and/or melon aphid *A. gossypii* (Glover) 33
(iii) The spirea aphid *A. spiraecola* (Patch)..... 34
(iv) The Black citrus aphid: *T. aurantii* (Boyer de Fonscolombe) 35
2.6.2.5. Environmental factors: 35
2.6.2.6. Virus-vector interaction..... 37
2.6.3. Genomic alteration of viral CTV population by aphid passage 39
2.7. Tristeza disease management 42
2.7.1. Prophylactic approaches..... 43
2.7.2. Host tolerance..... 44
2.7.3. Mild strain cross-protection..... 45
2.7.4. Pathogen derived resistance 46
Chapter 3: Overall objectives 48

Chapter 4: Materials and Methods 50
4.1. Molecular characterization of Mediterranean CTV isolates 50
4.1.1. Assessment of the genetic diversity of CTV by Multiple Molecular Markers 50
4.1.1.1. Total RNA isolation and complementary DNA (cDNA) synthesis . 51
4.1.1.2. Multiple Molecular Markers assays: 52
4.1.1.3. Cloning and Partial Genome Sequencing of the CTV-CPg 53
4.1.1.4. Single strand conformation polymorphism analysis (SSCP) 57
4.1.1.5. Computational-assisted sequence analysis 58
4.2. Experimental CTV aphid transmission trials 58
4.2.1. CTV virus sources 58
4.2.2. CTV aphid vector species 59
4.2.3. Transmission experiments 62
4.2.4. CTV detection and characterization of aphid-inoculated sub-isolates 64
4.2.4.1. Serological detection and discrimination of CTV isolates after aphid passage 64
4.2.4.2. Molecular detection and characterization of CTV sub-isolates after aphid passage: 65
4.2.4.2.1. Multiple SSCP analysis of the CTV vector inoculated sub-isolates 66
Chapter 5. Results and discussions 70
5.1. Molecular characterization of Mediterranean CTV isolates 70
5.1.1. Assessment of the genetic diversity of CTV by Multiple Molecular Markers 70
5.1.2. Partial genome sequencing and clustering patterns of CTV-CPg 76
5.2. CTV experimental aphid transmission trials 86
5.2.1. Virus sources 86
5.2.2.CTV vector transmission efficiencies 88
5.2.3. CTV detection and characterization of aphid-inoculated sub-isolates 91
5.2.3.1. Serological characterization of CTV sub-isolates after aphid passage 91
5.2.3.2. Multiple SSCP analysis of the CTV vector inoculated sub-isolates 93
5.2.3.3. Multiple Molecular Markers analysis of the CTV vector inoculated sub-isolates 103
5.2.3.4. Cloning and partial genome sequencing of the vector-inoculated sub-isolates 104
5.2.2.5. Nucleotide sequence variation after aphid passage 111
Chapter 6. Conclusion 114
Chapter 7. Bibliographical References 118

LIST OF FIGURES

Fig 1. World distribution of *Citrus tristeza virus* (EPPO, 2006) 5
Fig. 2. Genome maps of the family *Closteroviridae* genera 11
Fig 3. Schematic representation of the genome organization of CTV 12
Fig 4. Major field syndromes and greenhouse symptoms induced by *Citrus Tristeza Virus* 15
Fig 5. Schematic diagram of *Citrus tristeza virus* genome organization 53
Fig 6. Morphological discrimination between CTV aphid vector species under compound microscope 61
Fig 7. CTV aphid vectors rearing and transmission experiments of Mediterranean isolates. (A) and (B): Rearing of *A. gossypii* on Cotton and Melon plants respectively; (C) and (D): Rearing of *A. spiraecola* and *T. aurantii* on Viburum and Madame Vinous sweet orange plants, respectively; (E): Development of aphid populations under controlled hydrothermal conditions; (F): Infestation of a CTV infected source plants with aphids; (G): Acquisition feeding of *A. gossypii* from a CTV donor plant; (H) and (I): Inoculation feeding of virus-exposed aphids on healthy test plantlets and their maintenance under greenhouse conditions, respectively. 63
Fig 8. Dendrogram showing the clustering pattern of the CPg sequences from single infected Mediterranean CTV isolates and worldwide reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale. 77
Fig 9. Phylogenetic relationships of the CPg sequence from MAIB_Q5 Albanian Citrus tristeza virus isolate and standard reference strains. Numbers close to branches represent 79
Fig 10. Phylogenetic relationships of the CPg sequence from the Moroccan (MAIB_Q75) Citrus tristeza virus isolate and other reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale..... 80
Fig 11. Phylogenetic relationships of the CPg sequence from Croatian (MAIB_Q96) Citrus tristeza virus isolate and standard reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale..... 81
Fig 12. Phylogenetic relationships of the CPg sequence of Citrus tristeza virus isolate (MAIB_Q106) from Montenegro and standard reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale..... 83
Fig 14. SSCP patterns of CPg sequences from selected Mediterranean CTV sources submitted to aphid transmission experiments in 10% polyacrylamide gel electrophoresis 87

Fig 15. Symptoms of moderate to severe stunting of the aphid derived sub-isolates on Mexican lime. A, B and C, D, E, F and G: sub-isolates from SG29, MAIB_Q1294, MAIB_Q96, CTV2, MAIB_Q57 and MAIB_Q54, respectively; -C: Healthy control. 89
Fig 23. Multiple Molecular Markers profiles of the aphid transmitted isolates 103
Fig 24. CPg-SSCP patterns yielded from the Italian mild isolate (MAIB_Q1294) sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis 105
Fig 25. Phylogenetic analysis of CPg sequences yielded from the vector transmitted Mediterranean CTV isolates used before and after the aphid passage 106
Fig 26. CPg-SSCP patterns yielded from the Italian CTV-SY isolate (SG29) sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis 107
Fig 27. CPg-SSCP patterns yielded from the Palestinian CTV-SY isolate MAIB_Q54 sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis 109
Fig 28. Phylogenetic analysis of *p*23 sequences yielded from the vector transmitted Mediterranean CTV isolates used before and after the aphid passage 111

LIST OF TABLES

Table 1. Top ten world citrus producing countries (tons) (Anonymous, 2007) 4
Table 3. Occurrence of the major Citrus Tristeza Virus epidemics and estimated 6
Table 4. Oligonucleotide primers and their genomic positions used for Multiple Molecular Markers analysis (Hilf *et al.,* 2005) 52
Table 5. Legation protocol using *pGEM-T* Easy Vector (Promega, USA) 54
Table 6. Sequence oligonucleotide primers used for the amplification of the CTV genes 66
Table 7. Optimal conditions for adopted for CTV CE-SSCP trials 68
Table 8. Marker patterns of standard Citrus tristeza virus genotypes (Hilf *et al*., 2005) 70
Table 9. Genotype assignment of Mediterranean CTV isolates based on MMM analysis 71
Table 10. Summary of isolate genotypes determined by multiple molecular marker analysis 74
Table 11. Biological activities of the different tristeza CPg groups by APET analysis (Nolasco *et al*., 2008) 87
Table 12. Experimental vector transmission efficiencies of Mediterranean CTV isolates by 88
Table 13. MCA 13 MAb serotyping of CTV isolates by aphid transmission 92

List of annexes

ANNEX 1. Total nucleic acid extraction buffers 140
ANNEX 2. Buffers and gels used for electrophoresis 141
ANNEX 3. Bacterial media and solutions used for cloning and plasmid DNA extraction 142
ANNEX 4. Mounting medium for aphids 143
ANNEX 5. Identification key for worldwide citrus aphid species 144
ANNEX 6. Buffers used in serological assays: DTBIA and ELISA 145
Annex 7. CTV p25 nucleotide sequence of the MAIB_Q1294 isolate 146
Annex 8. Predicted p25 amino-acid sequence of the CTV MAIB_Q1294 isolate and its aphid derived sub-isolates 147
Annex 9. CTV p25 nucleotide sequence of the SG29 isolate and its aphid derived sub-isolates 148
Annex 10. Predicted p25 amino-acid sequence of the CTV SG29 isolate and its aphid derived sub-isolates 149
Annex 11. CTV p25 nucleotide sequence of the MAIB_Q54 isolate and its aphid derived sub-isolates 150
Annex 12. Predicted p25 amino-acid sequence of the CTV MAIB_Q54 isolate and its aphid derived sub-isolates 151
Annex 13. CTV p23 nucleotide sequence of the MAIB_Q1294 isolate and its aphid derived sub-isolates 152
Annex 14. CTV p23 nucleotide sequence of the SG29 isolate and its aphid derived sub-isolates 153
..... 154
Annex 16. Predicted p23 amino-acid sequence of CTV MAIB_Q1294, SG29 and MAIB_Q54 isolates and their aphid derived sub-isolates, respectively ... 155

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micrometer
A	Adenine
APET	Asymmetric PCR ELISA typing assay
APS	Ammonium Persulphate
ATP	Adenine Triphosphate
B.C.	Before Christ
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazol
BrCA	Brown citrus aphid
BSA	Bovine serum albumin
BYV	Beet yellow virus
C	Cytosine
CaCl2	Calcium chloride
cDNA	Complementary Deoxyribonucleic Acid
cm	Centimeter
CP	Coat Protein
CpD	Divergent CP analogue
CPm	Minor coat protein
cRNA	Complementary Ribonucleic Acid
CTP	Cytosine Triphosphate
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
dATP	Deoxyadenine Triphosphate
dCTP	Deoxycytosine Triphosphate
dGTP	Deoxyguanine Triphosphate
DNA	Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsRNA	Double stranded RNA
DTBIA	Direct tissue blot immunoassay
DTT	Dithiothreitol
dTTP	Deoxythymine Triphosphate
dUTP	Deoxyuracile Triphosphate
EDTA	Ethylene Diamino Tetra Acetic Acid (disodium salt)
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
Fig.	Figure
G	Guanine

g	Gram
GFP	Green fluorescent protein
gRNA	Genomic RNA
GLRaV-3	Grapevine leafroll-associated virus-3
GTP	Guanine Triphosphate
h	Hour
H2O	Water
HEL	Helicase
HCl	Hydrochloridric Acid
HS	Hierarchical sampling
HSP70	Heat Shock Protein 70
ICARDA	International Center for Agricultural Research in the Dry Areas
Kb	Kilo Base Pair
kDa	Kilodalton
LB	Luria Bertani
LIYV	Lettuce infectious yellows virus
LMTs	Low-molecular-weight tristeza sub-genomic RNA
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis software
mg	Milligram
mg/ml	Milligram per Milliliter
MGB	Minor groove binding
MgCl2	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
M-MLV RT	Moloney-Murine Leukemia virus Reverse transcriptase
MMM	Multiple Molecular Marker
MT	Methyl transferase
NaCl	Sodium chloride
NaI	Sodium iodide
nm	Nanometer
N°	Numbers
nt(s)	Nucleotide(s)
NTP	Nucleotide Triphosphate
oak-leaf pattern group	
ORF	Open Reading Frame
PAbs	Polyclonal antibodies
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pmol	Picomole
PVP	Polyvinylpyrrolidone
RdRp	RNA dependent RNA Polemerase

RFLP	Restriction Fragment Length Analysis
RNA	Ribonucleic Acid
RNAse	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SLS	Sodium lauryl sarcosyl
sec	Second
sgRNA	Sub-genomic RNA
SP	Stem pitting
Sweet potato chlorotic stunt virus	
spp.	Species
SSCP	Single Strain Conformation Polimorphosim
SSPE	Sodium chloride/sodium phosphate/EDTA buffe
ssRNA	Single Stranded Ribonucleic Acid
STET	Sodium chloride ethylenediaminetetraacetic acid Tris
SY	Seedling yellows
T	Thymine
Tab	Table
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethyl-Ethylenediamine
TNA	Total Nucleic Acid
Tris	Tris (Hydroxymethyl) aminomethane
U	Enzymatic Unit
Unweighted Pair Group Method with Arithmetic	
UTP	Uracile Triphosphate
UTR	Untranslated region
UV	Ultraviolet
V	Volt
V/V	Volume per Volume

Chapter 1. Introduction

Citrus trees are various species classified under the genus *Citrus* that belongs to the subtribe Citrinae, tribe Citreae, subfamily Aurantioideae and the family Rutaceae (Ben-Yehoshua *et al*., 1994). Among *ca*. 60 species in the genus *Citrus,* only ten are cultivated for their edible fruits and all believed to be native to the subtropical and tropical regions of Asia and the Malay Archipelago, prior to be widely spread from there around the world. They have been described from remote ages, and prototype forms of the most important species are not definitely known (Webber,1948). Curiously, the first member of the group to become known to European civilization was the citron (*Citrus medica* L.), mentioned about 310 B.C. by Theophrastus and was for several hundred years the only citrus fruit known. Then, came in order, the sour orange (*Citrus aurantium* L.), the lemon (*C. limon* [L.] Burm. f.), and the sweet orange (*Citrus sinensis* [L.] Osbeck), which was not known in Europe until approximately seventeen centuries later than the citron, as indicated by preserved literature.

1.1. Early introduction of *Citrus* spp. into the Mediterranean basin

Aubert and Vullin(1998) pointed out that the introduction of citron trees and related species into the Mediterranean area is very old and traces back to the East-West exchanges during the silk trade. Doubtless, Tolkowsky (1938) which recorded the history of citriculture and its special role in the ancient civilizations, gave evidence clearly indicating a much earlier introduction of the citron into Italy, since the time of Augustus. Hence, on the basis of careful examination of a Pompeian tile mosaic, he presented strong evidence that the orange tree, possibly of the sweet variety, was grown in Italy prior to the destruction of Pompei in 79 A.D. A tile floor mosaic found in a Roman villa near Tusculum (modern Frascati) indicates that soon thereafter lemons and limes were also known in Italy. By the

1800's, Citron has been successfully naturalized in Sicily, Sardinia and Kingdom of Naples, and from these regions that the Ligurians began to contend with the Venetians for the commerce in the northern districts, but also in the nearby countries by the ninth and tenth centuries (Webber, 1948).

Further to the gradual disintegration of the Roman Empire, the advancing Arab Empire spread through northern Africa and into Spain, entirely surrounding the Mediterranean Sea except for parts of the French and Italian coasts. Placed in a strategic situation, the Arabs also extended their conquests into Asia and Africa. As well, it was certain that the sour orange was known to the Arabs and they were instrumental in expanding its culture some time during the tenth century A.D. into Persia, Iraq, Syria, Palestine, and Egypt, and apparently later into northern Africa, Sicily, Sardinia, and Spain. Later on, also lemon was being commonly grown in those countries and evidently it was taken to the countries of northern Africa and into Spain, as was the sour orange.

Although the sweet orange had been introduced into Europe at least a century before the Portuguese reached China, it seems certain that the Portuguese contributed much to the spread and popularization of orange growing by introducing a superior variety to the other Mediterranean areas. This new variety, which later came to be known as the Portugal orange, evidently stimulated the industry much as the introduction of the Washington navel stimulated orange culture in California.

The mandarin orange (*Citrus reticulata* Blanco), was a native of China and south-eastern Asia. The first mandarin tree was brought to England from China in 1805, and it was spread from England first to Malta, and then to Sicily and continental Italy (Tolkowsky, 1938). The grapefruit (*C. paradis*i Macf.), early described under the name of "forbidden fruit" was probably originated as a mutation from the pummelo or shaddock (*Citrus grandis* L. Osbeck). The latter, may safely be considered as indigenous in the Malayan and Indian archipelagos and have spread from there to China and India, and thence to

Persia, Palestine, Europe and arrived in Spain by the middle of the twelfth century (Webber, 1948).

Closely related genus of *Fortunella* (kumquats), *Poncirus* and the Australian *Microcitrus* and *Eremocitrus*, are suggested to be included in *Citrus* spp., as well, most botanists now classify *Microcitrus* and *Eremocitrus* as part of the genus *Citrus* (Nicolosi *et al.,* 2000).

1.2. Worldwide Citrus industry

Citrus tree crop is produced all over the world. According to United Nations Conference on Trade and Development (UNCTAD), in 2004 there were 140 citrus producing countries. Around 70% of the world's total citrus production is grown in the Northern Hemisphere in particular countries around the Mediterranean Sea and the United States, although Brazil is also one of the largest citrus producers. China could be a major player in the orange juice and processed citrus markets, except for high tariffs on citrus that make domestic sale more profitable. Though citrus originated in southeast Asia, current citrus production is low due to lower than average yields, high production and marketing costs and problems with disease (Anonymous, 2004).

World production and consumption of citrus has witnessed a period of strong growth since the mid-1980s, whereby, production of the four major varietal classifications: oranges, grapefruit, tangerines, lemons and limes have all expanded rapidly (Anonymous, 2007).

As reported by the table follow (Table 1), China is becoming an important market for the world production of tangerines, but also grapefruit, while, Brazil and United States are considered as the two largest processed orange producing regions in the world.

Table 1. Top ten world citrus producing countries (tons) (Anonymous, 2007)

Country	Grapefruit	Lemons and limes	Oranges	Tangerines*	Other	Total
Brazil	72,000	1,060,000	**18,279,309**	1,271,000	-	**20,682,309**
China	547,000	745,100	2,865,000	**14,152,000**	1,308,000	19,617,100
USA	**1,580,000**	722,000	7,357,000	328,000	30,000	10,017,000
Mexico	390,000	1,880,000	4,160,000	355,000	66,000	6,851,000
India	178,000	**2,060,000**	3,900,000	-	148,000	6,286,000
Spain	35,000	880,000	2,691,400	2,080,700	16,500	5,703,600
Iran	54,000	615,000	2,300,000	702,000	68,000	3,739,000
Italy	7,000	546,584	2,293,466	702,732	30,000	3,579,782
Nigeria	-	-	-	-	**3,325,000**	3,325,000
Turkey	181,923	706,652	1,472,454	738,786	2,599	3,102,414
World	**5,061,023**	**13,032,388**	**63,906,064**	**26,513,986**	**7,137,084**	**115,650,545**

* Includes tangerines, mandarins and clementines.

According to the FAO statistics, global expected production of oranges in 2010 is of 64 MT, to be utilized as 35,7 MT fresh and 28,3 MT processed (Anonymous, 2001). Although, the two largest citrus producing countries: Brazil and the United States are expected to retain their leadership, their rapid expansion of orange production is expected to have a slow decline. First, Brazil is currently facing two major phytosanitary problems: citrus canker and citrus variegated chlorosis (CVC), in addition to emergent citrus greening and Leprosis diseases. Florida is also facing disease challenges from the citrus tristeza virus and citrus canker. As for lemons (*C. limon* L Burm. f.) and limes, these species are grown primarily for the fresh market with the juice from lemons and limes used primarily as a flavouring in beverages, essentially produced from India. They are generally produced in colder climates such as the western United States, Spain,

Italy, and Argentina, but they become also adapted to drier climates such as Egypt and Iran.

1.3. Citrus disorders and advance to Tristeza pandemics

The improvement of transportation systems, which was contemporaneous with an increased botanical and economic interest for citrus in many countries (Bar-Joseph *et al*., 1983), leaded to the active movement of budwood between continents and countries, but also enhanced the rapid dissemination of symptomless fastidious pathogens. To date, rather than nematodes, insects, bacterial and cryptogamic diseases, several viruses and virus-like agents, viroids and phloem-restricted prokaryotes have been extensively characterized, Among which, tristeza is one of the most devastating disease worldwide and its safe to predict its occurrence everywhere citrus is grown (Fig. 1) (Roistacher, 1991).

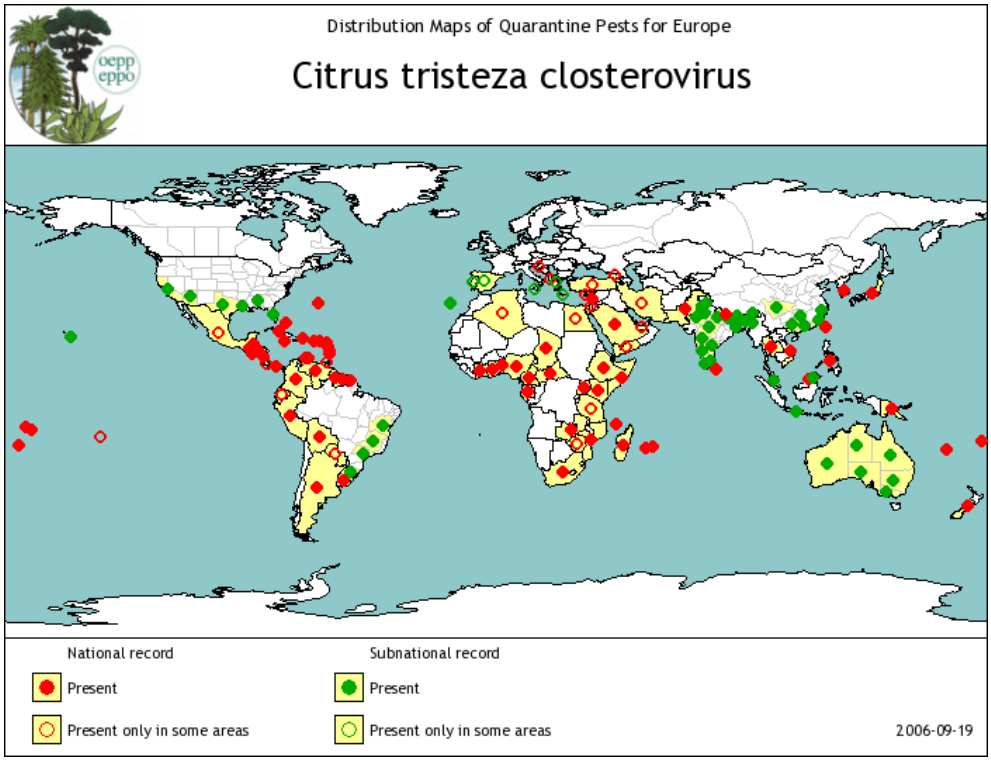


Fig 1. World distribution of *Citrus tristeza virus* (EPPO, 2006)

Heavy tristeza epidemics that started in Argentina since the 30's (Table 2), have continued to appear until today, resulting in over than 100 million killed trees and many million more in different stages of decline (Moreno and Garnsey, 2010). Within the Mediterranean Basin, repeated CTV epidemics dated from the 50's in Spain, where more than 40 million trees were killed and over than 5 million elsewhere. Recent outbreaks took place in Italy, where CTV foci have been depicted from the two major citrus producing districts of Apulia and Sicily (Davino *et al*., 2003 and 2005). Nevertheless, several Mediterranean countries where the CTV sensitive sour orange is continuing to be the major rootstock used, are so far suspected to greater potential for future outbreaks.

Table 2. Occurrence of the major Citrus Tristeza Virus epidemics and estimated number of trees on sour orange rootstock lost (Moreno and Garnsey, 2010)

Country	**Year**	**Trees killed (millions)**
Argentina	1930	18
Brazil	1937	10
California	1939	4
Florida	1951	5
Spain	1957	>40
Israel	1970	0,03
Venezuela	1980	4
Cyprus	1989	0,03
Cuba	1992	0,25
Mexico	1995	-
Dominican Republic	1996	-
Greece	2001	-
Italy	2002	0,45
Total		>85

In addition, CTV is a vector-borne virus, transmitted by several aphid species in a semi-persistent manner. The brown citrus aphid (BrCA) *Toxoptera citricidus* (Kirkaldy) is the most efficient vector especially in transmitting severe stem pitting inducing strains, and being recently established in Portugal and

Spain, represents a serious threat to the Mediterranean citrus industry. Moreover, the melon/cotton aphid *Aphis gossypii* (Glover), the spirea aphid *A. spiraecola* (Pagenstecher) and the black citrus aphid *T. aurantii* (Boyer de Fonscolombe) are endemic in this area and occur frequently as mixed aphid colonies. *A. gossypii* was reported as the most abundant in citrus plantings and the major vector of CTV in areas where the BrCA is absent, showing a high variable efficiency according to the virus isolate. *A. spiraecola* is less efficient vector, but since its populations are becoming so high, it may be a significant factor in CTV spread; as well, *T. aurantii* is likely to transmit only certain CTV isolates (Lee and Bar-Joseph, 2000). This alarming situation of the Mediterranean citriculture makes it urgent to enforce the use of selected varieties grafted on tristeza-tolerant rootstocks produced within official certification schemes. But, since these pathogen-free plants are likely to be infected by viruliferous aphids in the field, it is recommended to implement epidemiological investigations in order to elucidate the CTV increase over time and its spatial patterns, as well as to predict the disease dynamics and to prevent eventual outbreaks. In order to reach this goal, transmission trials of endemic and exotic strains by the aphid population composition should be coupled with rapid and reliable detection techniques of the viral targets by different serological and molecular tools.

Chapter 2. LITERATURE REVIEW: *Citrus tristeza disease*

From the earliests, Tristeza disease was one of the most devastating infectious diseases of citrus worldwide. The first reference of the disease was the Portuguese "A Podridao 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 "Podridao das radicelas", but also it was designated as "budunion decline" in California (Davino *et al*., 1998).

2.1. Origins of Citrus tristeza disease: Historical perspective and economic impact

Citrus is believed to be originated from Southeast Asia and tristeza disease was most probably associated with the citrus cultivated in China and Japan since ancient times (Bar-Joseph et *al*., 1989). In the 19th century, collar rot caused by *Phytophthora* spp. destroyed seedlings of sweet orange, tangerines and trees propagated from layerings in South Africa, thus, the history of tristeza epidemics was directly related to the movement of *Phytophthora* through the translocated soil into the Wardian cases or terrariums invented by Nathanial Ward in 1827 (Roistacher, 2001). In 1896, the Cape Agricultural Department issued a circular instructing the grafting of scions to sour orange rootstock which found to be tolerant to *Phytophthora* diseases, highly adaptable to all soil types and inducing good bearing and excellent fruit quality. This change in practice gained a good popularity throughout the Mediterranean area, North and South America (Fawcett, 1936; Webber, 1943). Later on, massive death of trees grafted on sour orange took place elsewhere and tristeza-decline epidemics occurred in South

Africa (1910), Java (1928), Brazil (1937) and Argentine (1938) (Herron, 2003). This feature was believed to be a "varietal incompatibility" for a long time; Webber was the leader in describing the problem in South Africa in 1924. As well, 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 (Webber, 1943). Grafting experiments concluded that sour orange did not injure sweet orange, but the latter produced some toxic substance able to injure the rootstock (Toxopeus, 1937).

Bitancourt (1940) tested numerous scion-rootstock combinations and postulated a theory suggesting the viral origin of the syndrome: a latent virus in sour orange could be transmitted via the bud union to sweet orange; Webber published the first comprehensive report on citrus diseases in the absence of any mention of tristeza-like symptoms (Webber, 1943). Although he highlighted the vector transmission of the "new disease" from symptomless carriers to susceptible plants, the virus nature of Tristeza disease was established in transmission experiments by Fawcett and Wallace (1946) and simultaneously, Meneghini (1946) proved its transmission by the aphid *T. citricidus*, the brown citrus aphid (Roistacher, 1991). The development of seedling lime indexing (Hughes and Lister, 1949) proved that the "Tristeza disease" in South America, "quick decline" in California, "stem pitting" of grapefruit in South Africa and Australia and "the decline and stem pitting of limes" in the Gold Coast of Africa were all caused by one virus: " The Citrus tristeza virus (CTV)".

The earliest serious epidemics of decline and death of citrus trees have been reported from Argentina in 1930 (Zeman, 1930), where 10 million trees were lost within 15 years. Similar outbreaks were reported from Brazil (1937) where over than 6 million trees were killed over a period of 12 years. In North America, Tristeza disease caused the loss of 3,5 million trees grafted on sour orange rootstock in California by 1939 (Wallace, 1956; Davino *et al*., 1998). In South America, the disease caused heavy losses in citrus trees in Uruguay (1940), Perù (1948), Venezuela (1949), and in Ecuador (1960) (Bové and Vogel, 1964).

Being dramatically worldwide spread, in some countries where serious epidemics have not been reported yet, CTV may occur symptomless for several years even in plants grafted on tristeza sensitive sour orange rootstock. Thus, CTV spread depends upon distribution of infected budwood, vectors, virus strains and temperatures (Roistacher, 1991). Within the Mediterranean area, heaviest CTV outbreaks started in Spain since the fifteen's and caused the death of over than 40 million citrus trees, then in Israel in 1970 (Moreno and Garnsey, 2010). Later on, CTV outbreaks occurred in Italy (2002) and have been depicted from the main citrus growing regions of Sicily and Apulia, resulting in the loss of thousands of sweet orange on sour orange trees (Davino *et al.,* 2003; 2004). CTV foci were pointed out from Algeria, Cyprus, Egypt, Italy, Lebanon, Morocco, Palestine, Malta, Greece, Syria and Turkey (Djelouah and D'Onghia, 2001a).

2.2. Causal agent: Citrus tristeza closterovirus taxonomy

Citrus tristeza closterovirus shows a phloem tropism in Citrus hosts and belongs to the Closteroviridae family (Karasev *et al.,* 1995). Members of Closteroviridae have characteristically flexuous filamentous rod-shaped virions, large single-stranded and positive-sense RNA (ssRNA) genomes. Within this family, three viral genera have been described based on their RNA genome and their vector transmission (Fig. 2). The genus Closterovirus, type species *Beet yellows virus* (BYV) have a monopartite RNA genome of up to 20Kb and 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) have bipartite genomes and are spread by whiteflies (Homoptera: Alyrididae); (Klaassen *et al*., 1995; Martelli *et al*., 2002).

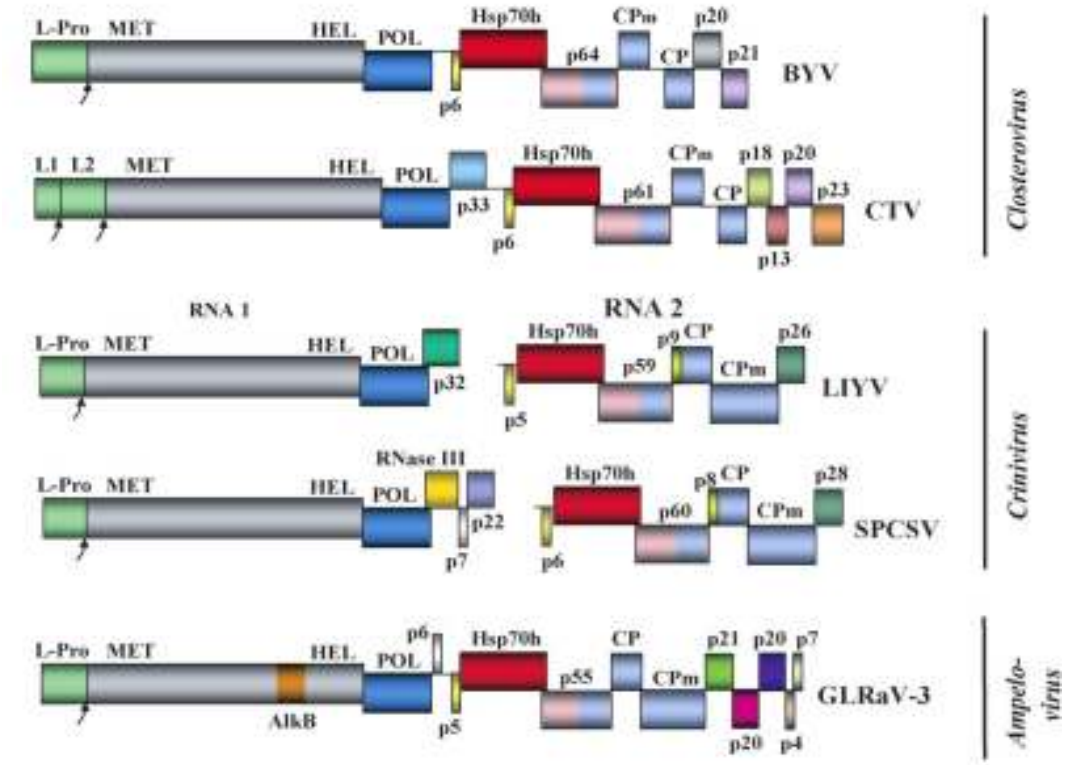


Fig. 2. Genome maps of the family *Closteroviridae* genera

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).

The evolution of Closteroviruses has been suggested on the basis of phylogenetic analysis of their replicative genes and the HSP70 homolog (HSP70h) (Karasev, 2000) which revealed the following three levels of gene conservation: **(i) RNA replication-related genes:** the product of the 5'-terminal ORF contains RNA methyltransferase (MET) and RNA helicase (HEL) domains (Fig. 3), while the RNA-dependent RNA polymerase (RdRp) is encoded by a downstream ORF that is presumably expressed via +1 translational frameshift (Karasev *et al*., 1995). Thus, translation of the closteroviral RNA genomes results in the production of MET-HEL-POL universally conserved polyprotein (Koonin and Dolja, 1993; Dolja *et al*., 2006).

(ii) Quintiple gene block: five downstream genes comprising a truly unique genetic module are not found outside the family Closteroviridae. These are encoding for a ~6-kDa hydrophobic protein (p6) that functions in cell-to-cell movement of the virus (Alzhanova *et al*., 2002), a ~65-kDa HSP70h, a ~60-kDa protein (p61), the minor capsid protein (CPm) and the major capsid protein (CP) (Dolja *et al*., 2006). **(iii) RNA silencing suppressors:** it was recently evidenced the production of some plant viral suppressors that counteract the powerful system of host defense against the parasitic RNAs during an infection. Thus, although CTV encodes for p20 and p21 like suppressors of RNA silencing, screening of the CTV genome revealed an additional suppressor, p23, having no homologues in the other closteroviruses (Lu *et al*., 2004; Dolja *et al*., 2006).

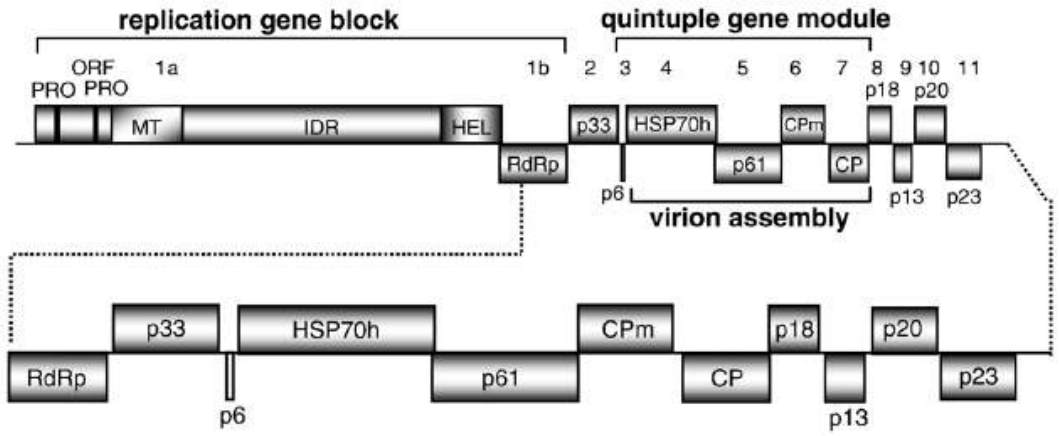


Fig 3. Schematic representation of the genome organization of CTV

(Satyanarayana *et al*., 2008)

2.3. Host range and field diagnosis

CTV replication is restricted to the phloem cells in most species and hybrids of the genus *Citrus* and large number of the Rutaceae; *Passiflora* spp. is the only non-Rutaceous host species (Garnsey *et al*., 1998; Roberts *et al*., 2001). Host resistance to CTV infection has been reported from some CTV relatives including *P. trifoliata* (L.) Raf., *Swinglea glutinosa*, *Severinia buxolia*, some

pummelo (*C. grandis* (L) Osb.) and some hybrids between *P. trifoliata* and sweet orange or grapefruit (Garnsey *et al*., 1997). However, CTV replication has been observed in *P. trifoliata*, pummelo or sour orange protoplasts, suggesting that only virus movement is impaired in these species (Albiach- Martí *et al.,* 2004).

Citrus tristeza virus (CTV) isolates differ in their pathogenicity on citrus plants depending on the virus strain, the variety of citrus, and the scion-rootstock combination (Yoshida, 1996). 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), while, economically damaging CTV infections can conversely induce quick decline (QD), seedling yellows (SY) and stem pitting (SP) symptoms (Fig. 4). It has been even evidenced that a field CTV isolate often harbour a mix of genomic variants belonging to different CTV strains (Gillings *et al*., 1993; Albiach-Martì *et al*., 2000a).

(i) Quick decline (QD): The name "Tristeza", *sensu stricto*, almost refers only to the decline disease known also as "sudden dieback", due to the interaction of CTV with citrus species propagated on sour orange rootstock (Moreno and Garnsey, 2010). Therefore, quick decline is one of the most dramatic reaction of CTV, whereby, the canopy of mature sweet orange, mandarin and grapefruit grafted onto sour orange, pummelo, or lemon rootstocks (but not rough lemon rootstock) suddenly become stunted, wilted, defoliated and then die within few months (Roistacher, 1991). Scion-rootstock incompatibility was turned back to the necrosis of the phloem cells in the cambium tissue of the sour orange rootstock, creating uneven distribution of carbohydrates in the scion and down in the rootstock responsible for unbalanced radial growth; this results in a kind of a bulge at the bud union girdling the tree in a chronic infection (Roistacher, 2006; Moreno and Garnsey, 2010). 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", it is generated by hyperplastic and/or hypertrophic medullar rays in the bark of sour orange species (Garnsey and Lee, 1988; Rocha-Penà *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. Rather than low yields, declining trees frequently show hypotrophic the root system in the origin of the mineral and water deficiencies in the canopy followed by wilting, chlorosis and dieback (Moreno and Garnsey, 2010).

(ii) Stem pitting (SP): Firstly found on Marsh grapefruit in South Africa in 1985, then, Navel orange industry was destroyed by severe stem pitting isolates in Perù (Roistacher, 1988; 2006). Severe SP infections may injure the main trunk, the small branches and the twigs of grapefruit and sweet orange regardless to the rootstock, by inducing deep pits in the wood under enlarged cheesy bark, accompanied by a general growth cessation of the trees and easy breakage of the twigs (Roistacher, 1991; Rocha-pena *et al*., 1995). At the anatomic level, SP syndrome originates from the interruption of the meristematic activity at limited areas of the cambium layer between the bark and the wood, resulting in irregular radial growth with local depression at the inactivated points (Schneider, 1959).

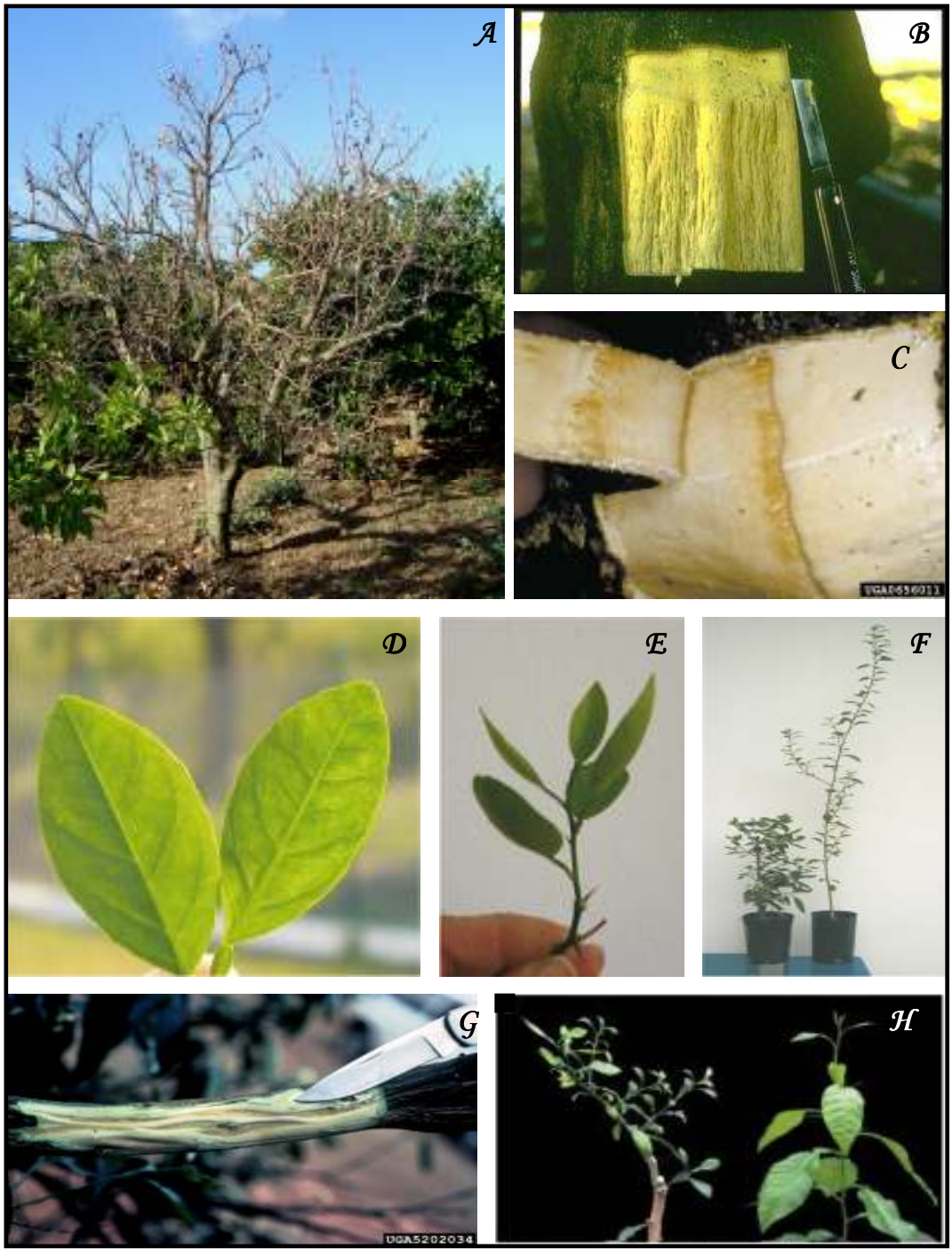


Fig 4. Major field syndromes and greenhouse symptoms induced by *Citrus Tristeza Virus*

(A) Decline of sweet orange grafted on sour orange rootstock; (B) Stem pitting (C) Inverse pitting/honey combing below the bud union on sour orange rootstock; (D) Vein clearing in Mexican lime seedlings in glasshouse conditions of 18-25°C; (E)Leaf cupping in Mexican lime leaves; (F) Stunting of young sweet orange on sour orange rootstock (healthy recipient-left, infected recipient-right); (G) Stem pitting injuries in grapefruit twigs; (H): Seedling yellow symptoms in sour orange seedlings.

Because tristeza SP form can not be avoided by graft-propagation on a different rootstock, unthrifty growth and chronic yield losses may in some instances be aggravated by severe permanent limitations for growing sensitive cultivars that may be lucrative for the local or international market (Moreno and Garnsey, 2010).

(iii) Seedling yellows (SY): A third form of CTV devastating symptom was firstly reported by Fraser (1952) as mostly observed in greenhouse grown seedlings of sour orange, lemon and grapefruit, as referred to seedling yellows syndrome (SY); it is characterized by general stunting, production of small and pale leaves, reduced root system and sometimes a complete cessation of plant growth (McClean, 1963; Roistacher, 1982). At genomic level, recent investigations showed that this symptom is induced by specific virulence determinants in the CTV genome (Satyanarayana *et al*., 1999 and 2001). Moreover, some observations suggested that in certain cases expression of SY could be modulated by some defective RNAs (Yang *et al*., 1999).

2.4. Evolutionary diagnosis

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*., 1987). 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).

2.4.1. Biological indexing

Glasshouse indexing of CTV isolates and the assessment of their biological reactions onto a panel of citrus indicator plants is the only method available to reliably determine the biological properties of a given CTV field isolate (Garnsey *et al.,* 1987; Gillings *et al*., 1993). Up to date, Mexican lime is still the appropriate universal indicator for detecting and differentiating most of

the CTV isolates; whereby, graft inoculation of the virus commonly elicits typical vein clearing and leaf cupping on the new flushes under relatively cool conditions (24-27°C day/18-21°C night), but also vein corking in the case of severe CTV infections. However, for the detection of more severe strains, seedlings of grapefruit, lemon and sour orange are highly sensitive to CTV seedling yellows; whereas, Duncan grapefruit and Madame vinous sweet orange seedlings provide satisfactory response to grapefruit and sweet orange stem pitting inducing strains, respectively (Roistacher, 1991; Lee *et al*., 1996). 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, etc (Niblett *et al*., 2000).

2.4.2. Serological diagnosis

CTV has been purified and polyclonal Antisera (Pab) have been prepared against a wide range of CTV isolates and used firstly in SDS-immunodiffusion tests (Garnsey *et al.,* 1979), then in conventional Enzyme Linked Immunosorbent Assay (ELISA) technique (Bar-Joseph *et al.,* 1979). Despite this made feasible the mass detection of CTV and some epidemiological studies (Cambra *et al.,* 2000a), it doesn't provide information regarding the severity of an isolate (Garnsey *et al*., 1981). For more accurate diagnosis of CTV, ELISA tests with specific polyclonal and monoclonal antibodies (MAb) are commonly used (Gumpf *et al*., 1987; Permar and Garnsey, 1988).

The monoclonal antibody MCA 13 was raised against T36 strain (Florida) and was highly useful as a strain specific reagent associated with severe sweet orange on sour orange decline in Florida (Permar *et al*., 1990), which was not the case in Spain and California (Niblett *et al*., 2000). Hence, CTV samples giving a cross reaction against MCA 13 (MAb) may harbor viral entities of severe nature, but it still ambiguous whether they contain SP or QD strains or a mixture of them. As well, non-reactive CTV samples with MCA 13 (MAb) can not be confirmed as

healthy nor containing a mild CTV strain (Niblett *et al*., 2000). Nevertheless, tests with MCA 13 can be preferentially used in conjunction with other reliable and sensitive techniques, such as the Direct Tissue Blot Immunoassay (DTBIA), which proved its convenience for large scale surveys owing to its efficient sensitivity and its simple practice under field conditions (Cambra *et al.,* 2000; Djelouah and D'Onghia, 2001a).

2.4.3. Molecular diagnosis

ELISA technique is convenient but limited in sensitivity of detection, especially with very low concentrations of CTV. Since most plant viruses have positive-sense ssRNA genome, detection of the dsRNA viral replicative form has been successfully used for the identification of virus groups. Although CTV dsRNA patterns has also been used to detect and discriminate between strains (Dodds *et al*., 1994; 1987a; Moreno *et al*., 1990), the variation of the dsRNA banding profiles in number and intensity according to the host plant in one hand (Jarupat *et al*., 1988), and the generation of defective RNAs of CTV in the other hand (Ayllon *et al*., 1999a), make dsRNA funding not always correlated with the pathogenic characteristics of CTV isolates.

More accurate assays based on the reverse transcriptase polymerase chain reaction (RT-PCR) techniques have been developed by several researchers (Mehta *et al*., 1997). Using this technique, Pappu *et al*. (1993) determined the highly conserved capsid protein's CP nucleotide sequence of several CTV strains, demonstrating its involvement in the symptom's expression. Knowledge of the full-length CTV nucleotide composition of the T36 isolate from Florida gave a revolutionary progress of CTV diagnosis as a starting point for the development of many genome-based procedures, including molecular hybridization (Hilf *et al*., 1999; Barbarossa and Savino, 2006), several RT-PCR based techniques (Nolasco *et al.,* 1993; Hung *et al.,* 2000; Roy *et al.,* 2005) and molecular discrimination

assays (Niblett *et al.,* 2000; Hilf *et al.,* 2005; Halbert *et al.,* 2004; Sieburth *et al.,* 2005; Huang *et al.,* 2004, Herrera-Isidrón, *et al.,* 2009).

Single-strand conformation polymorphism (SSCP) was described by Rubio *et al*. (1996) to detect minor base mutations in the CP nucleotide sequence of the CTV population; additionally, it has also been useful with *p*18, *p*20 and *p*27 genes (Febres, 1995). SSCP analysis supposes the denaturation of dsDNA complementary strands and their separation by non-denaturing polyacrylamide gel electrophoresis (PAGE). Difference in motility of the ssDNA fragments 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; 2000a). Several restriction fragment length polymorphism (RFLP) assays have been developed to discriminate between CTV isolates based on appropriate enzymatic digestion of cDNA specific restriction sites along the viral genome (Gilling *et al*., 1993).

Rather than the CP gene (CPg), a PCR-based strategy was implemented to discriminate between genetically similar but geographically distinct isolates throughout the amplification of similar sized DNA sequences (molecular markers), designed within the ORF 1 of the gRNA relatives to VT, T3, T30 and T36 reference strains (Ayllon *et al*., 2001; Hilf and Garnsey, 2000; Hilf *et al*., 1999). 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).

Meanwile, Cevik *et al*. (1996) were the leaders in analyzing in details the CPg sequences of several biologically and geographically diverse strains of CTV. After categorizing the different 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 co-infected with more than one strain of tristeza (Niblett *et al*., 2000).

2.5. Viral aspect and molecular features

2.5.1. CTV genome organization

The genome of Citrus tristeza closterovirus consists of a positive sense ssRNA genome of 19296 nucleotide, packaged in 11 nm × 2000 nm threadlike particles, organized in 12 open reading frames (ORFs) and two 5' and 3' unstranslated regions (UTRs) of about 107 and 273 nt, respectively (Bar-Joseph *et al*., 1979; Karasev *et al*., 1995). The 3' halves are highly conserved (90% of nucleotide homology), while much more dissimilarity is distinguished along the 5'-nontranslated region (homology as low as 42%) (Gowda *et al*., 2003).

The 5' half of the CTV genome encompasses the "replication gene block" encoding proteins associated with viral replication function (Klaassen *et al*., 1996; Peremyslov *et al*., 1998; Satyanarayana *et al*., 1999). The remaining 3' half of the genome contains at least 10 ORFs which are expressed via translation from 8 kb 3' co-terminal subgenomic RNAs (Hilf *et al*., 1995; Ayllon *et al*., 2003), encoding for the two capsid proteins (CP and CPm) of 25 and 27 kDa, respectively, in addition to p6, p13, p18, p20, p23, p33, p65 and p61 proteins. However, sgRNAs for p23 and p20 are more abundant and probably produce more proteins than other ORFs, with the possible exception of CP (Pappu *et al*., 1994; Karasev *et a*l., 1995).

A conserved "signature gene module" of five proteins within the 3' region was revealed from the Closteroviridae members. It encodes for a small (6 kDa) hydrophobic protein proposed to be a membrane anchor, which together with a protein closely related to the ubiquitous cellular heat shock induced chaperone (HSP70h), a protein of 60 kDa, CPm and CP coat proteins are involved in the

virion assembly of this closterovirus (Satyanarayana *et al.,* 2000). Therefore, the 25-kDa CP encapsidates the major part of the CTV particles (95% of virion) showing a highly conserved sequence composition (ca. 90%) and a strong relationship with symptoms expression. The remaining 5% of the total particle length are by contrast enclosed by the 27-kDa divergent CPm, resulting in a polar conformation termed rattlesnake-like virions (Agranovsky *et al*., 1995; Febres *et al*., 1996; Tian *et al*., 1999).

Despite that specific functions of the p27 have not been determined yet, examination of the involvement of gene products of CTV in virion formation showed that the coordinate action of p27 with each of p65, p61 and CP is essential for the efficient formation of viable virions. Additionally, CPm has been shown to initiate encapsidation of the genomic RNA from the 5' ends (Satyanarayana *et al*., 2000; 2004).

Closteroviridae members are the only viruses known to encode an HSP70h of cellular molecular chaperone-like proteins (Karasev, 2000). The latters are ubiquitous proteins that are involved in a wide range of cellular processes, even in unstressed cells, including protein folding, assembly and disassembly of multi-subunit complexes, translocation into organelles, and intercellular transport (Hartl, 1996; Bukau and Horwich, 1998; Feder and Hofmann, 1999). HSP70h and p61 genes appeared to be equally required for the proper formation of the viral particles, suggesting that they might co-evolve together. In addition, HSP70h and p61 analogues as components of the "signature gene module" of BYV have been reported to be essential for cell-to-cell movement (Peremyslov *et al*., 1999; Alzhanova *et al*., 2000).

Moreover, the *p*20 gene, a homologue of the *p*21 gene of BYV, and four genes encoding proteins have no homologue in other closteroviruses (p33, p18, p13 and p23) (Dolja *et al.,* 2006). Findings conducted by Gowda *et al*. (2000) pointed out that the p20 protein is localized with large amounts mainly in infected cells within the amorphous inclusion bodies accumulates in infected tissues and

has a high affinity for itself, suggested that this protein is a major component of the amorphous inclusion bodies.

The p23 protein has been reported as an RNA-binding protein involved in asymmetric accumulation of strands during the RNA replication (Lòpez *et al.,* 2000). More recently, p23 has been implicated in regulating the synthesis of plus and minus strands during RNA replication, with the basic residues and the zinc finger motif, being indispensable for asymmetrical accumulation of the plus strand (Satyanarayana *et al*., 2002). Ghorbel *et al*. (2001) found that in transgenic Mexican limes, over-expressing *p*23 products, displayed typical symptoms characteristic of CTV infection that were specifically associated to the *p*23 gene accumulation. Although this finding strongly suggests the involvement of *p*23 in the CTV symptom expression, the amino acid differences observed by other investigators between mild and severe isolates might have resulted from co-evolution with other genome regions responsible for the virus virulence (Sambade *et al*., 2003). Both of p20 and p23, in synergy with the CP, act as RNA silencing suppressors in *Nicotiana benthamiana* and *N. tabacum* plants, with *p*23 inhibiting intercellular silencing, CP intracellular silencing, and *p*20 both inter- and intracellular silencing (Lu *et al.,* 2004).

However, functions of p33, p18 and p13 proteins are so far unknown since experiments of mutants deletion within the correspondent ORFs did not affect the number and quality of virions generated. Therefore, it was deduced that they are not involved neither in virions replication nor in their assembly (Satyanarayana *et al.,* 1999 and 2000; Davino *et al.,* 2005).

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*., 1995). D-RNAs are variable between isolates and are thought to be correlated with SY symptom expression (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*., 1995).

2.5.2. CTV replication

Full-length replication mechanism of the ssRNA genome of CTV includes the synthesis of negative-stranded or complementary RNA molecule, from the parental infectious RNA which serves as a template for the positive-sense RNA progeny synthesis. Thus, infected phloematic host cells contain relatively large amounts of double-stranded replicative form RNA molecules (dsRNAs) corresponding to the genomic RNA, and to at least nine 3' co-terminal sub genomic (sg) RNAs (Hilf *et al*., 1995; Bar-Joseph *et al*., 2002). The RNA virion of CTV serves as a messenger to produce directly two polyproteins: MET-HEL-POL of ~349-kDa and a larger one of ~400-kDa relative to the RpRp (Karasev *et al*., 1995). The remaining 10 ORFs are expressed through a nested set of 3'-coterminal subgenomic RNAs (sgRNAs) used as messenger RNAs from which the 5′ proximal ORF get translated (Hilf *et al.,* 1995). An infectious replicon resulting from a CTV clone containing the entire 5' replication complex and a truncated 3' end with deletion of the ten ORFs, replicated successfully in *N. benthamiana* protoplasts, making in evidence the obligate government of the CTV replication process by ORFs 1a and 1b (Satyanarayana *et al*., 1999; 2002); whereas the early-expressed 3' most of the gene *p*23 is required for asymmetrical accumulation of plus and minus strands of both genomic and sgRNAs (Navas-Castillo *et al*., 1997).

Expression mode of the large ssRNA CTV genome was known by including proteolytic processing of the viral polyprotein, translational frameshifting, and formation of at least nine 3'-coterminal subgenomic RNAs (sgRNAs) ranging in size from 0,9 kb to more than 8 kb (Hilf *et al*., 1995); therefore, large diversity of RNAs get produced by CTV within infected cells. In

addition to the expected genomic RNA and its complementary copy, each 3' ORF controller element produced three different RNAs: a 3'-terminal plus-strand RNA that serves as messenger for the adjacent ORF and a relatively large amount of a corresponding minus-strand sgRNA, plus a 5'- terminal plus-strand sgRNA, apparently produced by termination near the controller element during genomic RNA synthesis (Gowda *et al*., 2001). Expression rates of *p*20 and *p*23 sgRNAs was shown to be higher than those of the two coat protein genes (Pappu *et al*., 1997).

Additionally, it has been evidenced from the VT strain of CTV the synthesis of unusual two small 5'-coterminal plus strand sgRNAs of *ca*. 0,8 kb known as low molecular weight tristeza (LMT1 and 2) RNAs and showing heterogenous 3' ends at nt 842 to 854 (LMT1) and nt 744 to 746 (LMT2), as well as a larger 11 kb one (LaMT), which were thought to be produced by premature termination during the gRNA synthesis (Mawassi *et al*., 1995; Che *et al*., 2001). Within the Closteroviridae family, similar sized 5' co-terminal sgRNA has been described from (BYV) (He *et al*., 1997) and a larger one from *Lettuce infectious yellows Crinivirus* (Rubio *et al*., 2000b). Both LMT RNAs are accumulated by several CTV strains more abundantly than gRNAs, while only LMT1 RNAs are produced by the replicons with all or most of the 3'genes deleted. It demonstrated so far that none of the 3' genes is necessary for production of the longer LMT1 RNAs, but it suggested their involvement in production of the shorter 5'-terminal LMT2 RNAs (Gowda *et al*., 2003), while no function has been assigned yet for larger LaMT molecules (Che *et al*., 2001).

A *cis*-acting element, that controls the production of the LMT1 RNAs, has been characterized (Gowda *et al*., 2003). Moreover, due to the absence of corresponding of minus strand molecules, it was suggested that the *cis*-acting element, which was expected to function as the ORF2 sgRNA promoter, might also act as the termination signal of the ORFs 1a and b on the 5' transcripts (Bar-Joseph *et al*., 2002).

2.5.3. CTV population structure and genetic diversity

Isolates of CTV differ widely in their biological properties. The latters highly depend on the structure of viral RNA populations relative to the different isolates. However, it was often found that a field isolate is a mixture of genomic variants belonging to different CTV strains, since genomes of RNA viruses are subjected to frequent changes due to their error-prone replication, but also to their vector transmission and graft-propagation to different hosts (Brlansky *et al*., 2003; Moreno *et al*., 1993a; Albiach-Martí *et al.,* 2000b; Sentandreu *et al.,* 2006).

Although environmental conditions may influence the outcome of the disease, the major factor is the viral strain: CTV strains differ according to their phenotype including the type and intensity of symptoms they could induce (Cerni *et al*., 2008). Moreover, some strains which are commonly referred as mild, do not produce noticeable symptoms in field conditions. Whereas, severe strains may have a devastating effect on trees, including quick decline on sour orange rootstock, stem pitting regardless to the rootstock, and seedling yellows of sour orange, grapefruit or lemons under greenhouse conditions (Nolasco *et al*., 2008).

As a first approach to study the molecular basis of the biological variability, most of CTV characterization researches were mainly conducted on the basis of the capsid protein (Gillings *et al*., 1993; Mawassi *et al*., 1993) and the 5' terminal gene sequences (Lòpez *et al*., 1998). Thus, some authors supported the existence of a strong relationship between the isolate phenotypic expression and minor variations over the CP gene sequence. Additionally, comparison of VT and T36 full-genome sequence composition indicated the high conservation over the 3'- UTR with respect to the gradual increase in nucleotide sequence divergence toward the 5' proximal termini. Interestingly, that polymorphism allowed the classification of the sequences into three groups, with intra-group sequence identity higher than 88% and intergroup sequence identity lower than 44%. The mildest isolates contained only sequences of group III represented by T318 isolate from Spain, whereas the most severe isolates contained sequences of

groups I and II represented by isolates T36 from Florida and VT from Israel, respectively (Lòpez *et al*., 1998).

Factors shaping CTV populations in the field include gene mutations, recombination events between diverged sequence variants, selection pressure, 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 RdRp is the basic mechanism in the origin of the genetic viral diversity (Domingo and Holland, 1994).

In addition, Citrus are long-living crop species that get repeatedly inoculated by field aphids with divergent CTV variants; therefore, changes in the haplotype population distribution and frequency of the isolates have been reported by SSCP after the vector inoculation (Ayllon *et al*., 1999b), as well as, the emergence of new sequence variants in the successive vector transmitted sub-isolates, able to increase the within-isolate genetic diversity (Ayllón *et al.,* 2006; Rubio *et al.,* 2001). 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 and 2003; Sambade *et al.,* 2007). Such changes were sometimes accompanied by a variation of the pathogenic behaviour of the isolates (Albiach-Martí *et al.,* 2000b; Brlansky *et al.,* 2003; Hermoso de Mendoza *et al.,* 1988a).

2.6. Epidemiology and vector transmission of Citrus tristeza virus

2.6.1. Concepts in CTV epidemiology

Occurrence of dramatic epidemics due to the CTV all over the world was turned back to the pathogenic interaction of the viral population with large reservoirs of susceptible hosts and rootstocks, but also to the adaptation of CTV isolates to the aphid transmission processes. Over the past 25 years, increasing awareness that the dynamics of CTV pathosystem are critically affected by the

complement of the overall vector population in the area and their ability to transmit particular isolates resulted in more researches for better understanding the spatio-temporal kinetic of CTV. Nevertheless, CTV pathosystem is complex; CTV isolates vary greatly in symptom expression and many interactions can occur due to various combinations of the virus, the host plant, aphid species and the environment (Gottwald, 2010). Since, emphasis is placed on the behavioural responses of vectors to environmental cues because vectors are responsible for almost all of the inter-plant spread that leads to epidemics of aphid-borne viruses (Irwin and Ruesink, 1986), the CTV pathosystem can be characterized mostly by the predominant vector species (Gottwald *et al*., 1996a; 1998).

Members of Closteroviridae family are transmitted in nature by three families of insects classified within the Order Homoptera, (including Aphididae, Pseudococcidae and Aleyrodidae species), provided by typical mouthparts suitable for piercing leaf tissues and sucking plant juice from the phloem (Karasev and Bar-Joseph, 2010). CTV vector-borne virus is transmitted by several species of aphids landing on Citrus trees. The main vectors associated with natural CTV spread are *T. citricidus* (Kirkaldy), *A. gossypii* (Glover), *A. spiraecola* (Patch), *T. aurantii* (Fons.)*, A. craccivora* (Koch) and *Mysus persicae* (Sulzer) (Dickson *et al*., 1956, Hermozo de Mendoza *et al*., 1988b, Rocha-Penà *et al*., 1995; Schwarz, 1965; Yokomi *et al*., 1994; Marroquin *et al*., 2004). The most efficient vector of CTV worldwide is the brown citrus aphid (BrCA) *T. citricidus* (Yokomi *et al.,* 1994; Gottwald *et al*., 1996b), so that the spatial and temporal dynamics appear to change as *T. citricidus* becomes a part of its pathosystem (Gottwald *et al.,* 1998). In spite that it is up to 25 times more efficient than *A. gossypii* in transmitting particularly severe CTV strains (Gottwald *et al.,* 1997; Roberts *et al.,* 2001), the melon aphid was reported as the main CTV vector in all tropical and temperate regions where *T. citricidus* does not occur (Roistacher *et al*., 1980; Roistacher and Bar-Joseph, 1984, Cambra *et al.,* 2000) such as California, Arizona, Texas, and undoubtedly the Mediterranean basin except of Madeira Island (Niblett *et al.*, 2000) and the Iberian peninsula (D'Onghia, 2005).

The spatio-temporal dynamic of plant viruses is strongly compromised by the abundance and feeding behavior of their vector species; in this sense, vectors "drive" epidemics (Irwin *et al*., 2000). In the case of CTV, relevant epidemiological differences are almost turned back to the differences in transmission efficiencies and in basic biology between *T. citricidus* and *A. gossypii* species. Citrus is the primary host of the BrCA, so, significant populations are expected at least once a year since it is a colonizer species. Newly molted adults, especially alatae, require several hours for the exoskeleton to harden and wings to expand. During that period, aphids feed continuously from the infected plant and become viruliferous. The latters preferentially move and transmit CTV over short distances to adjacent or nearby trees, resulting in a rapid and aggregated spatial pattern of Tristeza (Gottwald *et al.,* 1998).

However, Citrus is an alternative host for *A. gossypii* which builds up large colonies in other crops and CTV spreading is generally due to some occasional and brief migratory waves from surrounding orchards (Gottwald *et al.,* 1997). Because *A. gossypii* is a citrus migratory species, after feeding on the infected plant, only winged aphids fly and tend to land at about 100 to 200 m of distance from their take off point, often for several trees away. Consequently, 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 melon 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.,* 1997).

2.6.2. CTV aphid vectors

2.6.2.1. Taxonomic rank:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Homiptera

Sub-Order: Sternorrhyncha

Superfamily: Aphidoidea

Family: Aphididae (Blackman and Eastop, 2007).

From the systematic standpoint, aphids are Homopteran insects: known as sup-suckers provided with long and sophisticated piercing-sucking mouthparts for feeding directly from the plant sieve tube tissues. Then, Homoptera means "alike wings", referring to the fact that the frontal two wings are uniform and have a membranous texture throughout. Homopterans undergo simple metamorphosis, which means the young (nymphs) look similar to the adults and have like habits (young and adults feed and live in the same place) and the nymphs do not undergo pupation to achieve adulthood. Aphids can have winged (alatae) and wingless (apterous) adult forms. Aphids are considered adults when they have reproductive maturity. Then, they are classified in the sub-Order Sternorrhyncha given that their antennae are filiform and their beak arises between the front legs. Within this sub-Order, aphids have their own family: the Aphididae, which are distinguished by the presence of tube-like structures, "cornicules" or "siphunculi" as well as a rounded lobe called "cauda", protruding in the back ends of their bodies. The 6th antennal segment, cornicules and cauda are basically used to separate the different aphids at the genus and species levels (Wunderlich, 2000).

2.6.2.2. Morphology and biological behavior of CTV aphid vector species

Within the *Aphis* spp., *A. gossypii* is soft-bodied, varying from light yellow to dark green in color (Jones, 2004). Antennae are 6-segmented with a terminal process straight and longer than length base of the 6th antennal article, cornicules are black and legs are pale with black tips of the tibiae and tarsi. It is commonly confused with the spiraea aphid, but by examining the abdomen tips, *A. gossypii*

has a pale cauda with two or three pairs of lateral dusty setae, while *A. spiraecola* has a dark brown to black cauda with three to six pairs of setae (Blackman and Eastop, 1984).

Given that the BrCA is very close to the black citrus aphid, *T. citricidus* has four distinctive structures which separate it from *T. aurantii*: it is much larger, his upper wings are provided with a forked midvein (M2) and a light pterostigma; whereas *T. aurantii* is known by the dark pterostigma and once branched midvein in the alatae, and its banded antennae in all forms which is not present the case of *T. citricidus* (Stoezel, 1994).

The life cycle of most aphids is usually complex, the holocyclic species typically overwinter as fertilized eggs glued to stems or other parts of plants, which hatch in Spring into wingless viviparous females (Fundatrix). These reproduce parthenogenetically (without mating) giving origin to several generations of apterous viviparous females. Periodically, the nymphae develop wings and migrate to other plants. The return of autumn's shorter days and cooler temperatures is followed by the appearance of sexual males and oviparous females. After copulation, sexual females lay fertilized eggs, giving origin to the fundatrices for the following Spring (Day, 1996).

The biology of the major CTV vector species, including *T. citricidus*, *A. gossypii* and *A. spiraecola*, is sometimes characterized by this holocycle (Blackman and Eastop, 1984), however, they are normally anholocyclic reproducing permanently by parthenogenesis and overwinter as a mature parthenogenetic famale (Heie, 1980). The gradual development of wing pads in the larvae and the triggering of winged forms is under hormonal control and can be induced by environmental variations such as population density (crowding), food quality and photoperiod. Ecologically, the alternation of the host plant is a major determinant of aphids. Overwintering often took place on perennial woody species before migrating to fast growing annuals in the summer, and in the end of the season, the alatae migrate back to the original host plant for mating and lay-out of the overwintering eggs (Wunderlich, 2000).

Aphid species such as *T. citricidus* and *T. aurantii* are oligophagous, limited to Rutaceae and major pests of citrus, while the others are polyphagous and very damaging to a wide range of host plants, such as *A. spiraecola* in Asteraceae, Caprifoliaceae, Rosaceae, Rubiaceae and Rutaceae, as well as *A. gossypii* in cotton and Cucurbitaceae (Stoetzel, 1994).

2.6.2.3. Semi-persistent CTV vector transmission

Aphid species landing on Citrus spp. and described as CTV vectors at different transmission efficiencies include the BrCA (Yokomi *et al.,* 1994) followed by the melon-cotton aphid *A. gossypii*; but also some minor species can have a significant role in spreading the virus, such as: the spiraea or green citrus aphid *A. spiraecola*, the black citrus aphid: *T. aurantii, A. craccivora* and *M. persicae* Sulzer (Marroquin *et al.,* 2004).

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 (Costa and Grant, 1951; Bar-Joseph *et al.,* 1989) to 24 h; but, acquisition and inoculation periods of CTV by BrCA 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 loose their infectivity after two days feeding on a healthy plant and after molting (Roistacher, 2006). Previous investigations 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, using by ELISA (Cambra *et al*., 1982), or by conventional RT-PCR (Mehta *et al*., 1997) and real-time RT-PCR diagnosis of purified TNA targets (Bertolini *et al*., 2007).

Rather than aphids, There is also one report of a mealybug (*Ferrisia virgata* (Cockerell)) transmitting CTV after 30 h feeding on a CTV-infected plant (Hughes and Lister, 1953). It has been also transmitted from plant to plant throughout dodder (*Cuscuta americana* and *C. subglutinosa*) (Timmer *et al.,* 2000). Nevertheless, CTV is not a seedborne virus (Roberts *et al.,* 2001).

2.6.2.4. Major CTV aphid vector species

(i) The brown citrus aphid *T. citricidus* (Kirkaldy)

The brown citrus aphid *T. citricidus* (Kirkaldy) species, that means in Latin "citrus killer", has been probably originated from Southeast Asia, the home origin of Citrus, is readily considered as serious pest as well as a highly efficient vector of CTV worldwide (Yokomi *et al*., 1994). Since its introduction into the new world (South America) early last century and prior to its migration northward into most of the Islands in the Caribbean and Central America, damaging CTV strains has caused decline and death of tens of millions citrus trees grafted on sour orange rootstock in Brazil and Argentina (Knorr and DuCharme, 1951). The BrCA can survive a wide range of climates in the tropics, from Sub-Saharan Africa to areas in Australia, India, Sri Lanka and Asia (Roistacher, 1991). The Mediterranean Basin was free from *T. citricidus*, but its newly establishment in Portugal and Spain represents an inevitable threat of citriculture in the Mediterranean countries (Ilharco *et al.,* 2005). Even more alarming is the ability of *T. citricidus* to transmit preferentially severe stem pitting strains by contrast to the other vectors (Roberts *et al.,* 2001).

Despite that it is closely related to all *Citrus* spp., 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). One of the reasons making *T. citricida* such a good CTV vector may be the fact that it breeds exclusively on young citrus flush, therefore its population increases coincide with periods when tissue is most likely to have higher virus

titer (Yokomi *et al*., 2010). Then, 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.

(ii) The cotton and/or melon aphid *A. gossypii* (Glover)

Unlike the BrCA, *A. gossypii* is a cosmopolitan polyphagous species having a wide host range. 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, contaminates plants and produce with honeydew, and results in associated fungal growth on honeydew and finally transmission of viruses causing more than 50 plant diseases, such as *Cucumber mosaic virus* (CMV), *Water melon mosaic virus 2* (WMV 2) of Cucurbitaceae (Pitrat and Lecoq, 1980; Gray *et al.,* 1986). However, *A. gossypii* is the main vector of CTV in Spain, Israel, California (USA) and in all other important citrus-growing areas where *T. citricidus* does not prevail (Yokomi *et al*., 1989; Yokomi, 1992; Ballester-Olmos *et al.,* 1993; Gottwald *et al*., 1996a; Marroquín *et al*. 2004) having the potential to efficiently transmit some strains of CTV (Yokomi *et al*., 1989; Rocha-Penà *et al*., 1995). Dickson *et al*. (1956) reported that quick decline strains of tristeza were vectored by the melon aphid in an extremely inefficient manner. However, Martinez and Wallace later reported that the melon aphid

transmitted three seedling yellows CTV isolates at rates from 25 to 78% using small replicate sizes.

Recent investigations, focused on the sensitive detection and quantitation of CTV targets from single *A. gossypii* specimen by squash TaqMan real-time RT-PCR, showed that the virus acquisition rate from infected plants significantly increased from 10% viruliferous aphids after 1h to reach 20% - 34,7% after longer access periods of 24 and 48h respectively (Bertolini *et al*., 2008). Likewise, the mean value of acquired CTV targets were estimated to be 8,3 copies at the shortest acquisition access period, and raised between 35,9 and 38,2 viral copies after 24 and 48h acquisition probes, respectively. Interestingly, one hour feeding from the inoculum source seems to be enough for *A. gossypii* vectors to reach the phloematic tissues, in agreement with Limburg *et al*. (1997) hypothesis. The last suggested that BYV, another closterovirus, is acquired from sieve elements by aphids during phloem ingestion phase, correlating with the average time needed to reach the sieve elements with the minimum time needed for virus acquisition (20 min).

(iii) The spirea aphid *A. spiraecola* (Patch)

Almost cosmopolitan, the green citrus aphid, *A. spiraecola* Patch (= *A. citricola* van der Goot), has been present in Florida citrus since at least 1923. It has a wide range of hosts belonging to 20 different plant families, especially Caprifoliacea, Asteraceae, Rosaceae, Rutaceae and Apiaceae; where established heavy populations, often tended by ants, cause direct damage to young citrus trees including leaf distortion and shortening of terminal internodes. Interestingly, the spirea aphid is considered a serious pest because of its so far less efficient vectoring of plant viruses, including CMV, *Plum pox virus* (PPV), an isolate of *Alfalfa mosaic virus* (ALV) from Viburnum and *Zucchini yellow mosaic virus* (ZYMV) (Blackman and Eastop, 2007), as well as CTV (Yokomi and Damsteegt, 1991). Successful transmission trials conducted under experimental conditions

using some CTV isolates from Florida showed 29% transmission rate using *A. spiraecola* compared to 76% efficiency with *A. gossypii* (Yokomi and Garnsey, 1987). Later on, further investigation in Texas suggested that *A. spiraecola* is the major CTV vector having an overall transmission rate of about 8% from known CTV sources (Cutrer, 1998).

(iv) The Black citrus aphid: *T. aurantii* (Boyer de Fonscolombe)

By contrast to *T. citricidus*, the black citrus aphid has a cosmopolitan distribution, a wide host range and occasionally colonizes citrus species. 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).

2.6.2.5. Environmental factors:

Temperature has a paramount importance in the dramatic dissemination of CTV. Thus, higher viral titres have been revealed from CTV infected Californian field trees during the cooler months of February to April then from June to September (Dodds *et al*., 1987b). Likewise, better CTV aphid transmission rates have been described by Norman *et al*. (1968) when conducted by the cooler months of October to January and from Marsh to September in Florida. Similar investigations focusing on the impact of temperature in CTV transmissibility by the cotton aphid provided that cooler ambient conditions during pre-acquisition are favourable to build-up the virus titre resulting in significant higher transmission efficiencies. Furthermore, cooler temperatures following the vector inoculation also lead to keep the viral infectivity and to improve the transmission rate (Yokomi *et al*., 2010).

Additionally, citrus cultivars or varieties represent another critical factor governing the disease's dissemination regarding to their suitability for aphids landing and CTV acquisition. Comparative virus transmission studies in the Dominican Republic reported that higher transmission rates of CTV were obtained when the source and/or receptor plants are sweet orange varieties

compared to grapefruit and lemon species, furthermore, using Mexican lime and grapefruit as the recipients and 35 to 45 aphids per plant, 92 and 100% transmission was obtained, respectively (Roistacher and Bar-Joseph, 1984). Therefore, the probing behaviour of the aphid on the suitable citrus cultivars is marked by longer phloem ingestion associated with greater salivation frequencies resulting in higher transmission rates (Yokomi, personal communication). Within the Mediterranean basin, where *A. gossypii* and *A. spiraecola* are the most abundant aphid species in citrus groves (Hermozo de Mendoza *et al*., 1997), recent works carried out in Valencia (Spain) focused on the estimation of the number of aphids landing on field citrus trees in Spring 2000 showed that Clementine was the most visited host species followed by lemon and sweet orange (Marroquìn *et al*., 2004). This observation can be explained by the fact that these plant species are physiologically characterized by large area of the canopy, then, large number of young actively growing shoots per tree remain tender for longer than those of other citrus species. Additionally, it was also reported that Clementine and Satsuma mandarins were much more appreciated and received a significantly higher percentage of *A. gossypii* than *A. spiraecola*, whereas the proportions of the two species were not significantly different in grapefruit, sweet orange and lemon (Cambra *et al*., 2000 b).

Further detection assays of the viral RNA in individual aphid species suggested that approximately a quarter of all the aphid vectors captured from a visited tree carried RNA-CTV targets regardless to their ability to transmit the infection. Nevertheless, in mandarin and sweet orange species, the infection rate appeared proportional to the number of aphids landing on each tree, reflecting the high incidence and rapid spread of CTV in these citrus species in the last years (Cambra *et al*., 2000c).

2.6.2.6. Virus-vector interaction

Worldwide distribution and feeding behavior of aphids make them ideal vectors for dissemination of plant viruses, thus, over then 200 plant viruses are reported to be transmissible by aphids (Harris, 1977).

❖ **Non-persistent aphid virus transmission :** Foregut-borne transmission involves brief feeding on the epidermal layers of plant cells as the virus remains in the aphid for few minutes. Effectively, it is associated with the aphid's food canal within the maxillary stylets and involves the basal part of the feeding apparatus only (Harris *et al*., 1995). The non-persistent aphid transmission of filamentous potyviruses is mediated by the CP and a helper component-proteinase (*HC-Pro*) that is found in virus infected cells but not in virions. Hence, the direct interaction between the CP and *HC-Pro* creates a reversible binding of virions to the aphid foregut (Pirone and Blanc, 1996).

❖ **Persistent aphid virus transmission:** In circulative and propagative virus transmission, virus particles get acquired by aphids from the sieve tubes, diffused through the insect's hemocoel, translocated into the salivary gland and then injected with the saliva during the feeding from a recipient plant (Harris *et al*., 1995). Persistent transmission of luteoviruses generally involves an aphid-bacterial symbiosis; symbionts are located within specialized cells in the aphid known as "bacteriocytes" (Dixon, 1998). Symbionin, a bacterial protein get secreted into the insect's hemocoel by *Buchnera aphidicola* symbiont (Sub-Division: Probacteria), the first to be described from most aphids, forms a complex with the CPm of the virus within the aphid which is determinant for virus transmission (Van den Heuvel *et al*., 1997). Persistent transmission of citrus vein enation virus (CVEV) has been demonstrated in both *A. gossypii* and *M. persicae* (Hermoso de Mendoza *et al.* 1993).

❖ **Semipersistent CTV aphid transmission:** During the semipersistent's feeding process, repeated aphid's regurgitation for testing the quality of the plant sap that the virus comes into contact with a large area of the aphid's cuticular lining including the cibarial valve and pump, allowing the spread of the virus infection from insect to the plant and viceversa (Harris *et al*., 1995).

Differences between the non-persistent aphid-borne viruses and semi-persistent aphid borne-viruses are postulated to be related to retention of the virus in the foregut of the aphid (Harris, 1989). This seems to be related to the particular anatomical characteristics of the aphid species combined with virion physical dimensions, and additional specific virus-vector interactions such as those between the proteins on the surface of the virion and aphid foregut proteins. For potyviruses, the helper component protease (*HC-Pro*) is needed for aphid transmission of the virus (Pirone and Blanc, 1996). For *Cauliflower mosaic virus* (CaMV), helper proteins *P*2 and *P*3 are needed for the reaction between the virions and aphid cuticle (Woolston *et al*., 1987; Leh *et al*., 2001), and the virus has been reported as being preferentially acquired from the phloem by the aphid vectors (Palacios *et al.,* 2002).

However, precise information gathering the mechanism of Closteroviruses vector transmission is still scarce, but CTV proteases may be acting as reliable candidates for aphid transmission CTV-helper proteins. Therefore, CTV-CP, CPm, *HSP70h*, *p*61 and *p*20 are likely to be candidates involved in aphid transmission process since they are present on the outer surface of CTV virions. *HSP70h*, readily associated with cellular heat-shock protein, has been also reported to halt the cell's defenses against stylet penetration (Peng *et al*., 2001).

Recently, *in vitro* feeding experiment of the BrCA across artificial membranes on crude tissue preparations made from CTV-infected bark tissue permitted CTV transmission to virus-free receptor plants at low rates and showed the detection of *p*20, *p*27 and *p*25 proteins by Western blots from all crude tissue preparations. Moreover, Blocking the *p*20 function by feeding the aphids with the

correspondent antibodies significantly increased CTV transmission by the BrCA; suggesting that the inactivity of CTV *p*20 aids the virion transmission by *T. citricidus*. Thus, associated viral components or structural aggregations of viral protein complexes may be needed for CTV to be transmissible using *in vitro* BrCA acquisition feeding as documented by Herron *et al*., (2006).

To date, despite that it is little known about the virus-vector interaction mechanism of CTV, some studies showed that particular variant strains of CTV differ in their transmissibility by aphids (Bar-Joseph and Loebenstein, 1973; Raccah *et al*., 1978; 1980). From this standpoint, it was reported that severe strains of CTV were transmitted by BrCA with higher efficiency than the mild strains, with shorter acquisition and longer retention periods (Sharma, 1989). Regarding *A. gossypii*, it was reported to be the principle vector of CTV in California and has been readily associated with consistent spread of a stem pitting CTV isolate at the University of Riverside (CA) (Calavan *et al*., 1980; Roistacher *et al*., 1980; 1984). Similar transmission attempts by *A. gossypii* using three sub-isolates derived from the well known isolate SY568, obtained from a stunted and stem pitted Mineola Tangelo in California evidenced again a high potential of the cotton aphid in transmitting a severe honey combing sub-isolate (1B) at 50% of transmission level, compared to moderate sub-isolates which were transmitted at 30 to 43% of efficiency (Velazquez-Monreal *et al*., 2009).

2.6.3. Genomic alteration of viral CTV population by aphid passage

As for major RNA viruses, 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*., 1993b). Consequently, sub-isolates generated by these processes sometimes showed some divergence within the viral population of the source isolates, providing further explanation of the wide biological and pathogenic variability observed among CTV isolates over the time.

In this context, separation of CTV variants from the field isolates has been reported in several studies through aphid passage (Gillings *et al*., 1993; Brlansky *et al*., 2003; Albiach-Martì *et al*. 2000b). *T. citricidus* 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).

Therefore, aphid transmission sometimes alters the expression of symptoms and the pathogenic characteristics of an isolate (Hermoso de Mendoza *et al*., 1988; Moreno *et al*., 1993a, b; Broadbent *et al*., 1996), as well as their serological reactivity against monoclonal antibodies (Kano and Koizumi, 1991; Cambra *et al*., 1993). Using single BrCA transmission as a tool for separating the complex of CTV variants highlighted the detection of severe decline, MCA13 positive sub-isolates hidden within a mild inducing, MCA13 negative virus sources. Additionally, new severe sweet orange and grapefruit stem-pitting sub-isolates was risen from mild symptom producing isolates from Corsica (Brlansky *et al*., 2003).

Since, genetic data on the CTV populations before and after transmission are almost rare, these findings prompted more extensive molecular characterization of the genome population of several CTV isolates for better understanding the genomic drifts caused by aphid transmission bottlenecks (Ayllòn *et al.,* 1999b). Hence, double-stranded RNA (dsRNA) profile upon graft or aphid transmission to a new host have been documented (Moreno *et al*., 1993a, b), then, restriction fragment length polymorphism (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). Later on, Albiach-Martı´ *et al*. (2000b) also detected changes in the CTV genomic RNA population after

aphid transmission, by hybridization with a panel of cDNA probes to different genomic segments and the population of defective RNAs. Although these findings evidenced that changes in the viral population may occur in the transmission process, they did not deeply explain the mechanism involved behind these changes (d'Urso *et al*., 2000).

Single-strand conformation polymorphism analysis which has been efficiently processed 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 2000a). Thus, SSCP analysis of genes *p*18 and *p*20 showed that eventual changes after aphid transmission by *A. gossypii* may indicate a titre increase of certain sequence variants in the aphid transmitted sub-isolates and/or drastic reduction or disappearance of other variants previously present in the viral complex of the field isolate.

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 (Velazquez-Monreal *et al*., 2009). However, one of these new isolates (1B-a4) was predicted to be a mild strain. Through sequencing and comparison of the *p*65 region, two distinct sequences were recovered from the 1B inoculum source, showing highest sequence identities with well known mild and severe strains confirming its mixed nature; while, after the vector transmission, sequences from its corresponded mild sub-isolate 1B-a4 shared the most similarity with other known mild strains such as T385 and T30 and was symptomless when grafted on all indicator species; by contrast, sequences from other sub-isolates were most similar to known severe isolates (e.g., VT and NUagA) causing some degree of stem pitting, stunting, and vein corking (Velazquez-Monreal *et al*., 2009).

As documented by d'Urso *et al*. (2000), at least two factors could be in the origin of noticed alterations among the genomic RNA population in the transmission process: (i) uneven distribution of genomic RNA variants in different plant parts, as shown in the multiple infected isolate T388 aphid derived from a highly pathogenic isolate, illegally imported from Japan (Hermozo de Mendoza *et al*., 1988a), might result in aphids acquiring a different viral population depending on the probing site; and (ii) individual aphids 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. By these ways, some minor variants of the former viral population could become predominant by a founder effect and give rise to new populations with divergent characteristics.

2.7. Tristeza disease management

The ability to minimize the economic losses due to the disease damage depends upon the incidence and the severity of virus strains in a particular district (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). Hence, the best measures to avoid disease infection or dissemination in CTV-free areas include strict quarantine measures coupled with certification programmes 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). When CTV is still at low incidence and infected trees are grouped in limited foci, rapid eradication of infected trees is the most effective practice to avoid the inoculum dispersal and to delay an epidemic (Kyriakou *et al*., 1996; Gottwald *et al*., 2002). However, where the disease becomes endemic and the chances of natural dissemination are high, the use of CTV tolerant rootstocks and mild strain cross protection are the emphasis to extend the economic life of citrus trees without tristeza decline and/or stem pitting diseases (Garnsey *et al*., 1998).

Nevertheless, technical challenges posed by these undertaken options need to be solved. Thus, breeding strategies to incorporate resistance genes in commercial cultivars is becoming the best approach to avoid losses due to the pathogens (Moreno *et al*., 2008).

2.7.1. Prophylactic approaches

In areas where CTV is rare with limited natural spread, the emphasis has been on avoiding the virus throughout simple and inexpensive preventive efforts, mostly involving some forms of regulatory actions (Garnsey *et al*., 1998). Application of effective quarantine rules such as prohibiting any importation of plant material from high risky countries is mandatory in order to exclude the introduction of exotic and severe strains into the area (Frison and Taher, 1991). Plant material imported even from countries where CTV is not yet reported but may be occurring latently should be fumigated against vectors; fruits have to be free from peduncles and leaves, washed and waxed or fumigated (EPPO, 1990). But also, initial field plantings of introduced cultivars should be grown in isolated areas, propagated and regularly monitored for the presence of the virus (Garnsey *et al.,* 1998).

Nevertheless, large-scale surveys are of utmost importance to early depict eventual newly established foci of CTV into an area and the elimination of the virus reservoirs either from individual trees or entire plantings (Gottwald, 2010). If the disease incidence is low (<3%), rapid detection and eradication measures have met various degrees of success by maintaining the incidence of the disease at manageable levels; conversely, once a threshold of 5% incidence get reached, it becomes much more difficult to apply eradication to remain ahead of the new infection curve and others treatments get needed (Dodds *et al.,* 1994; Bar-Joseph *et al.,* 1989).

Quarantine measures should be enforced by a consistent certification program because the greatest hazard for CTV introduction is actually within the

Citrus industry. In countries where CTV is already widespread, the certification programs is essential to avoid the spread of severe CTV strains into new plantings (EPPO, 1990), as well as, to avoid damage from other graft-transmissible pathogens when tolerant rootstocks are replacing the sour orange (Garnsey *et al.,* 1998). Based on the European experience, a Mediterranean Research Network on Certification of Citrus (MNCC) have been established since 1995, promoted by the Mediterranean Agronomic Institute of Bari (CIHEAM/MAI-Bari) (D'Onghia, 2009).

2.7.2. Host tolerance

As reported by Moreno *et al*. (2008), propagation of citrus on tristeza-tolerant rootstocks is the only viable option to manage CTV without tristeza decline when eradication becomes unfeasible due to the efficient CTV dispersal by vectors. The most used tolerant rootstocks are *P. trifoliata* and its hybrids Carrizo and Troyer citrange (*C. sinensis* x *P. trifoliata*) as well as Swingle citrumelo (*C. paradisi* x *P. trifoliata*) and Rangpur lime (*C. limonia* Osb.). In this context, 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 co-existence of severe CTV strains and the BrCA vector (Bar-Joseph *et al*., 2010).
However, the most devastating tristeza-decline epidemics in the early 1930's caused catastrophic losses on sour orange rootstock, whose replacement by CTV-tolerant species have been counteracted by new problems (Bar-Joseph *et al*., 1989). Thus, a number of limiting factors makes more complicated the choice of suitable CTV-tolerant 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 (Romàn *et al*., 2004).

Despite that tolerant rootstocks can provide successful protection against QD epidemics, the presence of severe SP inducing strains heavily damage scions

regardless to the tolerance of the used rootstocks which suggested the need of constitutive resistance to the virus or cross protecting sensitive varieties with appropriate mild isolates.

2.7.3. Mild strain cross-protection

CTV cross-protection or pre-immunization consists of inoculating plants with protective mild isolates of the virus to afford protection against infections by severe strains (Gonsalves and Garnsey, 1989). In order to be selected for possible deployment within a management strategy, protective isolates should be picked up from field trees eliciting mild or no symptoms in all citrus cultivars under different temperature regimes and field site conditions. Such isolates should be easily and rapidly aphid transmitted and uniformly distributed within the inoculated plants, because any virus free tissue, even temporarily, provides an opportunity for an aphid to carry out a severe strain (Powell *et al*., 1992).

Successful cross-protection programs were firstly reported from Sao Paulo (Brazil) allowing the survival of more than 90 million protected trees in the last 30 years (Costa and Mùller, 1980); later from Australia (Broadbent *et al*., 1991), South Africa (van Vuuren *et al*., 1991), Florida and Venezuela with tristeza decline protection (Lee and Rocha-Penà, 1992; Ochoa *et al*., 1993). The real mechanism behind the cross protection between related virus strains is still unclear. Indeed, high accumulation of viral-specific small-interfering (si)-RNAs, revealed from CTV inoculated Mexican lime plants, 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 (Fagoaga *et al.*, 2006). Nevertheless, failure of cross-protection activities has been documented from other countries and diverse cultivars (Sambade *et al*., 2007) indicate that CTV could overcome the host defense process in some instances (Moreno *et al*., 2008) and suggest a strong association between the existent CTV strain, the host species and environmental

factors for a durable cross protection (Broadbent *et al*., 1991). Similar results dealing with 16-years protection of grapefruit on sour orange rootstock by some non-decline inducing CTV strains in Florida showed that the arrival of the BrCA hastened its breakdown within only 5 years. This suggested that, applying classical cross-protection where decline inducing CTV and its vectors are prevalent, is not a good management strategy (Powell *et al.,* 2003). The most plausible explanation for this approach is that some uneven distribution of CTV results in leaving certain parts of the plant susceptible to further infections. Failure of cross-protection could be accelerated further to the bombardment of the pre-immunized hosts by severe SP or SY-CTV variants, recorded to be easily transmissible by *T. citricidus* and *A. gossypii* vectors, respectively. Another approach is that a mixed CTV isolate can harbor a severe strain which does not necessarily incite severe symptoms, but on particular host and/or under specific environmental conditions, it may become dominant with disastrous consequences.

2.7.4. Pathogen derived resistance

Strain specific resistance to CTV has been commonly reported from some Citrus relatives of other genera within the Subfamily Aurantioideae including species of *Fortunella*, *P. trifoliata*, *Severinia buxifolia* and *Swinglea glutinosa* (Garnsey *et al*., 1987; Mestre *et al*., 1997; Yoshida, 1996). Although genetic improvement by the conventional breeding gained a good popularity in providing enough host immunity with response to their associated pathogens, it appeared to be inappropriate in citrus due to some limiting factors related to their physiological traits, such as their complex reproductive biology, their long-lasting juvenile period and especially their high levels of heterozygosity (Bernet *et al.*, 2004). Therefore, utilization of the transgenic plant strategy can be a viable alternative for insuring resistance to citrus varieties. The fact that hybrids between *P. trifoliata*, the only resistant species sexually compatible with citrus, and *Citrus* spp. is feasible, makes the resistance to CTV a suitable trait to be introduced into

the cultivated varieties, either by sexual hybridization or by genetic transformation after cloning of the resistance gene (Mestre *et al*., 1997).

This resistance has been associated with a single dominant locus (*Ctv*-R) which has been documented and mapped (Deng *et al*., 2001; Yang *et al*., 2003), and shown to be restricted to a 121 kb length fragment comprising 10 genes within the genome of trifoliate relative of citrus (Rai, 2006). To date, new approaches for the development of more durable CTV resistant plants have revolved around pathogen derived resistance (PDR) strategies. Transgenic Mexican lime plants inoculated with the CP gene from a severe and mild CTV strain, yielded varying degrees of resistance (Domìnguez *et al*., 2002). Within the fraction (10-30%) where the pathogenic CP highly was accumulated, transgenic lines appeared to be immune, conversely, when the CP titer was low, resulting lines showed a significant delay in virus accumulation and symptom expression.

Chapter 3: Overall objectives

Citrus tristeza is one of the most economically damaging viral disease affecting citrus all over the world. Isolates differing in the type and intensity of symptoms induced in different citrus species and their aphid transmissibility have been readily reported worldwide.

Within the Mediterranean basin, where the sour orange is the most predominant rootstock, and mild strains are still prevalent, the fact that extensive comparisons of CTV-CP gene sequences reported the occurrence of severe decline variants from many countries, made the situation alarming.

From an epidemiological standpoint, the recent establishment of *T. citricidus* in northern Portugal and Spain raised the risk for the rapid spread of severe CTV isolates in the area. Nevertheless, the second most efficient CTV aphid vector *A. gossypii* is pandemic and shown to have a potential to efficiently transmit some seedling yellows isolates from Spain and Israel.

Despite that many findings illustrated some genetic separation of CTV variants generated by aphid transmission bottlenecks, sometimes accompanied by a different pathogenic behaviour of the isolates, the mechanism involved during these changes is still largely unknown.

The current situation of the Mediterranean citriculture suggests to gather more information about the prevailing CTV variants and their severity. Thus, estimation of their genetic diversity and particularly the definition of their transmission properties is a key element for predicting disease impact and devising appropriate control strategies suitable to specific regions.

Taken into account these thoughts, the overall objectives undertaken by this work include:

- ✓ Evaluation of the genetic diversity of CTV Mediterranean isolates maintained *in-vivo* in the MAI-Bari, through genotype assignment by a set of multiple molecular markers and CP gene sequence analysis.

- ✓ Experimental transmission trials of some Mediterranean CTV isolates by the main aphid vector species: *A. gossypii, A. spiraecola* and *T. aurantii.*
- ✓ Study on eventual variations on Mediterranean CTV genetic structure after aphid transmission, including:
  - ➢ Response of the vector inoculated sub-isolates to MCA13 MAb;
  - ➢ Molecular characterization of transmitted isolates focusing on multiple genome regions by MMM analysis, SSCP on *p*25; *p*18, *p*20 and *p*23 genes and CPg partial genome sequencing;

Chapter 4: Materials and Methods

4.1. Molecular characterization of Mediterranean CTV isolates

The CTV genetic variation is unevenly distributed along the gRNA. The most conserved region being the 3'-UTR with 95% identity between isolates, whereas the 5'-terminus is more variable. Thus, characterization of different CTV isolates and assessment of their population diversity based on a single gene may not be conclusive for the entire genome. As well, full sequence comparison of the whole genome is not only difficult but also a very time consuming.

4.1.1. Assessment of the genetic diversity of CTV by Multiple Molecular Markers

By the use of the Multiple Molecular Markers technique, a "genotype profile" can be attributed to an unknown isolate based on its sequence specific amplifications with a set of primers designed from three different non-conserved regions of VT, T3, T30 and T36 CTV reference isolates (Hilf and Garnsey, 2000; Hilf *et al*., 2005).

*** Virus source populations**

A total number of 75 CTV sources originated from several Mediterranean countries, including Italy (24), Albania (10), Lebanon (10), Palestine (8), Montenegro(2), Algeria (2), Morocco (2), Trinidad (2), Croatia (1), Cyprus (1), China (3), Egypt (4), Iran (2), Syria (1) and Portugal (1) have been collected and maintained *in-planta* within the CTV collection of the MAI-Bari (Italy). Infected plants are kept in an insect-proof screenhouse under controlled conditions of 18-25°C. Each isolate is designated by the prefix (IAMB_Q) followed by a code number.

4.1.1.1. Total RNA isolation and complementary DNA (cDNA) synthesis

As described by Foissac *et al*., (2001), the total RNAs have been recovered from the CTV-infected plants, using a pool of 0,2 g of phloematic tissues in liquid nitrogen under aseptic conditions. After grinding in 1 ml of relative buffer (Annex 1), a volume of 500 µl of the extract were transferred to microcentrifuge tubes containing 100 µl of 10% Sodium Lauryl Sarcosyl (SLS) and incubated at 70°C for 10 min with intermediate shaking, then briefly chilled on ice for 5 min. After 10 min of full speed centrifugation, 300 µl of the collected supernatant was mixed with a solution containing a mix of 150 µl of absolute ethanol, 35 µl of re-suspended silica and 300 µl of 6 M Sodium Iodine solution (NaI). The mixture was then incubated at room temperature for at least 10 min with intermittent shaking and then centrifuged at 6000 rpm for 1 min. The pellet was recovered and washed 2-3 times by supplying 500 µl of washing buffer (Annex 1). By the end, the pellet get re-suspended in 150 µl of RNase-free water. Then, the total nucleic acids (TNAs) isolated were recovered after incubation for 4 min at 70°C and full speed centrifugation for 3 min, and finally stored at –80°C until use.

First strand cDNA was synthesised from 0,5 µg of RNA in a final volume of 20 µl of reaction mixture (4 µl of 5X first strand buffer, 2 µl of 0,1 M dithiothreitol, 3 µl of 10 mM deoxy-nucleotidetriphosphate (dNTPs); supplied by 1µl hexanucleotide random primer (0,5 µg/ µl concentration) and 0,8 µl of 200 U/µl Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) (Invitrogen, USA) following the manufacturer's instructions (Bethesda Research Laboratories, USA). Reaction mixtures were then 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.

4.1.1.2. Multiple Molecular Markers assays:

Amplification of CTV sequence specific molecular markers was performed using one universal (CTV-CP) and ten pairs of oligonucleotide primers as described in the table follow (Table 3).

Table 3. Oligonucleotide primers and their genomic positions used for Multiple Molecular Markers analysis (Hilf *et al.,* 2005)

Marker	Size bp	Oligonucleotide sequence (5′ to 3′)	Marker Position[a]
T36CP	671	(+) ATGGACGACGAAACAAAGAAATTG (–) TCAACGTGTGTTGAATTTCCCA	16,152-16,823
T36-5′	500	(+) AATTTCACAAATTCAACCTG (–) CTTTGCCTGACGGAGGGACC	1-500
T36K17	409	(+) GTTTTCTCGTTTGAAGCGGAAA (–) CAACACATCAAAAATAGCTAGT	4,865-5,273
T36POL	714	(+) TGACGCTAACGACGATAACG (–) ACCCTCGGCTTGTTTTCTTATG	10,791-11,508
T30-5′	594	(+) CGATTCAAATTCACCCGTATC (–) TAGTTTCGCAACACGCCTGCG	6-600
T30K17	409	(+) GTTGTCGCGCCTAAAGTTCGGCA (–) TATGACATCAAAAATAGCTGAA	4,848-5,256
T30POL	696	(+) GATGCTAGCGATGGTCAAAT (–) CTCAGCTCGCTTTCTCACAT	10,772-11,467
VT-5′	492	(+) AATTTCTCAAATTCACCCGTAC (–) CTTCGCCTTGGCAATGGACTT	1-492
VTK17	409	(+) GTTGTCGCGCTTTAAGTTCGGTA (–) TACGACGTTAAAAATGGCTGAA	4,824-5,232
VTPOL	695	(+) GACGCTAGCGATGGTCAAGC (–) CTCGGCTCGCTTTCTTACGT	10,745-11,440
T3K17	409	(+) GTTATCACGCCTAAAGTTTGGT (–) CATGACATCGAAGATAGCCGAA	4,871-5,279

[a:]T36, T30, and VT primer sequences and marker positions are from GenBank Accession Nos. AY170468, AF260651, and 56902, respectively. Genomic position of T3 marker was derived from pairwise alignment with the T36 genomic sequence.

The universal T36CP primers served as a positive control to confirm the infectivity of the studied isolates before to be characterized. While, the others were depicted from three noncontiguous regions (POL; 5'- and K17) within the most variable 5' co-terminal ends of four known CTV reference strains T36, VT, T3 and T30 (Fig 5).

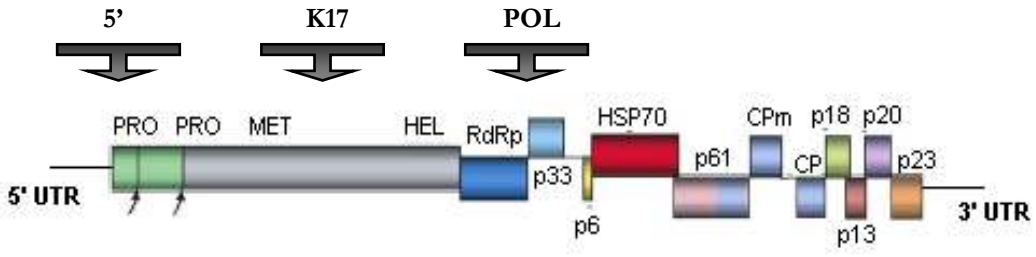


Fig 5. Schematic diagram of *Citrus tristeza virus* genome organization

Black blocks indicate the different ORFs and the positions of the sequence specific markers amplified as reported by Hilf *et al*., (2000).

Eleven PCR amplifications were carried out from each sample. CTV cDNA templates (5 µl) were submitted to a serial of PCR reactions in a final volume of 25 µl of reaction vessel containing 1X concentration of reaction buffer, 1.5 µM $MgCl_2$, 0,2 mM dNTPs, 0.2 µM of each of the sense and anti-sense sequence specific molecular markers and 0.6 U of *Taq* polymerase (Fermentas, UK). Amplification profiles comprised an initial denaturation at 95°C for 5 min, followed by 35 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 elongation, prior to be maintained at 4°C until analysis. PCR products were analysed by 1% agarose gel electrophoresis in TAE buffer (Annex 2), followed by Ethidium Bromide (200 ng/ml) staining and visualisation under UV lights.

4.1.1.3. Cloning and Partial Genome Sequencing of the CTV-CPg

Rather than analysis by multiple molecular markers tool, representative isolates from each group of genotype profiles has been depicted for further characterization focusing on the less variable CPg fragment. For this purpose, ten CTV isolates originating from Italy, Palestine, Lebanon, Egypt, Algeria and Albania showing single genotype patterns by MMM analysis, as well as five multiple infected isolates originating from Montenegro, Albania, Croatia, Italy and Morocco were submitted to further partial CPg cloning and sequencing.

Viral targets have been amplified with CTV-CP universal primers yielding 672 bp, then purified using the Wizard SV gel and PCR clean-up system kit (Promega Corporation ®, Madison, WI, USA) according to the manufacturer's instructions and eluted in 50 µl of elution buffer. Afterward, legation of the purified amplicons to the plasmid and their transformation of competent *Eschericha. coli DH5α* bacterial cells were performed following standard molecular biology protocols (Sambrook *et al*., 1989).

From each sample, 2 µl of eluted PCR products were inserted into a commercial *pGEM-T* (50 ng/µl) using the original TA Cloning Kit (Promega Corp, Madison, WI, USA). As recommended by the manufacturer's protocol, within each legation reaction, a Control Insert DNA (4 ng/µl) was included for assessing whether the legation proceeded efficiently, in addition to a background control to determine the number of blue colonies resulting from non-T-tailed *pGEM-T* Easy Vector (Table 4).

Table 4. Legation protocol using *pGEM-T* Easy Vector (Promega, USA)

	Standard reaction	**Positive control**	**Background control**
2X rapid ligation Buffer	5 µl	5 µl	5 µl
pGEM-T easy vector	1 µl	1 µl	1 µl
Purified PCR product	2 µl	-	-
T4 DNA Ligase	1 µl	1 µl	1 µl
Control Insert DNA	-	2 µl	-
H2O sterile	1 µl	1 µl	3 µl

In order to optimize the number of recombinants, legation mixtures were incubated for 1h at room temperature, then overnight at 4°C.

❖ **Preparation of *E. coli* competent cells:** In a strictly aseptic conditions, *E. coli* DH5α cells (Invitrogen, USA), were streaked across the surface of *Luria-Bertani* (LB) solid plate and incubated at 37°C for 16h (Annex 3). Then, a single colony was incubated into 2 ml of LB liquid medium overnight at 37°C

with shaking at 250 rpm. Additional growth of bacterial cells was performed by inoculating 50 µl of bacterial culture in 10 ml of LB liquid and incubating them at 37°C for 3h with shaking at 250 rpm. The culture was chilled in ice bath for 10 min to stop growth and then, was harvested by centrifugation at 6000 rpm for 10 min at 4°C. After discarding of supernatant, the bacterial pellet was gently re-suspended in 5 ml cold 0,1 M $CaCl_2$ (Annex 3) then stored on ice for 30 min. After 5 min centrifuged at 6,000 rpm, the $CaCl_2$-treated bacteria were re-suspended again in 0,5 ml of 0.1 M $CaCl_2$ and kept on ice for 2 hours before to get transformed. Aliquots were prepared and stocked at -80°C.

- **Transformation of *E. coli* competent cells:** In a sterile microfuge tube, 100 µl of competent cells suspension were added to a volume of 10 µl of legation mixture and kept on 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 45 sec, and immediately transferred on ice for 2-5 min. Afterward, cells were supplied by 600 µl of pre-warmed LB liquid (Annex 3) and incubated for 30 min at 37°C followed by additional 30 min at 37°C with agitation at 250 rpm. Once recovered, a brief centrifugation (2 min) was applied to eliminate the remaining liquid and 150 µl of bacterial suspension has been plated in LB-Agar medium supplied with 40 µl of 20 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Invitrogen, USA) and Ampicillin (50 mg/L), using a sterile bent glass rod. Inverted plates were grown overnight at 37°C.

- **Screening of bacterial colonies and recombinant DNA (*rec*DNA) extraction:**

Among the transformed *E. coli* cells, a number of white colonies suspected to harbour the recombinant plasmid with the target insert were picked up, then, re-inoculated and numbered in new LB-Agar plates with a sterile toothpick, and simultaneously, eluted in 35-50 µl of Triton buffer (1 % Triton X,

20 mM Tris HCl, pH 8.0 and 2 mM EDTA, pH 8.0) in a new microfuge tube. The latters were boiled for 10 min to release the suspected *rec*DNA from the bacterial cells.

In order to confirm the presence of the target insert, PCR reactions were performed with the same universal primers (CP) initially used, in a final volume of 25 µl reaction using 2 µl from the bacterial homogenate, as described before. Successfully amplified amplicons recovered from at least 10 recombinant clones per sample were subjected to SSCP analyses in comparison with the parental PCR product.

❖ ***rec*-DNA plasmid extraction:** *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 intermediate shaking (250 rpm). Bacterial suspensions were poured into a 1,5 ml micro-centrifuge tube, then centrifuged at 12,000 rpm for 30 sec and flowthrough were discarded. Dried pellets were re-suspended in 350 µl of STET buffer (Annex 3) and 20 µl of lysozyme (20 mg/ ml) by rigorous vortex. Lysis of the bacterial cells were favoured by boiling for 40 sec, and immediate chilling on ice and full speed centrifugation for 30 min. The pellet of bacterial debris and genomic DNA were remove with sterile toothpicks; plasmid DNA were extracted with 400 µl of 1:1 (v/v) of phenol/chloroform and 10 min of centrifugation.

Surfactant were gently recovered, transferred to new tubes and precipitated for 20-30 min on ice, in the presence of 125 µl of 5 M Ammonium Acetate solution (pH 3,5) and 1 ml of cold absolute ethanol followed by full speed centrifugation for 10 min. Pellets were washed using 500 µl of 70% cold ethanol and centrifuged again for 10 min at 13000 rpm. After surfactant elimination and drying into Vacuum pump for 1 h, it was re-suspended in 50 µl TE buffer (Annex 3) containing 1 µl of RNase A (10 µg/ µl), vortexed and conserved on ice until analysis.

❖ **Plasmid Enzymatic digestion and purification:** The presence of the specific insert inside the extracted plasmids were confirmed by an enzymatic digestion with the restricted endonuclease *Eco* RI (Promega Corp, Madison, WI, USA). Reactions were carried out in a total volume of 10 µl, in which 10 to 15 ng of plasmid DNA were added by 1 µl of 10X buffer H (Roche), 2U *Eco* RI and incubated for 1h 30 at 37°C. Quality and quantity of the digested products were confirmed by 1% agarose gel electrophoresis, followed by ethidium bromide (200 ng7ml) staining and UV lights visualization. Further purification of the selected plasmids was performed by precipitation with 30 µl of the PEG-NaCl solution (Annex 3) in ice for 1 h. Then, centrifuged at 14000 rpm for 5 min; after liquid phase removal, they were washed by 100 µl of 70% cold ethanol, centrifuged at 14000 rpm for 5 min, dried for 1h into a vacuum pump and the pellets were dissolved in 30 µl of sterile water.

4.1.1.4. Single strand conformation polymorphism analysis (SSCP)

Since SSCP technique proved its convenience with *p25* to detect single gene mutation (Niblett *et al.,* 2000; Hilf and Garnsey, 2000), 1 µl of the positive aliquots was mixed with 9 µl of denaturing buffer (95% formamide, 20 mM EDTA, 10 mM NaOH and a trace of Bromophenol-blue). Therefore, discrimination between sub-isolates differing by a few number of nucleotides was enabled by the denaturation of dsDNA for 10 min at 95°C and their immediate chilling on ice, followed by 10% non-denaturing polyacrylamid gel electrophoresis in 1x TBE buffer (Annex 2). The gel running took 3-4 hours at 4°C and 200V voltage. Afterward, classical colorimetric detection of the gel consisted on its fixation in (10%) acetic acid solution for at least 20 min, incubation in (1%) nitric acid solution for 3 min, then, its staining for 15-20 min in silver nitrate solution (Annex 2), separated by 3 min washing with distillated water; finally, a developing solution (Annex 2) was added and the gels were incubated till the appearance of the bands; this reaction was immediately stopped

by immersing the gel in acetic acid (10%) for 10 min for better conservation (Beidler *et al*., 1982).
SSCP analysis was applied to the PCR amplicons referred to the positive transformed bacterial colonies in order to exhibit the different haplotypes and to limit the number of clones to be sequenced.

4.1.1.5. Computational-assisted sequence analysis

Sequence haplotypes obtained from representatives *rec*DNAs were aligned using the BioEdit software package version 5.0.9 (Hall, 1999), and the default options of CLUSTALX 1.8 (Pearson and Lipman, 1988), a Windows interface for the CLUSTALW multiple sequence alignment program. Obtained sequences (Primm Company, Italy) were then confronted with other strain references available in NCBI Data Base and retrieved in the Gene databank entries: T30 (AF26065), VT (U56902), 28C (AF184118), Qaha (AY340974), T36 (AY170468), 19-21 (AF184114), T3 (DQ355053.1) and SY568 (AB046398).
Calculation of pair wise nucleotide distances between sequences and clustering were done using the Molecular Evolutionary Genetic Analysis software MEGA version 2.1 (Kumar *et al*., 2001) for the estimation of the nucleotide homology.

4.2. Experimental CTV aphid transmission trials

4.2.1. CTV virus sources

CTV isolates belonging to the MAI-Bari virus collection and maintained within the virology insect-proof glasshouse were selected to be assessed for aphid transmissibility.

In the lack of enough information regarding the biological activities associated with each isolate, an extrapolation between the CPg features obtained from some fully characterized CTV isolates and their confrontation with available sequence data (Hilf *et al*., 2005; Nolasco *et al*., 2008), enabled the selection of representative isolates for each standard CTV group. In addition to a CPg-SSCP

analysis, a number of accessions have been selected for the transmission by aphids. Infected CTV source plants were graft inoculated on Mexican lime and sweet orange seedlings and maintained under greenhouse conditions. At least three replicates by virus source were adopted as "donor plants (D.P)" during the aphid transmission trials.

4.2.2. CTV aphid vector species

Within the BrCA free Apulian orchards, both of *A. gossypii* and *A. spiraecola* were almost the most prevailing aphid species landing on citrus trees (Yahiaoui *et al*., 2009), in accordance with previous data postulated from Italy (Davino *et al*., 2004) and from some Mediterranean area (Marroquin *et al.,* 2004). In order to conduct aphid transmission experiments, virginopares of *A. gossypii*, *A. spiraecola* and *T. aurantii* species were picked up from citrus plantings. Prior to initiate any experiment, field aphids were maintained on virus-free "Carrizo" citrange (*P. trifoliata* (L.) x *C. sinensis* (L.)) for 48h aiming to prevent eventual field infections (Yokomi, personal communication). At the same time, some specimens of the collected aphids were subjected to rigorous mounting on slides as described by Heikinheimo (1988) to make feasible their taxonomic identification.

4.2.2.1. Aphids mounting

* ***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 (Ilharco and Lemos, 1981). Aphids were then transferred in test tubes containing 10% KOH solution and moved to a water bath at 85-90°C for about 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:*** Mainly 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 polyvinylalcohol-lactic acid PVA (a mounting medium developed by Ossiannilsson (Danielsson, 1985), (Annex 4) and placed on a microscopic slide. Antennae and legs were arranged under a stereoscopic microscope with magnifications of 60-120 X. A clean cover slip was finally put in the middle of the slide to seal the sample.

* ***Labelling:*** Essential information was written on two standard labels 23 x 23 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.

4.2.2.2. 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 25X to 400X magnification (Annex 5) prior to be reared and adopted in the CTV transmission experiments (Fig 6).

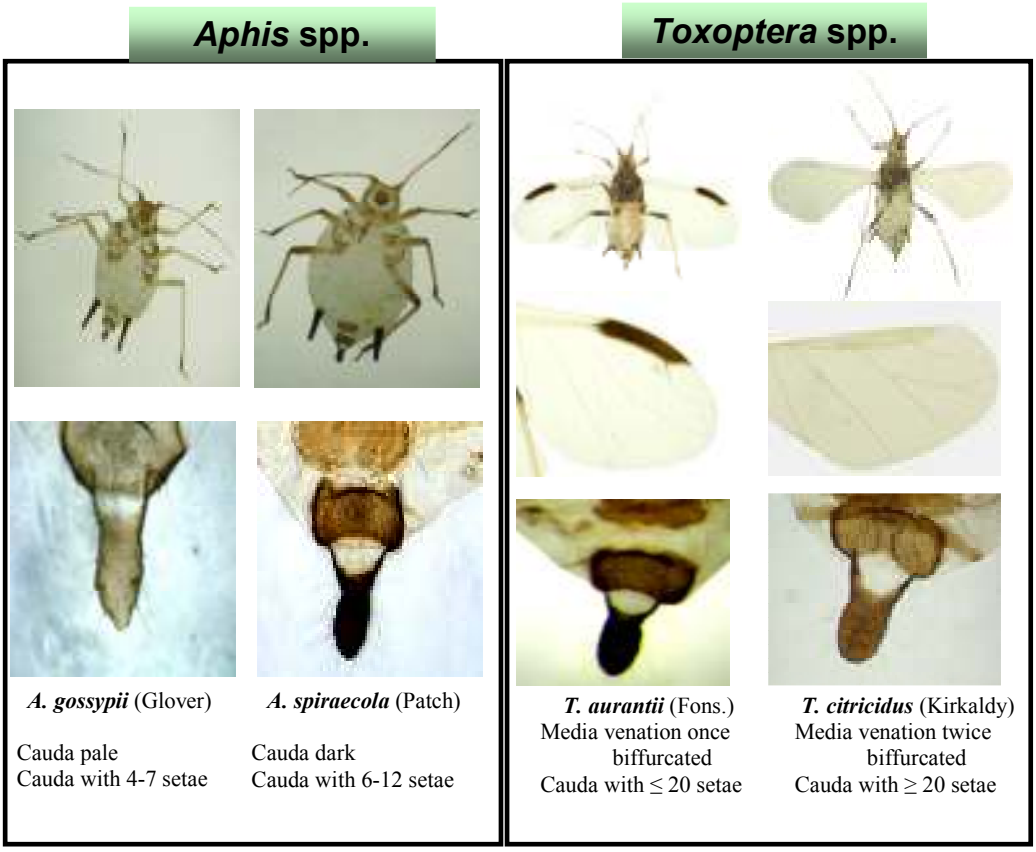


Fig 6. Morphological discrimination between CTV aphid vector species under compound microscope

4.2.2.3. Aphids rearing

A. gossypii species is interchangeably designated as the common melon and cotton aphid, besides that previous works (Yokomi *et al*., 1989) demonstrated noticeable differences in CTV transmission efficiency between the two vector biotypes. Comparative trials of aphid rearing were conducted both on cotton and melon species under similar conditions; consequently, it was observed that the life cycle of the cotton biotype was much more brief with respect to the melon one. Thus, the metamorphosis of the preimaginal instars were more accelerated and the number of progeny generated after one week gaps of time was much more important when grown on cotton with respect to the melon plants (Fig. 7), enabling the virus transmission experiments of several isolates within a restricted gaps of time. This observation can be explained by the fact that cotton host

species is physiologically characterized by producing large number of soft leaves within few days, and plant tissues that remain tender for longer than those of the cucurbitaceous species.

During these experiments, rearing of the aphids was performed in a growing chamber within the MAI-Bari facilities. Therefore, colonies of *A. gossypii* were successfully reared on 'Celia' cotton plantlets (*Gossypium hirsutum*) under controlled conditions [23:16° C (day/night) and a photoperiod of 16/8 h, as reported by Bertolini *et al*., (2008). While, populations of *A. spiraecola* were maintained on Viburum plant species (*Viburnum suspensum*); and *T. aurantii* aphids on healthy citrus plantlets.

Pure progeny were generated for each species starting from one single virginopare, in order to avoid the development of mixed aphid populations, but also, to do not transfer any parasitoids and/or predators previously co-surviving with the collected field aphids into the rearing plants. The growing chamber was equipped with an air ventilation system, supplementary electric power, a thermo-hygrometer apparatus, cages with 200 μm mesh diameter and abundant yellow aphid traps. Plants used as aphid hosts should be weekly renewed and a special care is to be taken in order to avoid different enemies infestations that frequently occur, such as thrips, mites, fungi, etc.

4.2.3. Transmission experiments

The methodology of aphid inoculation was partly based on the protocol described by Roistacher (2006). One leaf fully infested with aphids, allowed to acquire the virus from new flushes of the selected donor plants during 24 to 48 h acquisition access period (AAP), was divided into small pieces containing 5-10 individuals. Afterward, virus exposed aphids were transferred by the same way to a number of healthy receptor plants for 48h of inoculation access period (20-30 replicates/isolate/vector species). After 24 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 50 to 60 days of post-inoculation as pointed out by Velazquez-Monreal *et al*. (2009).

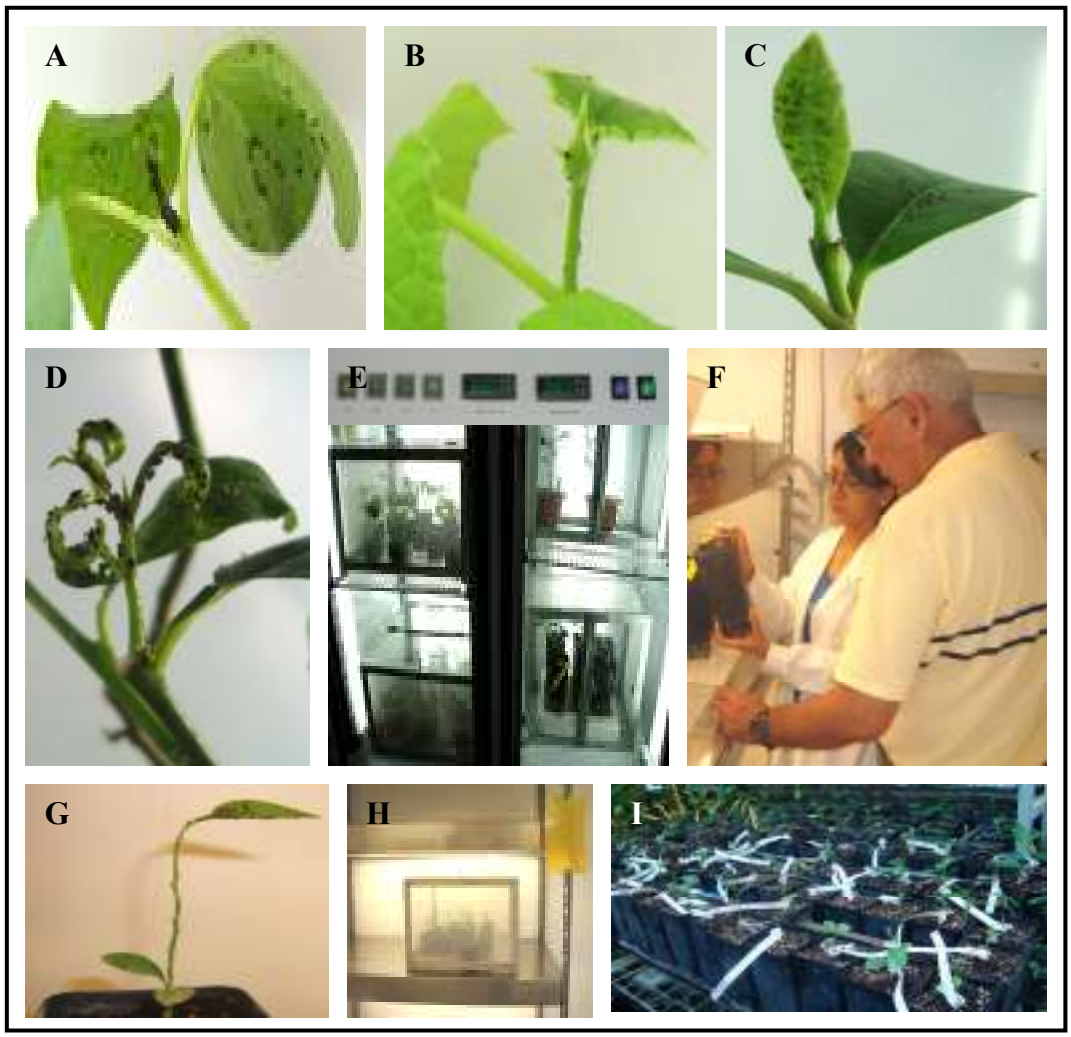


Fig 7. CTV aphid vectors rearing and transmission experiments of Mediterranean isolates. (A) and (B): Rearing of *A. gossypii* on Cotton and Melon plants respectively; (C) and (D): Rearing of *A. spiraecola* and *T. aurantii* on Viburum and Madame Vinous sweet orange plants, respectively; (E): Development of aphid populations under controlled hydrothermal conditions; (F): Infestation of a CTV infected source plants with aphids; (G): Acquisition feeding of *A. gossypii* from a CTV donor plant; (H) and (I): Inoculation feeding of virus-exposed aphids on healthy test plantlets and their maintenance under greenhouse conditions, respectively.

Regarding the "receptor hosts", an average number of 25 virus-free 2-6 month-old Mexican lime plantlets were used for each transmission test by isolate and by vector species. Nevertheless, in some cases, further experiments were

carried out by adding a number of sweet orange and/or grapefruit seedlings. Vector-exposed plants are designated by the suffix ($T\_{A.g}$; $T\_{A.s}$ and $T\_{T.a}$) corresponding respectively to *A. gossypii*, *A. spiraecola* and *T. aurantii* aphid species used, in addition to the isolate code.

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

4.2.4.1. Serological detection and discrimination of CTV isolates after aphid passage

Soon after the appearance of new flushes (approximately 2 months), aphid inoculated plants were visually monitored for the appearance of typical CTV symptoms and subjected to Direct Tissue Blot Immuno-Assay tests (Djelouah and D'Onghia, 2001b) aiming to confirm the virus transmission. Tissue blot assays were repeated regularly each a couple of weeks; and were carried out using Plantprint kit (Spain) applied to plant stems and leaf petioles, as follow:

(i) Membrane printing: Fresh clean cuts were made in each indicator with a sharp and sterile razor and the sections were pressed carefully on the nitrocellulose membrane and left to dry for few minutes. **(ii) Membrane blocking:** The printed membranes were blocked in 1% bovine serum albumin (BSA) solution, and incubated for 2h at room temperature or overnight at 4°C with a slight agitation. Then, it was washed three times with PBS Tween (Annex 6) for 3 min each. **(iii) Addition of monoclonal antibodies (MAbs):** linked to Alkaline Phosphatase (AP). After discarding the albumin solution, the blocked membrane was incubated for 2-3 h with 3DF1+3CA5 mixture of monoclonal antibodies conjugated with Alkaline Phosphatase (Plantprint), then washed. **(iv) Membrane development and reading:** The membranes were incubated in the substrate buffer by BCIP-NBT (Sigma fast tablets). Results were evaluated by microscopic observation (10X to 20X) for detection of the purple-violet

precipitation inside the sieve tubes indicating presence of infections. Transmission efficiencies were calculated and expressed as a percentage of the total number of plants inoculated with viruliferous aphids.

Aiming to evaluate the aggressiveness of the studied 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*., (1990); ELISA plates were coated with polyclonal antibodies Pabs (Agritest, Italy) diluted (1:250) in coating buffer (Annex 6) and incubated for 2h at 37°C. After washing 3X3 min the plates with PBS-Tween washing buffer (Annex 6). Fresh leaf samples taken either from the source inoculum or the sub-isolates, were grinded in extraction buffer (Annex 6) and added to the plates; two wells were filled with 100 µl each, in addition to a negative and a positive controls. After an overnight incubation at 4°C and washing, 100 µl of single MAb diluted in conjugate buffer (1:2500) was distributed by rows and incubated for 2h at 37°C. After washing, 100 µl of anti-mouse diluted in conjugate buffer (1:1000) were added and incubated again for 2 h at RT, then washed. Development of the results was done by adding 100 µl of *P*-nitrophenyl phosphate prepared as 1 mg/ml of substrate buffer (Annex 6). The absorbance reading of the plates was done up to 2h at RT in a conventional ELISA plate reader at 405 nm.

4.2.4.2. Molecular detection and 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 vector inoculated plant tissues, cDNA first strand synthesis and reverse transcription were implemented following Foissac *et al*., (2001) instructions, and PCR reactions were carried out with universal CTV-CP forward and reverse primers yielding 672 bp length amplicons as mentioned before (4.1.1.2.).

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

- **Conventional SSCP trials**

For the purpose of revealing eventual nucleotide mutations after the aphid passage, a serial of RT-PCR amplifications were performed, focusing on four genomic regions of CTV including the less variable p25, p18, p20, and p23. cDNA templates (5 µl) have been amplified in a final volume of 25 µl of reaction vessel containing 1x Taq polymerase buffer (Fermentas, UK), 1 µM $MgCl_2$, 0,5 mM dNTPs, 0,2 µl of Taq polymerase (5U/ µl) and 0,2 µM of each sense and anti-sense primers. Other than universal T36CP primers reported before (Hilf and Garnsey, 2000), amplifications conditions respective to both of p18 and p20 (Sambade *et al*., 2002; 2007) pairs of primers (Table 5) consisted on 7 min first denaturation at 95°C; followed by 35 cycles of 95°C denaturation 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 elongation prior to maintenance at 4°C.

Table 5. Sequence oligonucleotide primers used for the amplification of the CTV genes encompassing p18; p20; p23 and p25 proteins.

Primer names	Polarity	Oligonucleotide sequence (5'~ 3')	Binding site	Size
P25	+ -	ATGGACGACGAAACAAAGAAATTG TCAACGTGTGTTGAATTTCCCA	16,152-16,176 16,802–16,823	672
P18	+ -	TTCTATCGGGATGGTGGAGT GACGAGATTATTACAACGG	16811-16830 17217-17235	425
P20	+ -	CGAGCTTACTTTAGTGTTA TAATGTCAAACTGACCGC	17767–17785 18269–18286	520
P23	+ -	ACTAACTTTAATTCGAACA AACTTATTCCGTCCACTTC	18347–18365 19026–19044	698

*Binding site of the primers is given on the positive strand of gRNA of isolates T36 (U16304).

Whereas, amplification of primers specific to p23 gene, located on the sequence of CTV isolate T36 (U16304), were proceeded with the following conditions: 2 min at 94°C; then 40 cycles of 15 s at 94°C, 15 s at 55°C and 30s at 72°C; followed by 2 min final extension at 72°C (Sambade *et al*., 2003). Resulted amplicons were checked by electrophoresis on a 1% agarose gel.

SSCP analysis was performed by denaturing dsDNA flunking the different amplified genomic fragments in the presence of formamide and fractioning both DNA strands by electrophoresis in a 10% non-denaturing polyacrylamide mini-gel (Annex 2). Under the appropriate conditions previously described, small changes in the nucleotide sequence may alter conformation of ssDNA and, consequently, its electrophoretic mobility. Resulted patterns were then compared with that derived from their correspondent inoculum sources.

- **Capillary Array Electrophoresis Single Strand Conformation Polymorphism (CAE-SSCP) trials:**

CAE-SSCP is an evolutionary screening method for the detection of unknown and previously identified mutations based on the principle of sequence specific mobility of single-stranded DNA in a native polymer. As described by Larsen *et al*., (2007), successful PCR amplicons have been firstly diluted (1:30) using sterile water. In each well of the plate, samples denaturation was performed by adding 10 µl of deionized formamide and 0,25 µl of marker (GeneScan ROX standard size) to a volume of 1 µl of diluted DNA, then, mix have been heated for 5 min at 95°C and transferred immediately on ice. Prior to be loaded onto the Genetic Analyser instrument, each plate were supplied with a previously analysed control (a known wild-type sample), diluted to the same extend as the samples to run in parallel. CAE-SSCP have been performed on a previously calibrated ABI PRISM 3100 Genetic Analyser programmed to run in non-denaturing conditions at both 18°C and 30°C for higher sensitivity, using the GeneScan polymer and the genetic analyser buffer with EDTA (Applies Biosystems, Japan). During the

capillary electrophoresis, ssDNA fragments had different migration patterns according to their sequence specific secondary structures as in classical SSCP. Afterward, sample patterns were automatically analysed at both temperatures, aligned with relation to established migration standards (red peaks) and compared to the known wild-type sample data. Subsequently, mutations corresponded to differences of the peak signals detected from the analysed samples compared to the control profile.

Standard Protocol for CE-SSCP analysis have been optimized for the analysis of CTV are reported by the table (6) follow:

Table 6. Optimal conditions for adopted for CTV CE-SSCP trials

Standard parameters	Optimized conditions
Oven Temperature	24 °C
Poly Fill Vol	4840 steps
Current Stability	5.0 µAmps
Pre Run Voltage	15.0 kVolts
Pre Run Time	180 sec.
Injection Voltage	3.5 kVolts
Injection Time	12 sec.
Voltage Number of steps	10 nk
Voltage Step Interval	60 sec.
Data Delay Time	1 sec.
Run Voltage	15.0 kVolts
Run Time	1600 sec.

4.2.4.2.2. Multiple Molecular Markers analysis of the CTV vector inoculated sub-isolates

The same cDNA preparations were subjected to MMM analysis as described before (4.1.1.2), using only of the most specific markers of each genotype group including T36POL, T30POL, VTPOL and T3K17, as documented by Hilf *et al*. (2005).

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

Based on the SSCP analysis, amplicons displaying diversified patterns depicted from the p25 and p23 genomic regions were considered different genomic variants (haplotypes). Once purified, PCR targets were legated into the vector *P*-GEM-T overnight at 4°C, and used to transform *Escherichia coli* cells following the manufacturer's instructions (TA cloning kit; Invitrogen, Carlsbad, CA), previously mentioned (4.1.1.3). *rec*DNA targets were subjected to additional PCR reactions with the correspondent primers used initially, then, a number of 20-30 clones were separated by SSCP analysis. Selected plasmids were purified and digested with *Eco* RI (Promega Corp., Madison, WI) to check for appropriate inserts prior to be sequenced in both directions using an ABI 3730 xl DNA Analyzer (Applied Biosystems Inc., Foster City, CA).). Resulting consensus sequences were compared with their correspondent source isolates, as well as other CTV references SY568, T30, T36, VT, T385, and NUagA using BLAST analyses (available online from the National Centre for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Chapter 5. Results and discussions

5.1. Molecular characterization of Mediterranean CTV isolates

5.1.1. Assessment of the genetic diversity of CTV by Multiple Molecular Markers

Following the synthesis of 672 bp amplicons referred to the CTV-CPg, cDNA templates from the different CTV isolates have been subjected to PCR re-amplifications using a subset of linked sequence specific markers. Obtained results have almost been compared to the standard genotype profiles yielded by each of the reference CTV isolates (Table 7).

Table 7. Marker patterns of standard Citrus tristeza virus genotypes (Hilf *et al*., 2005)

Origin	Strain	CP	T36-POL	T36-K17	T36-5'	T30-POL	T30-K17	T30-5'	VT-POL	VT-K17	VT-5'	T3-K17
Florida	T36	1[a]	1	1	1	0	0	0	0	0	0	0
Florida	T30	1	0	0	0	1	1	1	0	1	1	0
Florida	T3	1	0	0	0	0	0	0	1	0	1	1
Israel	VT	1	0	0	0	0	1	0	1	1	1	0

[a] 1: Positive amplification signal; 0: lack of amplification

As a result, a specific "genotype profile" has been attributed for each of the 75 CTV isolates according to its correspondent amplification signals observed (Table 8). This finding ascertained the occurrence of field isolates harbouring multiple genomic variants that can be turned back to the impact of the graft/aphid co-infection processes in generating/separating some of which, resulting in a high genetic diversity of the CTV sequence composition.

Table 8. Genotype assignment of Mediterranean CTV isolates based on MMM analysis

Origin	Code	CP	T36-POL	T36-K17	T36-5'	T30-POL	T30-K17	T30-5'	VT-POL	VT-K17	VT-5'	T3-K17	Genotype
Albania	MAIB_Q3	1	1	1	1	0	0	0	0	0	0	0	T36
	MAIB_Q5	1	0	0	1	0	0	0	0	0	0	0	T36
	MAIB_Q90	1	1	1	1	0	0	0	0	0	0	0	T36
	MAIB_Q91	1	1	1	1	0	0	0	0	0	0	0	T36
	MAIB_Q92	1	1	1	1	0	0	0	0	0	0	0	T36
	MAIB_Q93	1	1	0	1	0	0	0	0	0	0	0	T36
	MAIB_Q94	1	1	0	1	0	0	0	0	0	0	1	T36+T3
	MAIB_Q95	1	1	0	1	0	0	0	0	0	0	1	T36
	MAIB_Q132	1	1	1	1	0	0	0	0	0	0	1	T36+T3
	MAIB_Q133	1	1	1	1	0	0	0	0	0	0	1	T36+T3
Montenegro	MAIB_Q106	1	0	0	1	1	0	1	1	0	0	0	T36+VT+T30
	MAIB_Q107	1	1	0	0	1	1	1	0	0	0	0	T36+T30
	MAIB_Q108	1	1	0	0	1	1	1	0	0	1	1	T3+T36+T30
	MAIB_Q109	1	0	0	1	1	0	0	1	0	0	0	T36+VT+T30
Trinidad	MAIB_Q30	1	0	0	1	1	0	0	0	1	0	0	T36+T30
	MAIB_Q31	1	0	0	1	1	1	0	0	0	1	0	T3+T30
Palestine	MAIB_Q40	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q44b	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q48	1	0	0	0	0	0	0	1	1	0	0	VT
	MAIB_Q49	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q51	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q52	1	0	0	0	0	0	0	0	1	1	0	VT
	MAIB_Q53	1	0	0	0	0	0	0	0	1	1	0	VT
	MAIB_Q54	1	0	0	0	0	0	0	0	1	0	0	VT

Origin	Code	CP	T36-POL	T36-K17	T36-5'	T30-POL	T30-K17	T30-5'	VT-POL	VT-K17	VT-5'	T3-K17	Genotype
Lebanon	MAIB_Q4b	1	0	0	0	0	1	0	1	1	1	0	VT
	MAIB_Q6	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q7b	1	0	0	0	0	1	0	1	1	1	0	VT
	MAIB_Q12b	1	0	0	0	0	0	0	0	1	1	0	VT
	MAIB_Q125	1	0	0	0	0	0	0	0	1	1	0	VT
	MAIB_Q126	1	0	0	0	0	0	0	1	1	1	0	T3+VT
	MAIB_Q127	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q128	1	0	0	0	0	0	0	1	1	1	0	VT
	MAIB_Q130	1	0	0	0	0	0	1	1	0	0	0	VT+T30
	MAIB_Q131	1	0	0	0	0	0	0	1	1	1	0	VT
Egypt	MAIB_Q57	1	0	0	0	0	0	0	1	0	0	1	T3+VT
	MAIB_Q61	1	1	0	0	1	1	0	1	0	0	0	T36+VT+T30
	MAIB_Q81	1	0	0	0	0	1	0	1	1	0	1	T3+VT
	MAIB_Q82	1	0	0	0	1	0	1	1	1	0	1	T3+VT+T30
Algeria	MAIB_Q76	1	0	0	0	1	1	0	0	1	1	0	T30
	MAIB_Q79	1	0	0	0	1	0	1	0	0	0	0	T30
Italy	MAIB_Q21	1	1	0	0	0	1	1	1	1	1	0	VT+T30
	MAIB_Q32	1	0	0	0	0	1	0	1	1	1	1	T3+VT
	MAIB_Q37	1	0	1	0	0	0	0	0	0	0	1	T3 +T36
	MAIB_Q102	1	0	0	0	1	1	1	1	0	1	1	T3+T30
	MAIB_Q103	1	0	0	0	1	1	1	0	1	1	0	T30
	MAIB_Q110	1	0	0	0	1	1	1	0	1	1	1	T3+T30
	MAIB_Q111	1	0	0	0	1	1	1	1	1	1	0	VT+T30
	MAIB_Q112	1	0	0	0	1	1	1	1	1	1	0	VT+T30
	MAIB_Q113	1	0	0	0	1	1	1	0	1	0	0	T30
	MAIB_Q114	1	0	0	0	1	1	0	1	1	0	1	T3+T30

Origin	Code	CP	T36-POL	T36-K17	T36-5'	T30-POL	T30-K17	T30-5'	VT-POL	VT-K17	VT-5'	T3-K17	Genotype
Italy	MAIB_Q116	1	0	0	0	1	0	1	1	1	1	1	T3+VT+T30
	MAIB_Q117	1	0	0	0	1	1	1	0	1	1	1	T3+T30
	MAIB_Q118	1	0	0	0	1	1	1	0	0	0	0	T30
	MAIB_Q119	1	0	0	0	1	1	1	0	1	1	1	T3+T30
	MAIB_Q120	1	0	0	0	1	1	1	1	1	1	1	T3+VT+T30
	MAIB_Q122	1	0	0	0	1	1	1	1	1	1	0	VT+T30
	MAIB_Q123	1	1	0	0	1	1	0	1	0	0	0	T36+VT+T30
	MAIB_Q124	1	0	0	0	1	1	0	1	1	0	1	T3+VT+T30
	MAIB_Q134	1	0	0	0	1	1	1	1	1	1	0	VT+T30
	MAIB_Q135	1	0	0	0	1	1	1	1	1	1	0	VT+T30
	MAIB_Q142	1	0	0	0	1	0	0	1	0	1	1	T3+T30
	MAIB_Q143	1	0	0	0	1	0	1	1	0	1	1	T3+T30
	MAIB_Q144	1	1	0	0	1	0	1	1	0	1	1	T3+VT+T30
	MAIB_Q1294	1	0	0	0	1	0	1	0	0	0	0	T30
	Sg29	1	0	0	0	1	0	0	1	0	0	1	T3+VT+T30
China	MAIB_Q25	1	0	0	0	0	1	0	1	1	1	1	T3+VT
	MAIB_Q26	1	0	0	0	0	0	0	1	1	1	1	T3+VT
	MAIB_Q27	1	0	0	0	0	0	0	1	1	1	1	T3+VT
Croatia	MAIB_Q96	1	0	0	0	0	1	1	1	0	1	1	T3+VT+T30
Cyprus	MAIB_Q73	1	0	0	0	1	0	0	0	0	0	0	T30
Portugal	CTV2	1	0	0	0	0	0	0	0	0	0	0	Unknown
Iran	MAIB_Q97	1	0	0	0	1	1	0	1	1	1	0	VT+T30
	MAIB_Q98	1	1	0	0	0	0	1	0	0	0	0	T36+T30
Morocco	MAIB_Q74	1	0	0	0	1	1	0	1	0	1	1	T3+T30
	MAIB_Q75	1	0	0	0	1	0	0	1	1	1	0	VT+T30
Syria	MAIB_Q505	1	0	0	0	0	0	0	1	1	1	0	VT

[a] 1: Positive amplification signal; 0: lack of amplification

As reported from the table above, CTV isolates showing genotype patterns identical to one single standard marker profile represented one third (*ca*. 40%) of the global virus collection. While, over than 60% of the accessions were identified as mixed infections yielding two (43,5%) or three (17,5%) genotypes. From this standpoint, the concept of CTV as a population of genetic variants like all of the RNA viruses previously described by Domingo and Holland (1994) has been evidenced.

Analysis of the genotypes obtained showed that the incidence of the "VT, T36 and T30" single genotypes were estimated to 22%, 10% and 8% respectively, in the absence of pure T3 isolates, as described by the table below (Table 9).

Table 9. Summary of isolate genotypes determined by multiple molecular marker analysis

Origin	Albania	Algeria	China	Croatia	Cyprus	Egypt	Italy	Iran	Trinidad	Montenegro	Morocco	Palestine	Lebanon	Syria	Portugal	Total
N° isolates	10	2	3	1	1	4	24	2	2	4	2	8	10	1	1	74
T36	7															7
VT												8	8	1		17
T30		2			1		3									6
T3+T36	3						1									4
T36+T30								1	1	1						3
T3+VT			2			2	1						1			6
VT+T30							6	1			1		1			9
T3+T30							7		1		1					9
T36+VT+T30						1				2						3
T36+VT+T3							1									1
T3+VT+T30			1	1		1	5									10
T3+T36+T30										1						1
UNKNOWN															1	1

Furthermore, the most common double genotype mixtures were "VT+T30" and "T3+T30" with equivalently 9 occurrences, as well, "T3+VT+T30" was the most

frequent multiple genotype profile with 7 occurrences. Interestingly, despite that VT and T30 were the most stable components over the Mediterranean area, since incidence of "VT, T30 and VT+T30" genotypes represented ca. 42% of the whole CTV collection, the incidence of genotype profiles arisen from the studied CTV isolates reflected some regional diversity of CTV genotypes between and within the Mediterranean countries.

Thus, the T36 decline-inducing genotype was typically reported from all of the isolates originating from Albania, Montenegro and Trinidad regions. A number of 7 out of 10 Albanian isolates reacted perfectly with the T36-5', T36K17 and T36POL markers which are specific to the T36 standard isolate, and were considered as pure T36 genotypes, while the remaining isolates from the same group reacted also with T3K17 molecular marker (Table 9). Conversely, combined patterns of T36 and T30 specific markers were exclusively reported from Montenegro and Trinidad accessions. Then, MMM analysis showed that T30POL marker reacted positively with most of the Italian studied isolates, (22 out of 24 accessions), indicating the wide occurrence of the T30–like genotype in the area. Furthermore, two accessions originating from Algeria (MAIB_Q76 and MAIB_Q79) and 3 from Italy (MAIB_Q103, MAIB_Q113 and MAIB_Q118) that shared identical marker profile to the T30 mild strain from Florida, were considered as pure "T30 genotypes" (Tab.) even in the lack of some non specific amplifications, such as T30K17, VT-5' and VTK17.

Similarly, VTPOL molecular marker has been readily amplified from almost all of the isolates from the Middle East area, suggesting the high spread of the severe VT genotype strain in that region. Thus, the 8 Palestinian accessions, the 8 Lebanese isolates as well as the 1 Syrian isolate have been assigned typical "VT" genotype profiles.

5.1.2. Partial genome sequencing and clustering patterns of CTV-CPg

The basis of the current classification is mainly the comparative analysis of the MMM typing and the CPg sequencing, since many authors supported the existence of a consistent relationship between virus phenotype and coat protein gene sequences (Pappu *et al*., 1993; Rubio *et al*., 2001; Zemzami *et al*., 2002; Nolasco *et al*. 2009). Hence, RT-PCR templates yielding 672 bp and flanking the CTV-CPg fragment, originating from many countries, such as Albania, Algeria, Palestine, Lebanon, Montenegro, Syria sharing single genotype profiles identical to each of the standard strains (T36, T30, VT and T3), as well as accessions reacting with more molecular markers were subjected to classical SSCP trials prior to be cloned. Afterward, *rec*DNA from the resulting clones were re-amplified with CPg universal primers and analysed by SSCP. Almost, one SSCP pattern was the most predominant from each sample and only representative haplotypes have been selected to be sequenced .

CPg sequencing of the MAIB_Q76 isolate from Algeria and MAIB_Q1294 from Italy, representatives of the T30 genotype profile, confirmed the results obtained by MMM, since they shared the group-type of mild strains and showed respectively 92% and 99% as highest nucleotide homology with both of T30 and T385 reference isolates.

However, highest nucleotide similarity of 98%-99% with both of T36 and QAHA decline-inducing strains has been revealed from four Albanian accessions MAIB_Q3, MAIB_Q93, MAIB_Q90, and MAIB_Q5 despite that the later reacted also with T3K17 molecular marker. Meanwhile, other two isolates, MAIB_Q106 from Montenegro and MAIB_Q96 from Croatia, showed 97% of nucleotide similarity between them, but clustered closed to this group showing only 94-95% of nucleotide identity with both of T36 and QAHA strains as well as all of the Albanian isolates.

Regarding the VT type-group, CPg sequencing from all of the Palestinian isolates in addition to one isolate from Lebanon and one other from Syria that

were assigned a "VT genotype" patterns by MMM typing, confirmed their clustering closed to the VT reference strain (Israel) showing 98% of nucleotide similarity (Fig. 8), except of the MAIB_Q52 (Palestine) that revealed only 96% of inter and intra-group homology as highest value. Within the T3 cluster, an Egyptian isolate MAIB_Q57 which yielded the VT-POL and the T3K17 differential marker between VT and T3 genotypes, has been assigned a "T3" genotype profile which was confirmed again by CPg sequencing revealing 98% of sequence nucleotide identity with T3 (Florida) reference strain.

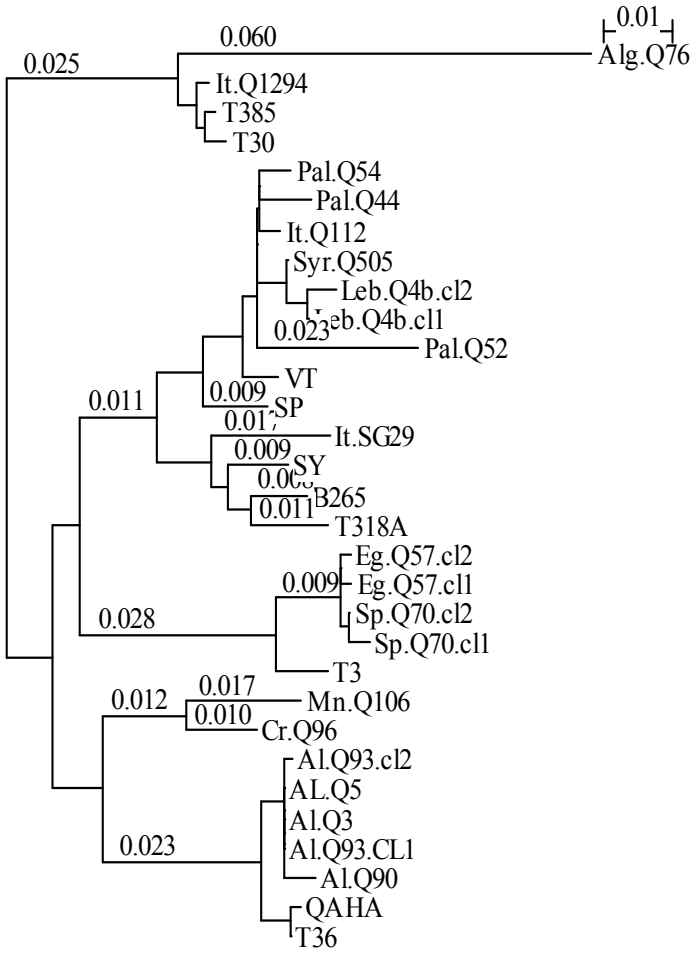


Fig 8. Dendrogram showing the clustering pattern of the CPg sequences from single infected Mediterranean CTV isolates and worldwide reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale.

Rather than accessions for which it was assigned single genotype profiles by MMM analysis, two isolates reflecting a recombination between two patterns have been studied. Among the Albanian isolates, four accessions yielded also positive amplification of the T3K17 marker (MAIB_Q5, MAIB_Q91, MAIB_Q94 and MAIB_Q132) suggesting mixed "T36+T3" genotypes. The isolate MAIB_Q5 has been subjected to p25 cloning and a number of 13 clones has been sequenced. As a result, the within-isolate nucleotide similarity between the different clones ranged from 96% to 99%. Compared to the standard strains, the whole clones illustrated 98% - 99% sequence identities with both of T36 and QAHA references; whereas, lower values which have been obtained with the others, such as T3 (91% -92%) although that it reacted positively with T3K17 marker noticing the presence of T3 variant within its 5' terminal genome sequence. This result suggests that the amplification from the T3K17 marker is not sufficient enough to assign a "T3 genetic profile" to an unknown isolate, taken into account that it has been reported maximum nucleotide sequence identities (95% – 98%) of this marker for B14, B211 (Republic of China), etc, by contrast to the T3 type isolate of this group (85% – 87%) as described by Hilf *et al*. (2005).

As illustrated in the Figure (9), MAIB_Q5 joined the group of Q8 (EU660917) and 59Mcl22 (EU579396) isolated from Albania, as well as the two Tunisian isolates K2cl3 (EU579406) and K4cl22 (EU579409). The latters, clustered in Group 1 unequivocally related to decline symptoms on sweet orange trees grafted on sour orange as described by Nolasco *et al*. (2008), and extrapolated from previously published data (Hilf *et al*., 2005).

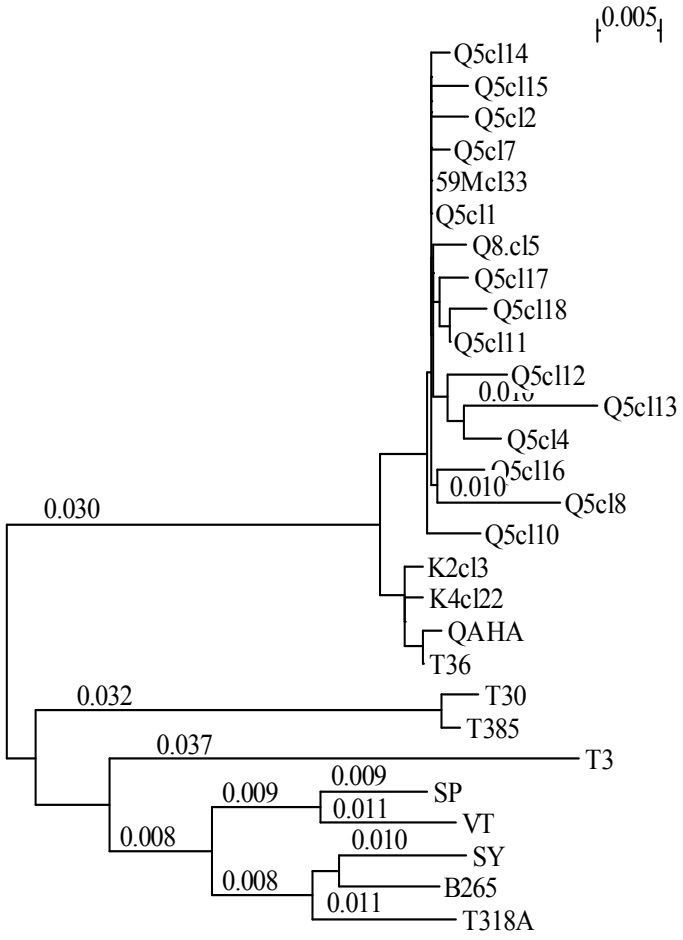


Fig 9. Phylogenetic relationships of the CPg sequence from MAIB_Q5 Albanian Citrus tristeza virus isolate and standard reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale.

Moreover, considering that a noticeable number of genotype combinations yielded "VT + T30" profile, CPg fragment from one Moroccan isolate (MAIB_Q75) have been *TA*-cloned and 7 haplotype clones have been sequenced. As a result, two divergent variants showing only 90% - 92% within-isolate sequence homology have been emerged (Fig. 10): (i) clones n° 2, 3, 4, 5 and 6 formed together a unique group revealing 98% - 99,5% of intra-group nucleotide similarities between them and comparable values (99%) with the

C257cl2 from Argentina (AY750757). While, clones n° 1 and 7 appeared to be very similar to each other, they clustered close to the 398cl2 from Morocco (EU579434) and showed maximum similarities (98% - 99%) with severe strains including CTV-0002 from China (AJ518841), AR126 from USA (DQ363389), SP (EU857538) from New Zealand, but also to 28C isolate (AF184118) from Portugal which yielded a "VT" genotype by MMM characterization as previously postulated by Nolasco *et al*.(2008).

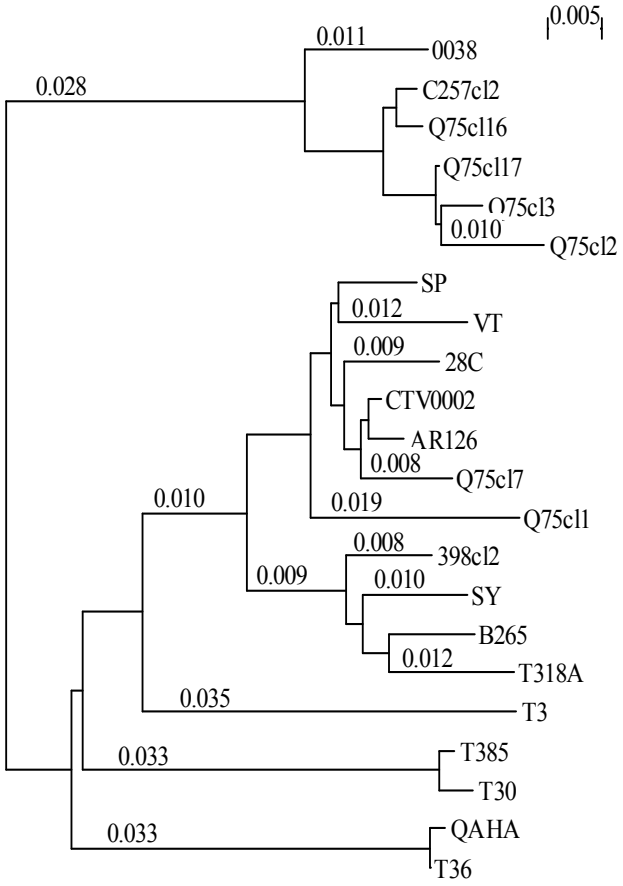


Fig 10. Phylogenetic relationships of the CPg sequence from the Moroccan (MAIB_Q75) Citrus tristeza virus isolate and other reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale.

Among the isolates harbouring a mixture of three different genotype elements by MMM typing, the following combinations: "T3+VT+T30" and "T36+VT+T30" are the most common. Therefore, CPg amplicons corresponding

to two isolates originating from Croatia (MAIB_Q96) and from South Italy (SG29) showing the first genotype pattern have been sequenced.

Regarding the Croatian isolate, all of the cloned haplotypes shared together the Croatian group designated as "unusual", showing highest intra-specific nucleotide similarity between each other, but also 99% of sequence identity with some other isolates already characterized, such as: 440cl6 (EU579416), 445cl6(EU579423), 42J (EU66092), 44J (EU660922) and 389cl3 (EU579433) from Morocco (Fig. 11).

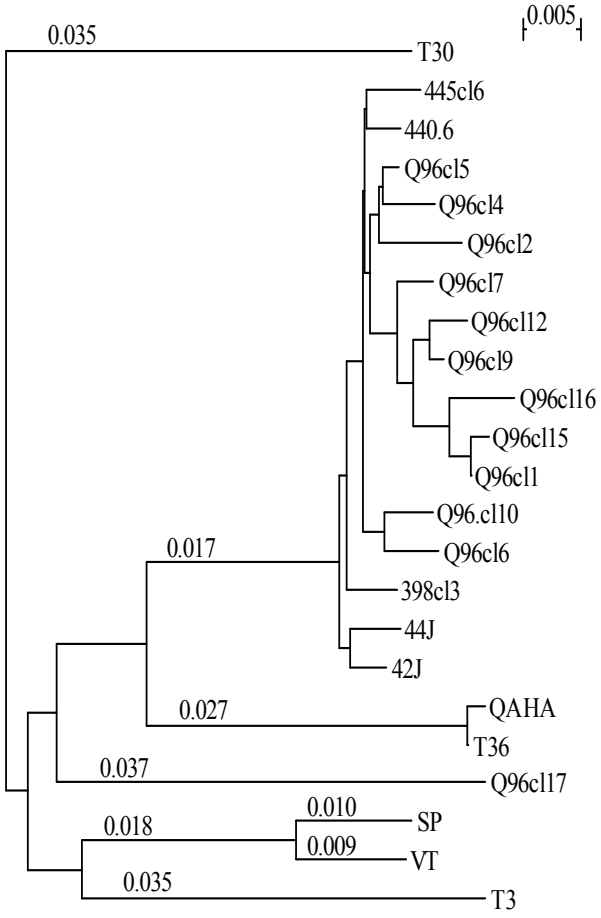


Fig 11. Phylogenetic relationships of the CPg sequence from Croatian (MAIB_Q96) Citrus tristeza virus isolate and standard reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale.

As mentioned by Nolasco and colleagues (2008), those Croatian accessions yielded both "VT+T30" and "VT+T3" genotypes by MMM using only POL specific markers; while in this work MAIB_Q96 cross-reacted also with T30-5', T30K17, VTPOL and T3K17 markers.

In addition, 5'UTR molecular characterization of the Sicilian SG29 isolate by MMM revealed also a recombinant virus infection having "VT+T30+T3" genotype profile, since T30POL, VTPOL and T3K17 specific markers have been successfully amplified from it. Further p25 gene sequencing showed a highest nucleotide similarity with SY568 strain (97,3%); B265 (96,7%) and T318 (96,5%) aggressive strains (Fig. 8). This funding evidenced the aggressiveness of the studied isolate, and also confirmed the marker analysis results which were in agreement with those previously reported by Hilf *et al*., (2005) indicating that SY568 was a mixed infection of "VT+T30" genotype; bearing in mind that "T3" virus strain is so far associated with seedling yellows symptoms (Yokomi, personal communication).

As for the group illustrating the triple genotype combination "T36+VT+T30", one representative isolate from Montenegro (MAIB_Q106) that yielded successfully each of T36-5', T30POL, T30-5' and VTPOL markers have been submitted to p25 sequencing. Consequently, 5 haplotype clones that were arisen from this isolate showed over than 97% as maximum nucleotide identity between each other and with the T3cl22 (EU660907) isolate from Montenegro, but only 95,2% similarity with T36 reference strain (Florida). However, higher sequence homology values have been obtained by comparison with 44J (EU660922), 446cl7 (EU579424) and 440cl4 (EU579415) from Croatia, reaching 97,9%; 98,2% and 99,1%, respectively (Fig. 12). The Croatian group-type of CTV showed mostly "T30+VT" genotype profiles when characterized by the standard specific markers restricted only to the POL region of the 5'UTR, while T36-5' marker appeared to be also relevant to the T36 genotype group.

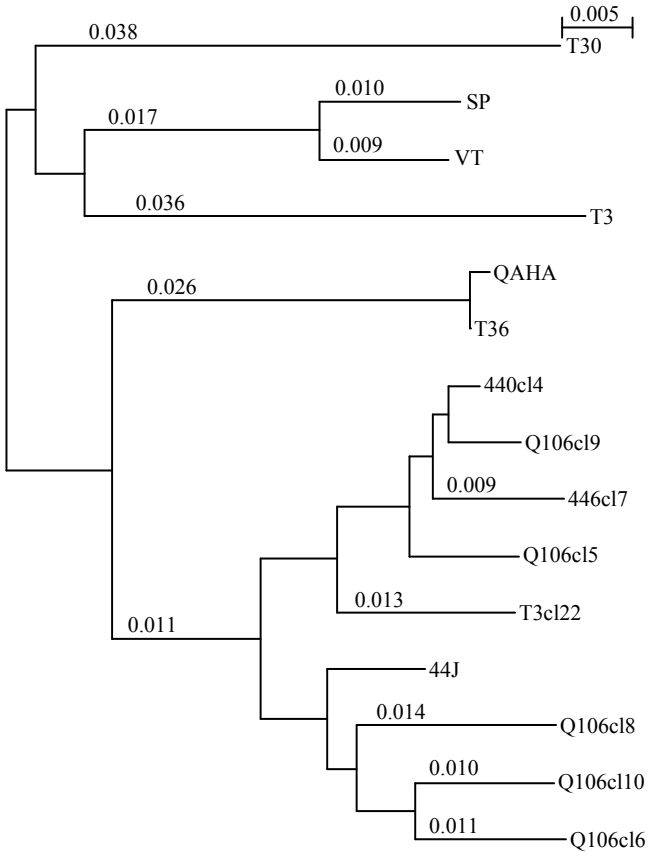


Fig 12. Phylogenetic relationships of the CPg sequence of Citrus tristeza virus isolate (MAIB_Q106) from Montenegro and standard reference strains. Numbers close to branches represent the phylogenetic distances. Horizontal bar represent nucleotide distance scale.

Summary*: Geo-phylogenetic relationship estimation of CTV in the Mediterranean Basin*

The occurrence of CTV infections geographically distant but genetically similar highlights the high genetic variability of the viral population structure from the Mediterranean area between and within countries. Moreover, it confirms the impact of the illegal exchange of infected plant propagation material in the disease spread over large distances. Information provided by MMM typing applied to a significant number of CTV accessions from different origins and completed with a phylogenetic relationship analysis of CTV-CPg sequences

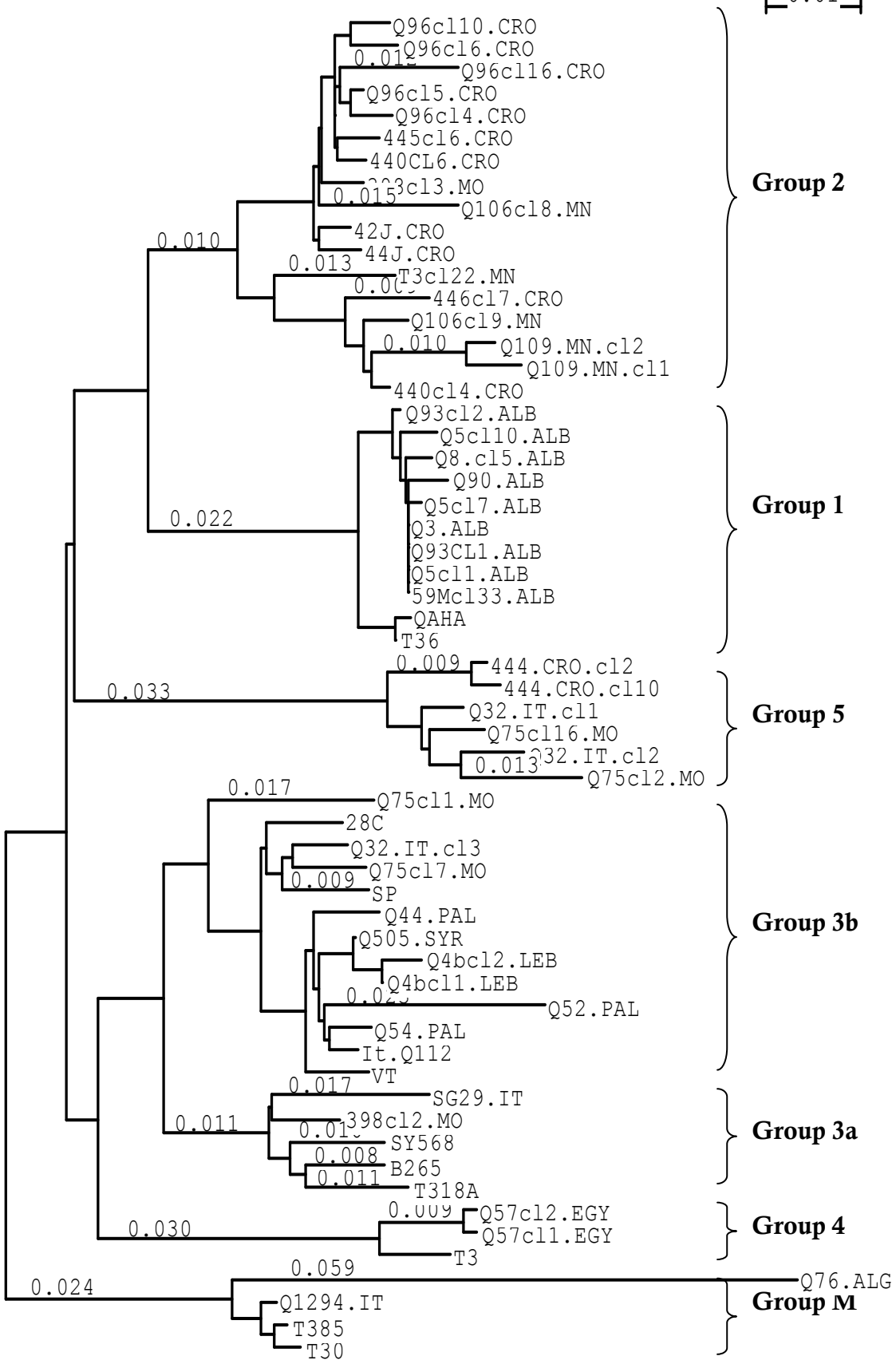


Fig 13. Neighbour-joining phyllogenetic tree of Mediterranean *Citrus Tristeza Virus* CPg population sequences analysis. Sequences are named after the CTV isolate followed by the clone number and isolate origin

showed a clear categorization of the CTV populations within the Mediterranean basin, and reflected the frequent occurrence of some relevant CTV variants. In the lack of enough data from the field, the "CTV-T30" variant appears to be a stable component within the Mediterranean virus population, occurring as pure and multiple infections clearly confirmed by the high nucleotide similarity revealed from the MAIB_Q1294 and MAIB_Q76 originated from Italy and Algeria, respectively.

However, highest occurrence of the "T36 genotype" have been predominantly depicted from Albania and Montenegro, even as single genotype profiles, but also as multiple combinations with other types. This funding suggests the predominance of the CTV-T36 population within the East Adriatic region as an endemic sequence variant. Being in accordance with previous investigations and by extrapolation with results obtained by asymmetric PCR-ELISA (APET) typing system developed by Nolasco *et al*., (2008), the current group harboring the T36CP gene haplotypes appears to be associated unequivocally to quick decline symptoms on sweet orange trees grafted on sour orange. Furthermore, unusual group-type delimited by isolates from Croatia and Montenegro displayed the occurrence of mixed CTV variants and a high genetic variability within the East Adriatic region, previously reported to be associated with a high pathogenic potential (Cerni *et al.*, 2009).

Meanwhile, VT genotype represented a major element on the Middle East region, even by MMM typing or by CPg sequencing, noticing the predominance of the "CTV-VT" as a relevant haplotype population in the current area. Concordant results were obtained by confrontation with APET system enabling their clustering within the Gr 3b associated with moderate quick decline and severe grapefruit stem pitting inducing strains. However, SG29 Italian isolate showing highest homology with SY568 reference clustered together in the Gr 3a, encompassing severe sweet orange stem pitting inducing haplotypes. Rather than VT-like isolates, wide distribution of other CTV genotypes of severe nature is not excluded from the Mediterranean area. From this standpoint, haplotype clones

emerging from Egypt (MAIB_Q57), Morocco (MAIB_Q75) and Italy (MAIB_Q32) were classified with the Gr 4. and 5 whereby isolates were formerly associated with severe forms of decline and sweet orange stem pitting symptoms. Moreover, SY haplotypes are also associated with other groups of biological activities, such as Gr 1, Gr 3a, Gr 3b and Gr 4 (Nolasco *et al*., 2008).

5.2. CTV experimental aphid transmission trials

5.2.1. Virus sources

The aphid transmission trials were conducted by the use of a number of ten isolates representatives of each Mediterranean CTV population category and originating from different countries, including Italy (MAIB_Q1294, MAIB_Q32 and SG29), Albania (MAIB_Q3), Palestine (MAIB_Q54), Croatia (MAIB_Q96), Egypt (MAIB_Q57), , Morocco (MAIB_Q75), Montenegro (MAIB_Q109) and Madeira Island (CTV2). The last has been kindly provided by Prof. Nolasco (Table 10).

Based on their molecular features, they showed a high genetic polymorphism related to their CPg sequences and associated to different pathogenic behaviours. Thus, rather than T30-like isolate from Italy (MAIB_Q1294) known to induce barely syndromes on the most common citrus species, other studied sources readily represented each of the T36 decline group of CTV (MAIB_Q3), the SY568 cluster (SG29), and the most severe sweet orange and/or grapefruit stem pitting inducing strains, such as the MAIB_Q54 and MAIB_Q57 representing the VT and T3 groups, respectively. Furthermore, the remaining isolates have been used as typical CTV populations from the different unclassified virus-types occurring in the East Adriatic and the South Mediterranean areas.

Table 10. Biological activities of the different tristeza CPg groups by APET analysis (Nolasco *et al*., 2008)

Isolate code	Origin	Scion	Rootstock	Associated biological activities
MAIB_Q3	Albania	Meyer lemon	Sour orange	Gr 1. T36 (Florida): a quick decline inducing strain (Hilf *et al*., 2005)
MAIB_Q96	Croatia	Satsuma	Sour orange	Gr 2. Mediterranean mild strain (Zemzami *et al*., 2002); Sw Or stem pitting (Niblett *et al*., 2000)
MAIB_Q109	Montenegro	Satsuma	Troyer Citrange	
M1294	Italy	Mexican lime (seedlings)		Gr M. T30 (Florida), 25-120 (Spain): a mild protective strain causing mild vein clearing but no stem pitting on Mexican lime
SG29	Italy	Mexican lime (seedlings)		Gr 3a. Madeira strain (8): severe sweet orange stem pitting (Nolasco *et al*., 2008)
MAIB_Q54	Palestine	Sweet orange	Rough lemon	Gr 3b. 28C: moderate quick decline and severe grapefruit stem pitting
MAIB_Q75	Morocco	Common Grapefruit	Sour orange	Gr 3b. 28C: moderate quick decline and severe grapefruit stem pitting; Gr 5. B249: severe sweet orange stem pitting and possibly grapefruit stem pitting
MAIB_Q32	Italy	Meyer lemon	Sour orange	
MAIB_Q57	Egypt	Common Lemon	Troyer Citrange	Gr 4. T3: seedling yellows and decline
CTV2	Portugal	Mexican lime		Gr 5. B 249: severe sweet orange stem pitting and possibly grapefruit stem pitting

Likewise, the CPg amplified from the selected virus sources have been submitted to SSCP analysis. Correspondent patterns showed that 8 isolates showed only one couple of cut-bands in 10% non-denaturing polyacrylamid gel (Fig. 14), suggesting that they area single infected. Whereas, multiple SSCP profiles resulted from both of MAIB_Q32 and MAIB_Q75 isolates which were considered as mixed CTV infections.

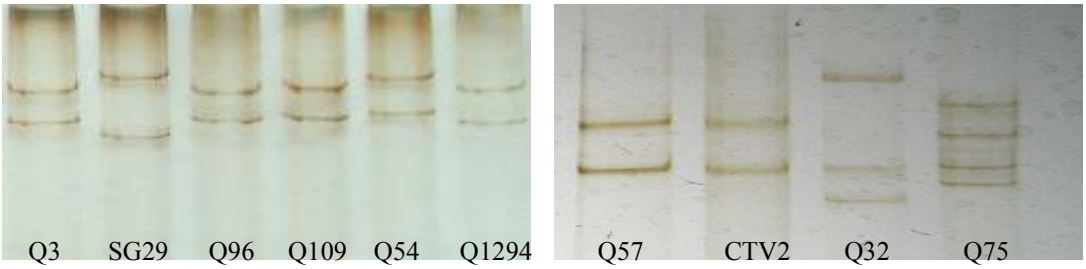


Fig 14. SSCP patterns of CPg sequences from selected Mediterranean CTV sources submitted to aphid transmission experiments in 10% polyacrylamide gel electrophoresis

Successful CTV graft-inoculated plants were detected by DTBIA tests, and an average number of three donor plants per each virus source, reacting positively to DTBIA tests and showing typical CTV syndromes of vein flecking, leaf cupping and stunting were selected for the aphid transmission trials. Therefore, infected plants were forced for actively developing tender shoots.

5.2.2.CTV vector transmission efficiencies

Aphid-inoculated plantlets have been submitted to preliminary screening by DTBIA, prior to be confirmed by conventional RT-PCR analysis. As reported in the table (Table 11), diversified virus transmission abilities have been denoted in association with the vector species and the virus isolates governing the aphid transmission experiments.

Table 11. Experimental vector transmission efficiencies of Mediterranean CTV isolates by *A. gossypii* and *A. spiraecola*

CTV isolate	Origin	*A. gossypii*		*A. spiraecola*	
		Infection	Transmission rate (%)	Infection	Transmission rate (%)
SG29	Italy	15 /29	51,7	1 /30	3,3
M1294	Italy	15 /30	50	1 /35	2,8
CTV2	Portugal	10 / 32	31,2	0/ 32	0
MAIB_Q54	Palestine	5 / 27	18,5	1 / 40	2,5
MAIB_Q57	Egypt	3 / 27	11	0 / 27	0
MAIB_Q96	Croatia	2 / 26	7,7	1 / 35	2,8
MAIB_Q109	Montenegro	4/25	16	2 /40	5
MAIB_Q3	Albania	0 / 50	0	0 / 50	0
MAIB_Q75	Morocco	0/40	Failed	0/30	Failed
MAIB_Q32	Italy	0/35	Failed	0/30	Failed

* Average of 5-10 aphids per test
* Infection: number of positive infected plants / the total number of replicates

❖ **CTV transmission by *A. gossypii:*** Using the cotton aphid, the most predominant CTV vector species in the Mediterranean area, a wide range of virus transmission values have been obtained. Thus, the Albanian isolate MAIB_Q3 that showed a high genetic homology with the T36 quick decline strain (Florida) has not been experimentally transmitted by *A. gossypii*, even after 6 months of aphid inoculation to 50 Mexican lime recipient plants. This result confirmed again

previous investigations describing the poor aphid transmissibility of this isolate even by the use of the most efficient vector *T. citricidus* (Dickson *et al*., 1956). Conversely, moderate to high transmission values have been successfully reported from the remaining CTV isolates. As shown in the table (Table 11), the highest transmission efficiency have been revealed from the Sicilian isolate SG29 reaching 52% value. Therefore, this result reflects evidently the potential impact of the combination *A. gossypii*-SY virus haplotype in the rapid spread of such a severe form of infection in the studied area. In spite that this funding has been recorded since the earliest studies using this vector in California (Martinez and Wallace, 1964; Calavan *et al*., 1980), it was perfectly reconfirmed in accordance with the recent investigations conducted by Velazquez-Monreal *et al*., (2009) showing 50% transmission efficiency of a seedling yellows isolate from California. Intensity of symptoms caused by the aphid transmitted sub-isolates on Mexican lime seedlings were generally similar, including some moderate to severe stunting, pronounced leaf cupping and very intense vein clearing (Fig. 15)

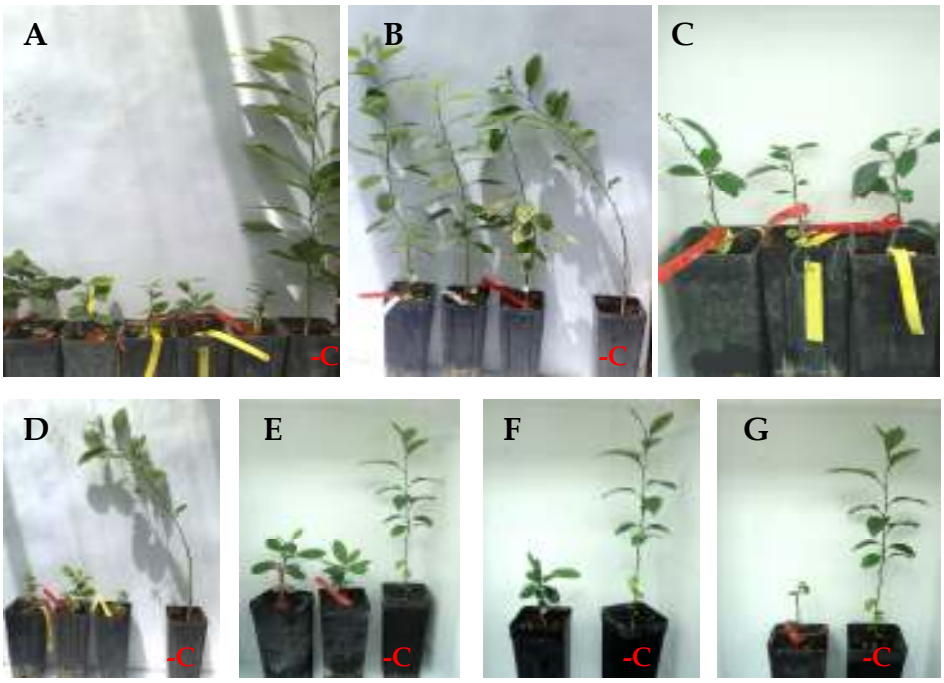


Fig 15. Symptoms of moderate to severe stunting of the aphid derived sub-isolates on Mexican lime. A, B and C, D, E, F and G: sub-isolates from SG29, MAIB_Q1294, MAIB_Q96, CTV2, MAIB_Q57 and MAIB_Q54, respectively; -C: Healthy control.

Interestingly, the most diffused Apulian isolate MAIB_Q1294 that clustered closely to the T30 mild CTV strain (Florida) was found to be easily transmissible by *A. gossypii* local biotype at 50% transmission rate. As well, aphid inoculated sub-isolates showed noticeable syndromes of discernable stunting and vein clearing on Mexican lime seedlings (Fig. 15).

Regarding the remaining isolates, average transmission rates ranged from 7,7% to 31% corresponding respectively to the Croatian (MAIB_Q96) and the Portuguese (CTV2) isolates as shown by the table (Table 11). Previously characterized as severe Mediterranean infections, transmission trials from those CTV isolates induced high mortality of the young tested plantlets and yielded fewer leaving infected sub-isolates which were accompanied by noticed stunting syndromes.

❖ **CTV transmission trials by *A. spiraecola* and *T. aurantii*:** Despite that the spirea and the black citrus aphids are more serious pests on Citrus species than *A. gossypii*, they are so far less efficient at vectoring CTV. Thus, comparative transmission tests of CTV isolates of different genotypes under the same conditions, but using both of *A. gossypii* and *A. spiraecola* vectors, showed the low efficiency of the latter in transmitting CTV infections.

As expected, negative results have been obtained from MAIB_Q3 isolate (Albania), but also from MAIB_Q57 and CTV2 as reported by the table above (Table 11). However, 2.5% to 5% transmission efficiencies have been obtained from the remaining accessions. Similar findings suggesting an overall transmission rate of 8% by the spirea aphid of known CTV isolates from Texas (Cutrer, 1998). Moreover, taken into consideration the scarcity of the black citrus aphid *T. aurantii* over more than 10 monitored groves during the last three years (Yahiaoui *et al*., 2009), transmission trials have been carried out to few isolates, including the T36-like isolate (MAIB_Q3), the T30-like isolate (MAIB_Q1294) and the SY severe isolate (SG29). No aphid transmission has been obtained from the Albanian and Sicilian isolates, while only 1 out of 52 (*ca*. 2%) replicates have been successfully transmitted from the Italian mild isolate (MAIB_Q1294),

confirming again the weak vector efficiency of *T. aurantii* in transmitting CTV isolates.

Regarding both of MAIB_Q32 and MAIB_Q75 isolates, first transmission experiments by *A. gossypii* using 20 Mexican lime plantlets/test were rejected due to the low virus titer on the donor plants used for aphid inoculation. Later in spring (June-September), similar tests have been carried out directly using the mother virus source and both of the spirea and the cotton aphids as vectors. Unfortunately, inefficient transmission values have been reported again even by adopting 15 replicates from each of Madame Vinous sweet orange, Duncan grapefruit and Mexican lime by test. So far, the most plausible explanation of this result may be that the aphid transmission cannot be optimized unless the vector population increases coincide with periods when tissue is most likely to have higher virus titer, as reported by Dodds *et al*., (1987). Furthermore, these results may support Roistacher and Bar-Joseph's hypothesis (1984) suggesting that virus source plants such as grapefruit or lemon cultivars are not almost appreciated by aphids and often result in low transmission rates.

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

5.2.3.1. Serological characterization of CTV sub-isolates after aphid passage

DASI-ELISA immunoassays were applied to the infected donor isolates and their correspondent aphid inoculated sub-isolates, the overall response of the studied CTV accessions against MCA13 MAb seems to remain unchanged after the aphid transmission (Table 12) indicating that no appreciable variation occurred on the epitopic conformation of CTV by aphid passage. The T36 decline isolate from Albania reacted perfectly against the CTV strain-discriminating MAb as expected and was used as positive control. As well, other genotype accessions, including VT, T3 and SY-like isolates exhibited positive immuno-reactions with MCA13.

Table 12. MCA 13 MAb serotyping of CTV isolates by aphid transmission

Origin	Isolate	MCA13 MAb
Italy	MAIB_Q1294 (DP)	-
	MAIB_Q1294.T$_{A.g}$ (1)	-
	MAIB_Q1294.T$_{A.g}$ (2)	-
	MAIB_Q1294.T$_{A.g}$ (3)	-
	MAIB_Q1294.T$_{A.g}$ (4)	-
	MAIB_Q1294.T$_{A.g}$ (5)	-
	MAIB_Q1294.T$_{A.g}$ (6)	-
	MAIB_Q1294.T$_{A.s}$ (1)	-
	MAIB_Q1294.T$_{T.a}$ (1)	-
	SG29 (DP)	+
	SG29.T$_{A.g}$ (1)	+
	SG29.T$_{A.g}$ (2)	+
	SG29.T$_{A.g}$ (3)	+
	SG29.T$_{A.g}$ (4)	+
	SG29.T$_{A.g}$ (5)	+
	SG29.T$_{A.g}$ (6)	+
	SG29.T$_{A.g}$ (7)	+
	SG29.T$_{A.g}$ (8)	+
	SG29.T$_{A.g}$ (9)	+
	SG29.T$_{A.g}$ (10)	+
	SG29.T$_{A.s}$ (1)	+
Croatia	MAIB_Q96 (DP)	+
	MAIB_Q96.T$_{A.g}$ (1)	+
	MAIB_Q96.T$_{A.g}$ (2)	+
	MAIB_Q96.T$_{A.s}$ (1)	+
Palestine	MAIB_Q54 (DP)	+
	MAIB_Q54.T$_{A.g}$ (1)	+
Egypt	MAIB_Q57 (DP)	+
	MAIB_Q57.T$_{A.g}$ (1)	+
Portugal	CTV2 (DP)	+
	CTV2.T$_{A.g}$ (1)	+
	CTV2.T$_{A.g}$ (2)	+
Albania	MAIB_Q3 (DP)	+

Whereas, the T30-type isolate from Italy (MAIB_Q1294) was not recognized by MCA13 MAb as well as its relative aphid generated sub-isolates. Consequently, this antibody can be a useful tool to indicate the potential aggressiveness of CTV which could not necessarily be a decline-inducing strain, as reported by Zemzami *et al.,* (2007). Being most widely applied to distinguish

among mild and severe isolates, it has been shown that reaction with MCA13 MAb depends on the presence of a phenylalanine residue at position 124 in the CP. Analysis of the sequence database shows that this residue is present in all groups except in the mild one, in which it is substituted by tyrosine (Pappù *et al*., 1993).

5.2.3.2. Multiple SSCP analysis of the CTV vector inoculated 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. Thus, an initial screening by conventional and capillary array SSCP, structure of the different dsDNA populations have been analysed having as a penalty to detect single nucleotide mutations of CTV after aphid passage.

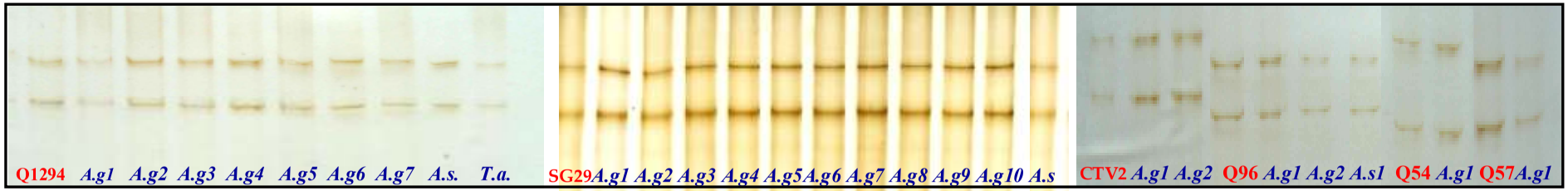
- **Nucleotide mutation screening by conventional SSCP analysis:** As illustrated by the CPg SSCP trials (Fig. 16: CTV-*p*25), similar patterns have been yielded from the RT-PCR amplicons of 672 bp length fragment amplified from the CPg of infected plants before and after the aphid transmission. The lack of new patterns within the p25 targeted fragment from the aphid derived sub-isolates evidenced that the major CPg viral populations remained highly conserved regardless to their transmission abilities by the different vector species. As well, haplotypes from sub-isolates generated by the different aphid species (MAIB_Q1294, SG29 and MAIB_Q96) showed that no differential variations have been associated with vector species.

Similar analysis performed on the p18 region showed detectable variations from some SG29 aphid transmitted sub-isolates, which was not the case

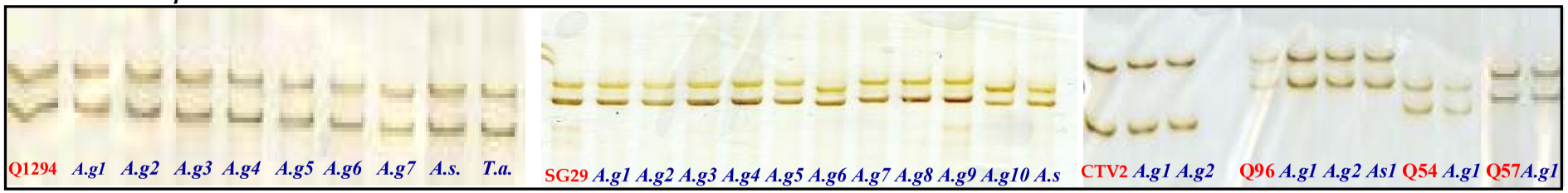
for the other studied isolates (Fig. 16: CTV-*p*18). Hence, rather than the predominant sequence profile herited from the parental virus source (frequency 7/11), new haplotypes have been arisen from the 6th replication (SG29_T$_{A.g.}$6), in addition to a second one that appeared from both of the sub-isolates SG29_T$_{A.g.}$10 and SG29_T $_{A.s.}$1 transmitted respectively by the cotton and the spirea aphids from the same isolate.

Nevertheless, cDNA sequences flunking *p*20 and *p*23 gene fragments shared identical SSCP profiles from all the isolates before and after their aphid transmission processes. This can be turned back for the fact that in single infections, recombination phenomena between virus variants seems to be less frequent with respect to that in mixed ones.

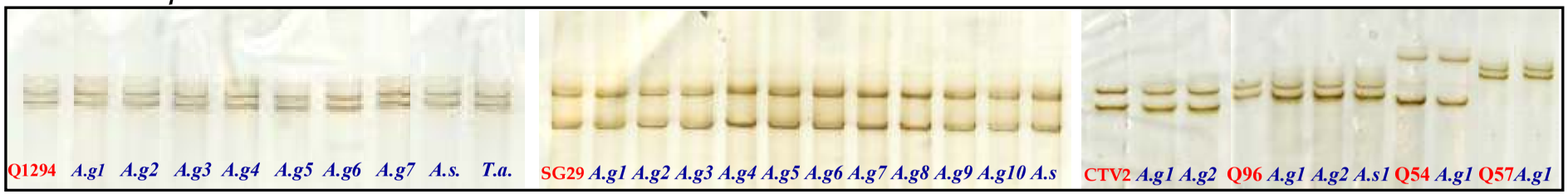
❖ CTV-*p*25



❖ CTV-*p*18



❖ CTV-*p*20



❖ CTV-*p*23

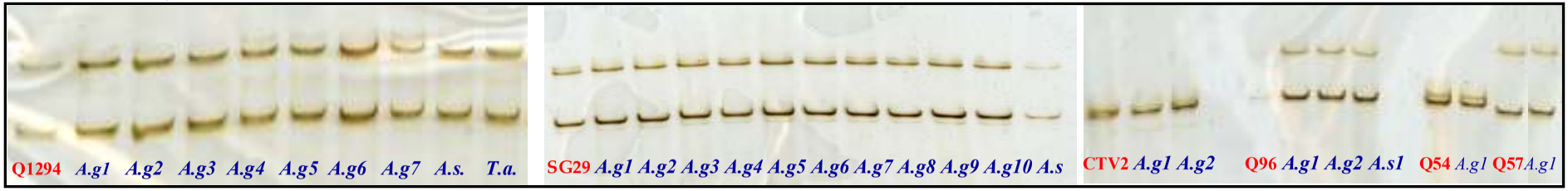


Fig 16. SSCP patterns of CTV-*p*25, *p*18, *p*20 and *p*23 sequences yielded from Mediterranean isolates and their aphid-transmitted sub-isolates.

Q1294, SG29, CTV2, Q96, Q57 and Q54: donor plants of isolates MAIB_Q1294, SG29, CTV2, MAIB_Q96, MAIB_Q57 and MAIB_Q54, respectively; *A.g, A. s* and *T.a*: *A. gossypii, A. spiraecola* and *T. aurantii* generated sub-isolates respectively.

- **Nucleotide mutation screening by CAE-SSCP analysis:** Since *p*18 is one of the most non-conserved regions within the CTV genome, more accurate CAE-SSCP tests have been applied to all of the studied samples. As a result, three different chromatograms have been exhibited from the analysed SG29 isolate and its sub-isolates (Fig. 17). By coincidence with haplotypes yielded by conventional SSCP, the parental panel was the most frequently transmitted, which was clearly separated from the SG29_$T_{A.g}$.6 sub-isolate one in one hand and the common one between SG29_$T_{A.g}$.10 and SG29_$T_{A.s}$.1 in the other hand. By contrast to the isolate MAIB_Q1294 (Fig. 18), minor nucleotide variations from the same genomic region occurred on the CTV2 following the transmission by *A. gossypii* have been readily detected by CAE-SSCP and resulted in new peak signals different from those yielded by the donor plant (Fig. 19). This can prove the reliability and the higher sensitivity of the CAE-SSCP technique by comparison to the classical one.

Generally, optimal sensitivity of SSCP trials can be reached when the fragment's sizes is of about 200 bp by the conventional way (Kahlon, 2005) and 400 bp when CAE is applied (Larsen *et al*., 2007). Thus, *p*23 amplicons of about 700 bp length have been hardly denaturated and repeated trials for the dsDNA discrimination have been obtained only when 15% polyacrylamide gel and 25°C temperature have been set up during the electrophoretic migration (Selma *et al*., 1999). From this standpoint, CAE-SSCP technology was shown to be an ideal supplement to solve problems of sequence denaturation and proved its convenience with the CTV-*p*23 sequence, even in the lack of emerged mutations in the isolates analysed in the present work (Fig. 20, 21 and 22).

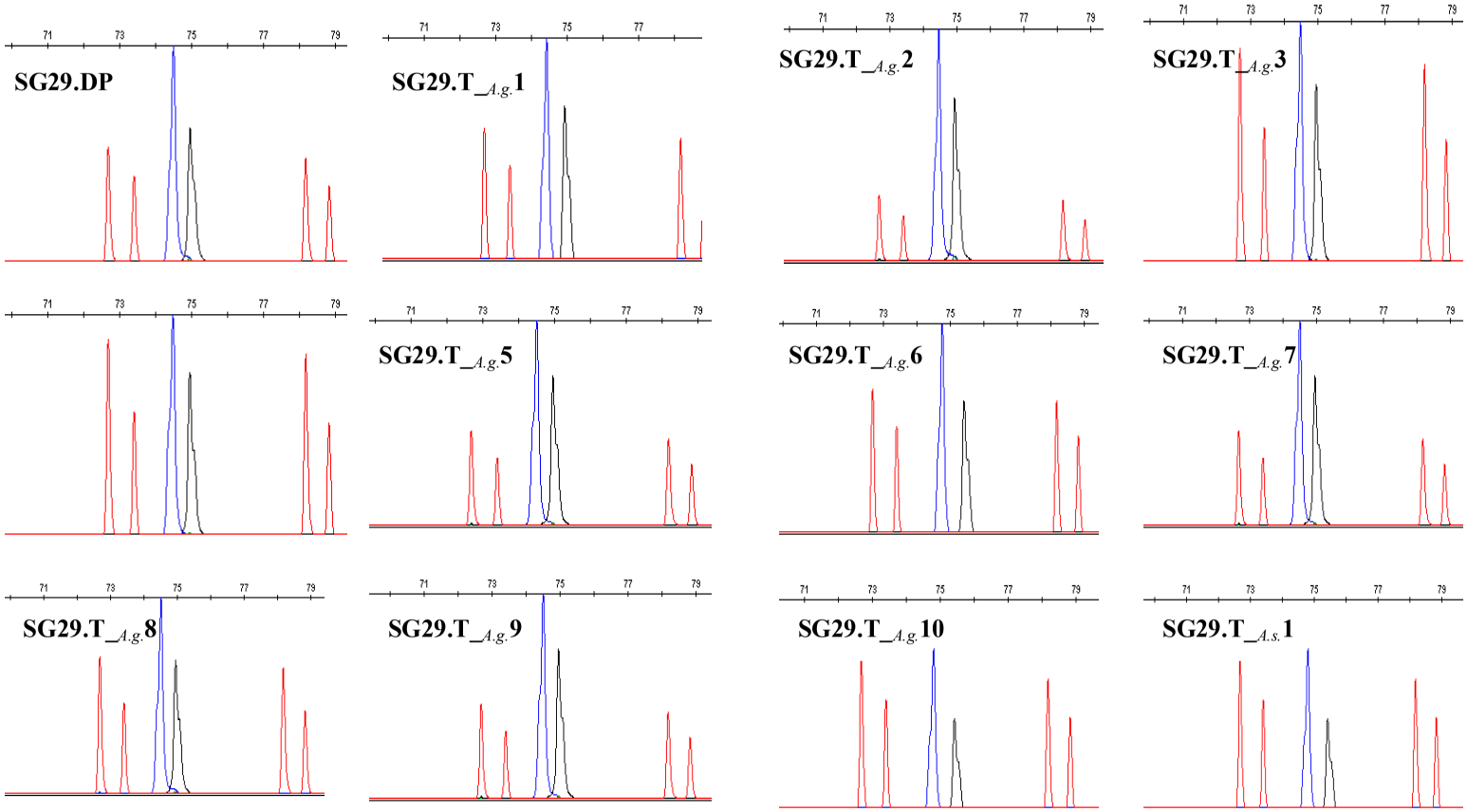


Fig 17. CAE-SSCP profiles of CTV *p*18 sequences from the Italian SG29 isolate and its associated aphid transmitted sub-isolates.
SG29 D.P: SG29 Donor plant; SG29.T_*A.g.*1 to SG29.T_*A.g.*10: SG29 *A. gossypii* derived sub-isolates; SG29.T_*A.s.*1: SG29 *A. spiraecola* derived sub-isolate

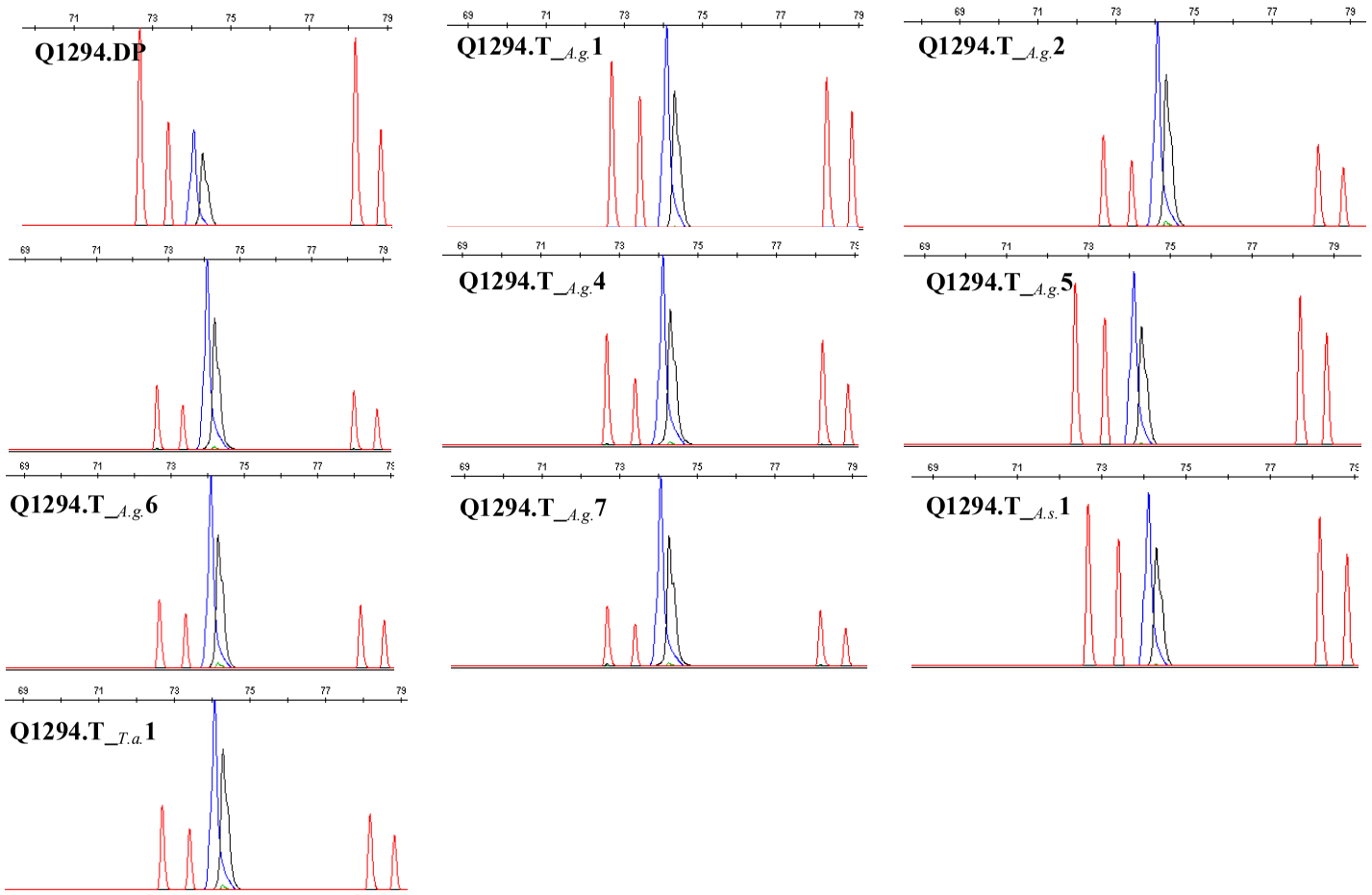


Fig 18. CAE-SSCP profiles of CTV *p*18 sequences from the Italian MAIB_Q1294 isolate and its associated aphid transmitted sub-isolates. Q1294 D.P: Q1294 Donor plant; Q1294.T_*A.g.*1 to Q1294.T_*A.g.*7: Q1294 *A. gossypii* derived sub-isolates; Q1294.T_*A.s.*1: Q1294 *A. spiraecola* derived sub-isolate; Q1294.T_T.a.1: Q1294 *T. aurantii* derived sub-isolate.

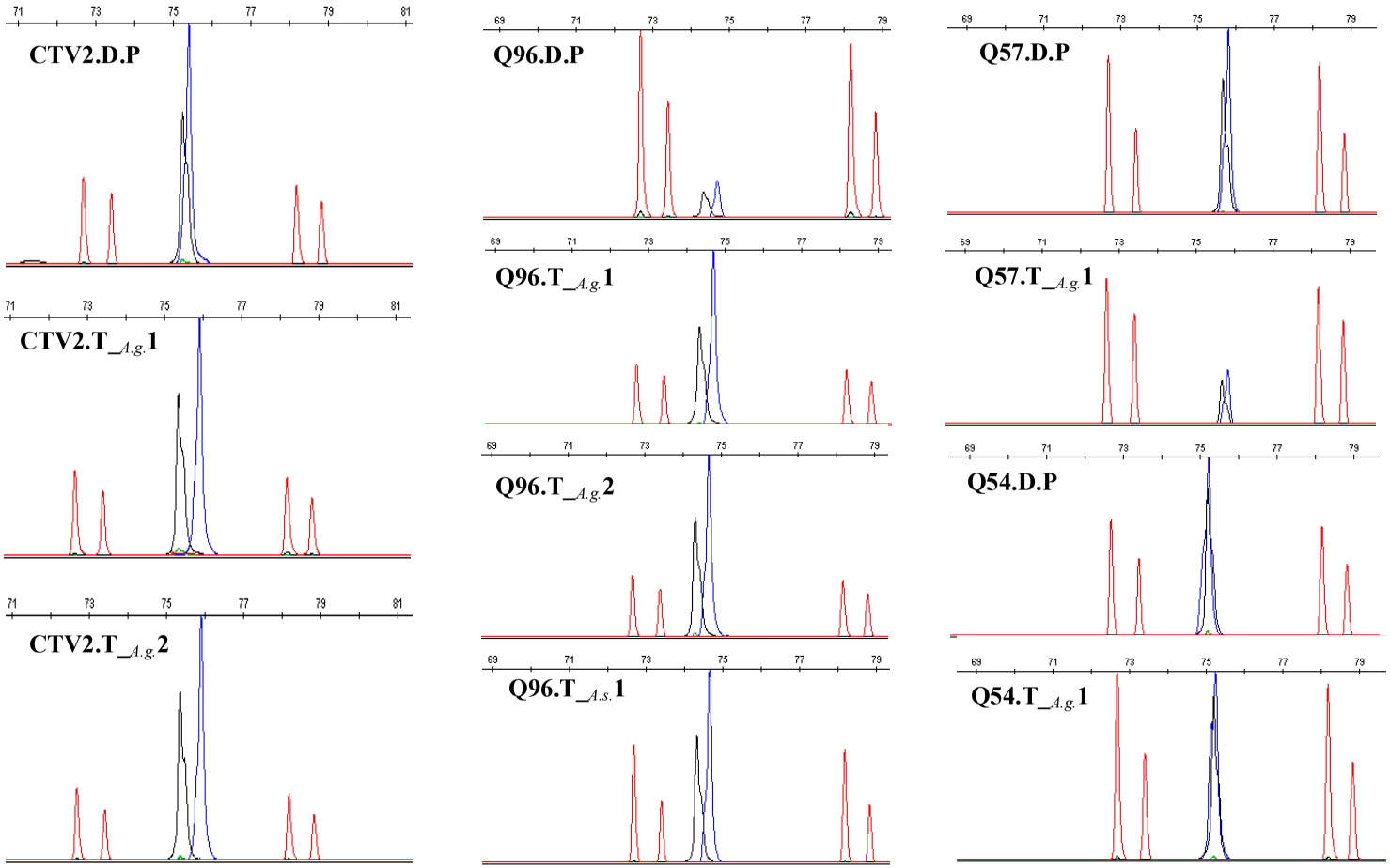


Fig 19. CAE-SSCP profiles of CTV *p*18 sequences from the isolates CTV2, MAIB_Q96, MAIB_Q57 and MAIB_Q54 and their associated aphid transmitted sub-isolates. CTV2 D.P:CTV2 Donor plant; CTV2.T_*A.g.*2: CTV2 *A. gossypii* derived sub-isolates; Q96 D.P: MAIB_Q96 donor plant; Q96.T_*A.g.*1 and 2: MAIB_Q96 *A. gossypii* derived sub-isolates; Q96.T_*A.s.*1: MIAB_Q96 *A. spiraecola* derived sub-isolate; Q57 D.P and Q57.T_*A.g.*1: MAIB_Q57 donor plant and *A. gossypii* derived sub-isolates, respectively; and Q54 D.P and Q54.T_*A.g.*1: MAIB_Q54 donor plant and *A. gossypii* derived sub-isolates, respectively.

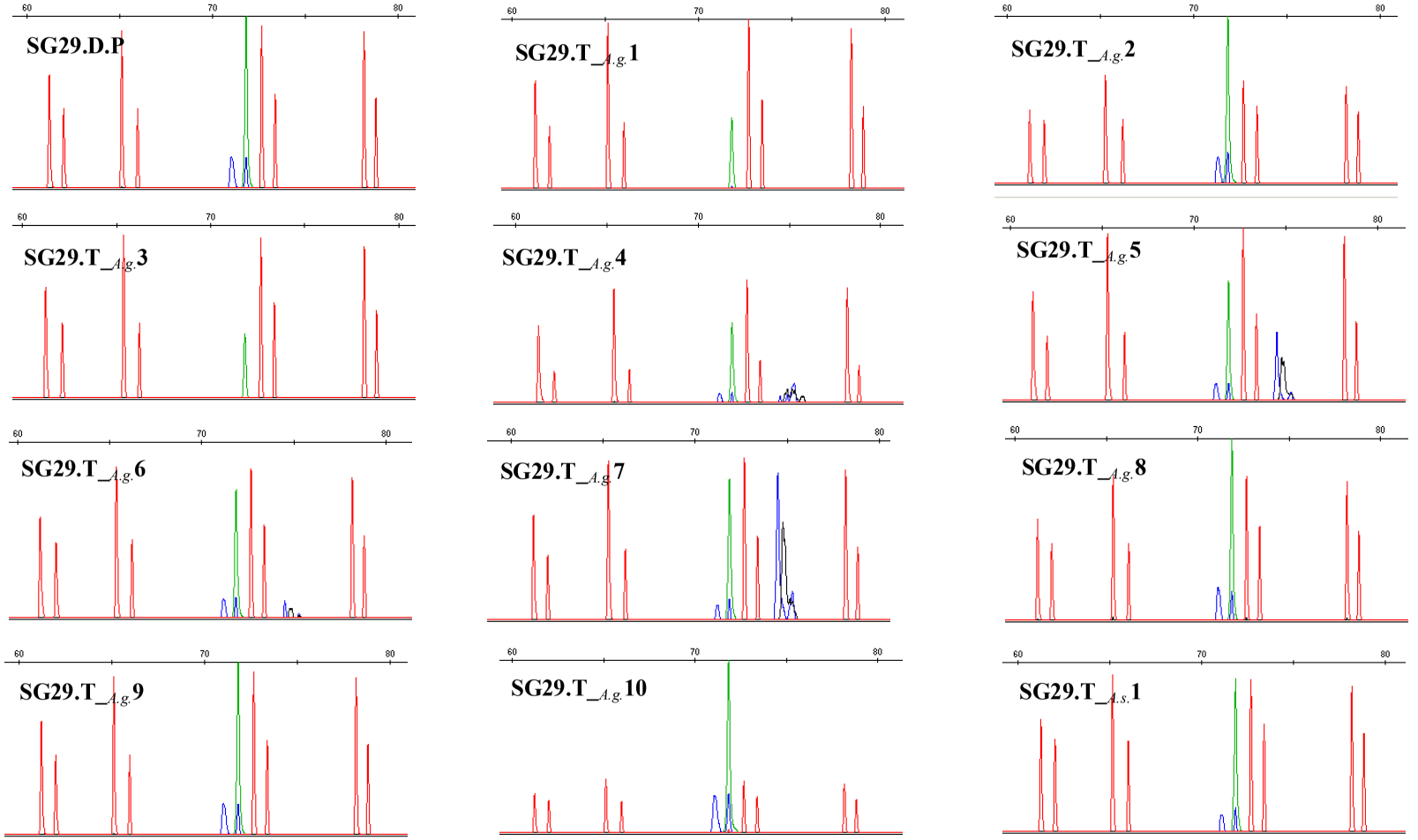


Fig 20. CAE-SSCP profiles of CTV *p*23 sequences from the Italian SG29 isolate and its associated aphid transmitted sub-isolates.
SG29 D.P: SG29 Donor plant; SG29.T_*A.g.*1 to SG29.T_*A.g.*10: SG29 *A. gossypii* derived sub-isolates; SG29.T_*A.s.*1: SG29 *A. spiraecola* derived sub-isolate.

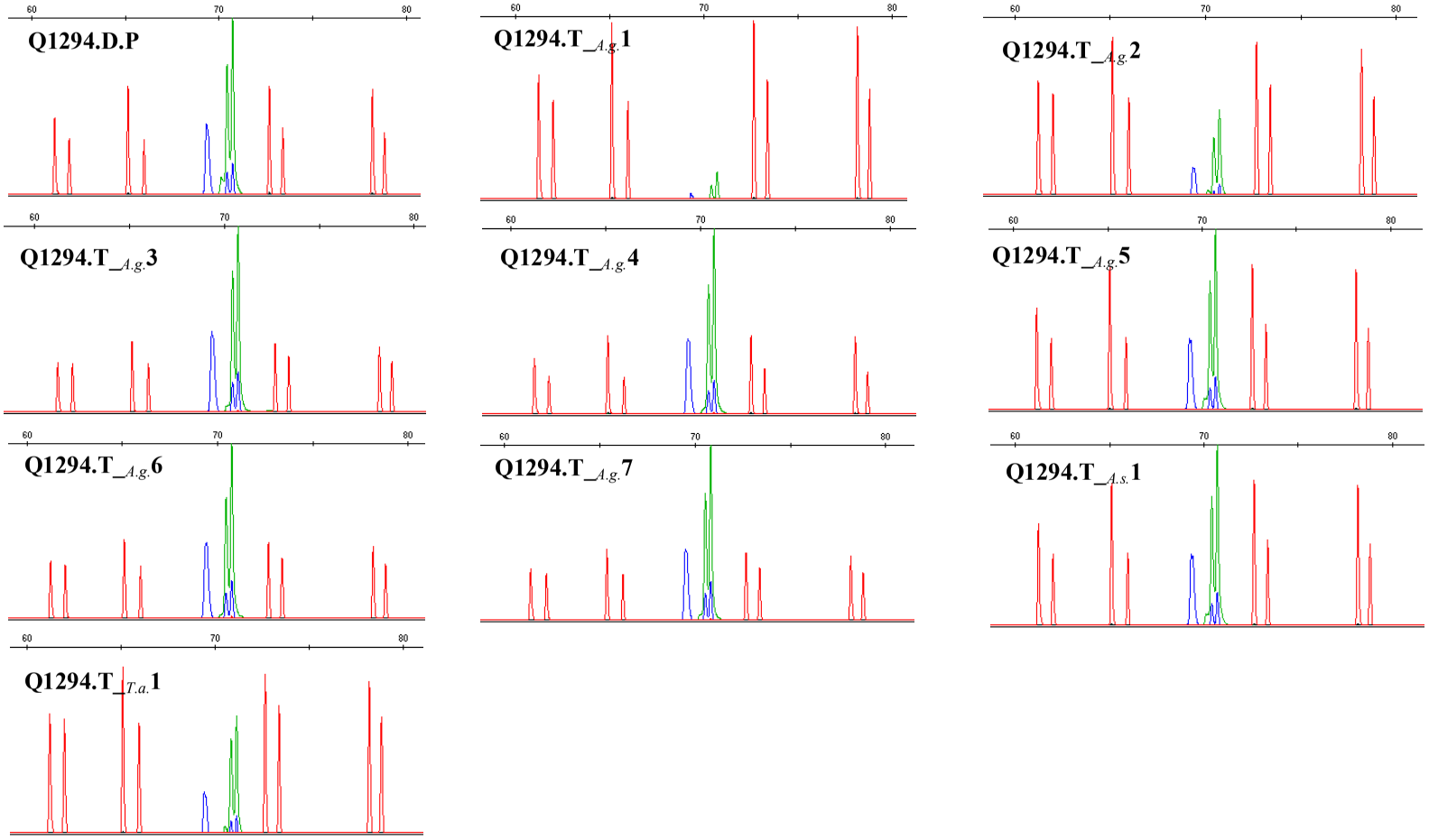


Fig 21. CAE-SSCP profiles of CTV *p*23 sequences from the Italian MAIB-Q1294 isolate and its associated aphid transmitted sub-isolates. Q1294 D.P: Q1294 Donor plant; Q1294.T_*A.g.*1 to Q1294.T_*A.g.*7: Q1294 *A. gossypii* derived sub-isolates; Q1294.T_*A.s.*1: Q1294 *A. spiraecola* derived sub-isolate; Q1294.T T.a.1: Q1294 *T. aurantii* derived sub-isolate.

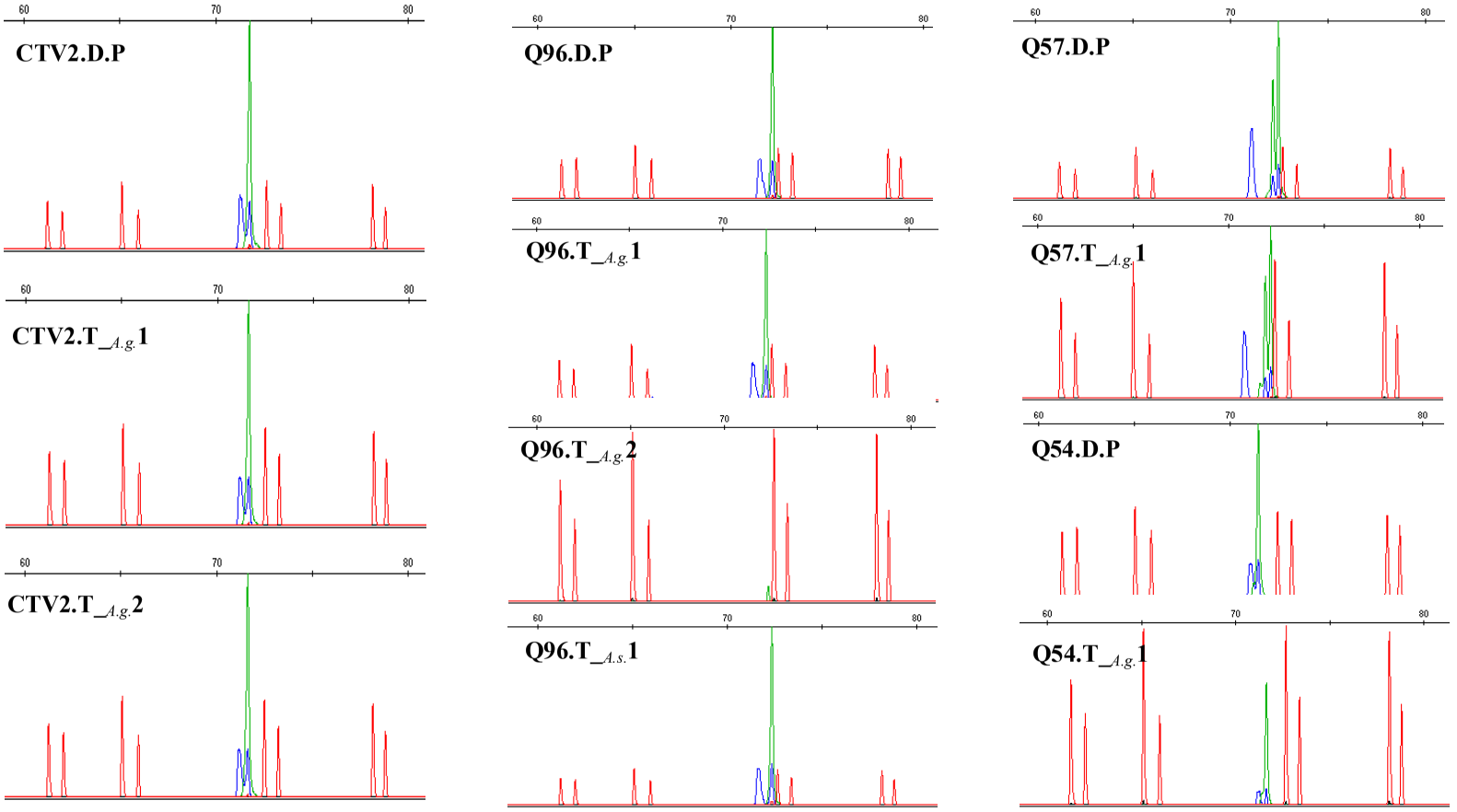


Fig 22. CAE-SSCP profiles of CTV *p*23 sequences from the isolates CTV2, MAIB_Q96, MAIB_Q57 and MAIB_Q54 and their associated aphid transmitted sub-isolates. CTV2 D.P:CTV2 Donor plant; CTV2.T_*A.g.*2: CTV2 *A. gossypii* derived sub-isolates; Q96 D.P: MAIB_Q96 donor plant; Q96.T_*A.g.*1 and 2: MAIB_Q96 *A. gossypii* derived sub-isolates; Q96.T_*A.s.*1: MIAB_Q96 *A. spiraecola* derived sub-isolate; Q57 D.P and Q57.T_*A.g.*1: MAIB_Q57 donor plant and *A. gossypii* derived sub-isolates, respectively; and Q54 D.P and Q54.T_*A.g.*1: MAIB_Q54 donor plant and *A. gossypii* derived sub-isolates, respectively.

5.2.3.3. Multiple Molecular Markers analysis of the CTV vector inoculated sub-isolates

Taken into account that comparison of single gene sequences is insufficient to be representative of the entire genome, the 5' divergent region of the different CTV aphid transmitted isolates have been analysed only with MMM from the POL region highly specific to the four standard isolates T36, T30, VT and T3.

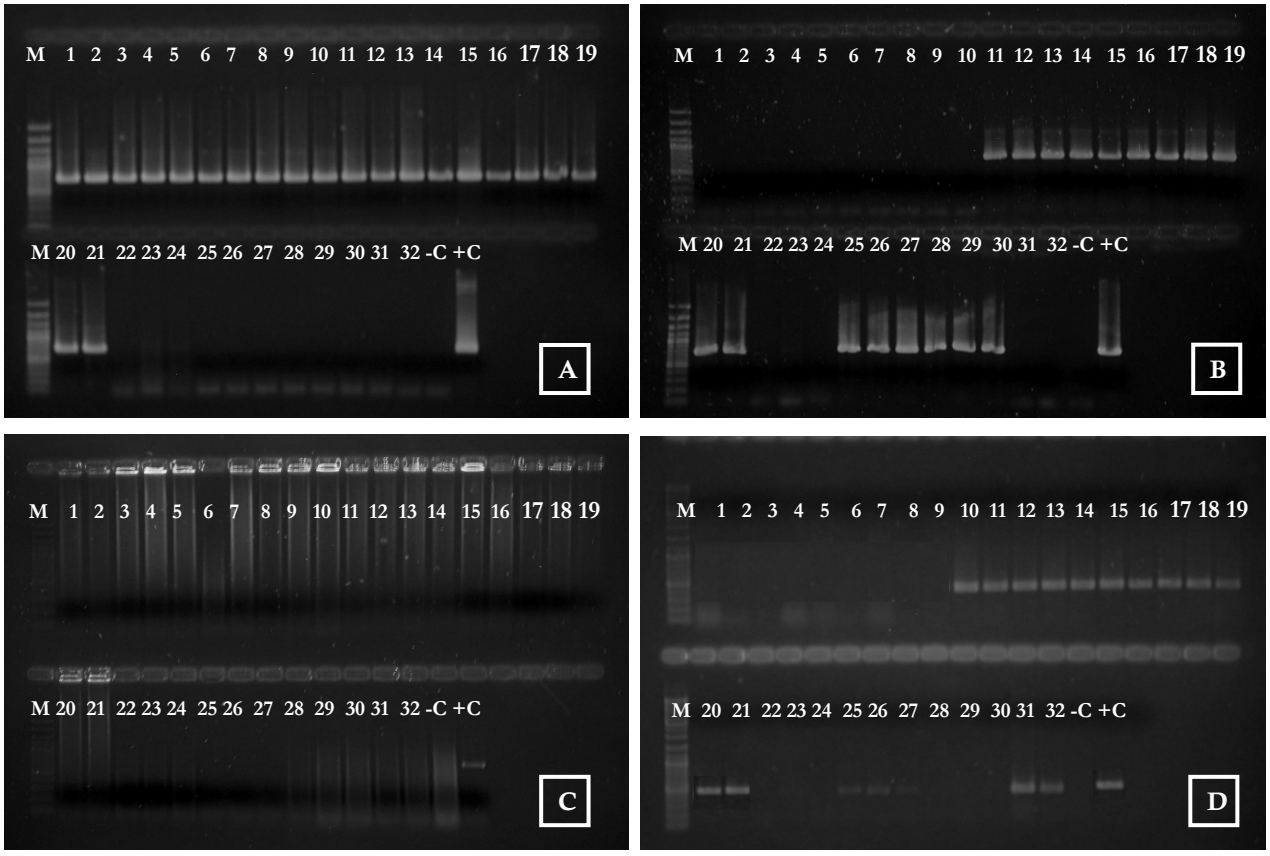


Fig 23. Multiple Molecular Markers profiles of the aphid transmitted isolates

Marker amplifications are: (A): T30POL; (B): VTPOL; (C): T36POL and (D): T3K17. Lane M: 100 bp DNA Ladder. Samples: 1-9: MAIB_Q1294 and relative sub-isolates; 11-21: SG29 (IT) and relative sub-isolates; 22-24: CTV2 (POR) and sub-isolates; 25-28: MAIB_Q96 (CRO) and relative sub-isolates; 29-30: MAIB_Q54 (PAL) and *A. gossypii* sub-isolate; 31-32: MAIB_Q57 (EGY) and *A. gossypii* sub-isolate; -C: healthy control; +C: positive control.

As shown by the figure above (Fig. 23), T36POL marker has been yielded only from the from Albanian isolate (MAIB_Q3) known to be decline inducing, as expected. The 695 bp VTPOL marker have been amplified from the Croatian (MAIB_Q96), the Palestinian (MAIB_Q54) and the Italian (SG29) isolates, before and after their aphid transmission. Amplicons from the Egyptian isolate (MAIB_Q57) remained T3K17 reactive even after the transmission by aphids, as did the MAIB_Q1294 isolate homologous to the T30 strain, as well, sub-isolates from the SG29 SY-type isolate shared the same genotype as the parental one. The isolate CTV2 (Madeira Island) gave only too slight amplification from the T30POL marker (696 bp), and negative signals from the other molecular markers used. The same isolate did not gave any positive PCR signal when analysed by Nolasco and colleagues (2008) using the following markers as described by Hilf's system.

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

Since nucleotide sequencing provides precise information leading with specific gene mutations but still an expensive technology, a number of three isolates including MAIB_Q1294, SG29 (Italy) and MAIB_Q54 (Palestine) have been submitted to further sequence analysis flanking two genome regions CPg and p23, in correlation with data obtained by SSCP trials.

The sequences were compared among them and with the nucleotide sequences of reference isolates SY568, VT, T30, T36 and T3 retrieved from the GenBank (AB046398, EU937519, EU937520, EU937521 and DQ355053.1 accession numbers, respectively).

(i) CPg sequencing

From the MAIB_Q1294 isolate, CPg targets recovered from the virus source and a number of three sub-isolates derived from the different aphid vectors have been *TA*-cloned and sequenced. As a result of SSCP analysis, new

haplotypes have been arisen following the transmission by each of the aphid vector species with the high frequency of the parental haplotype profile (I) herited from the donor plant (Fig. 24).

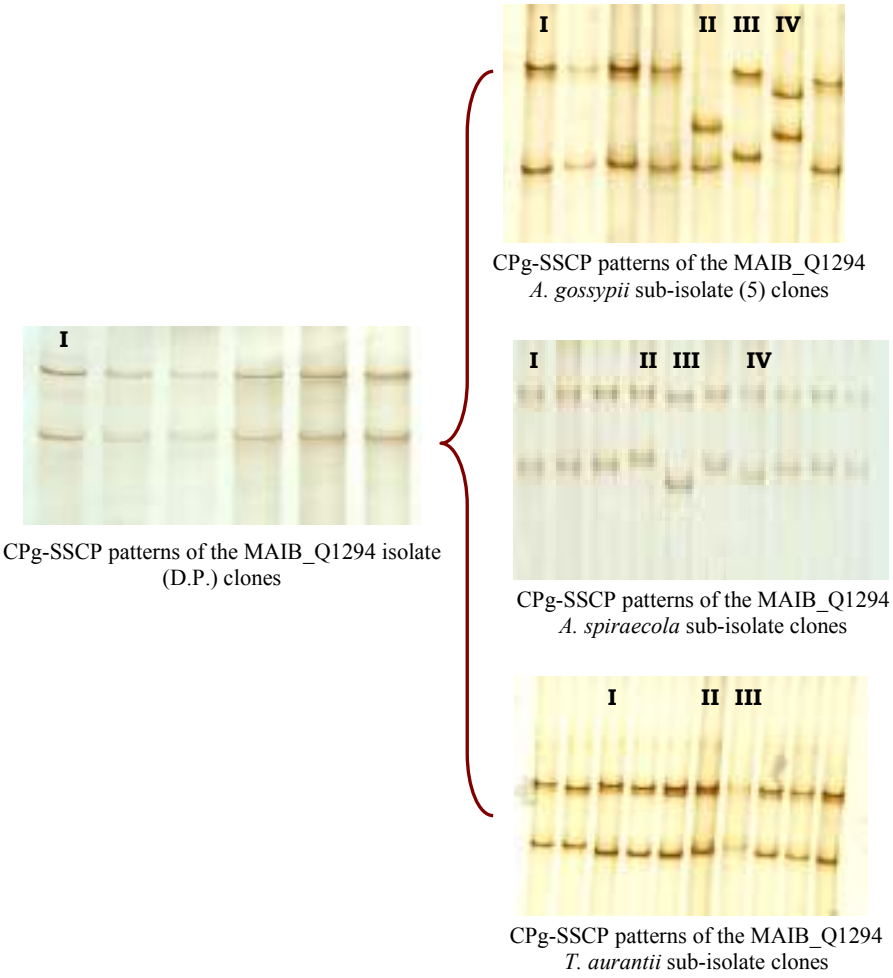


Fig 24. CPg-SSCP patterns yielded from the Italian mild isolate (MAIB_Q1294) sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis

Sequences obtained were aligned and compared among them and with the nucleotide sequences of reference isolates SY568, VT, T30, T36, T3, QAHA, SP, T318A, B265 and T385 (Fig. 25).

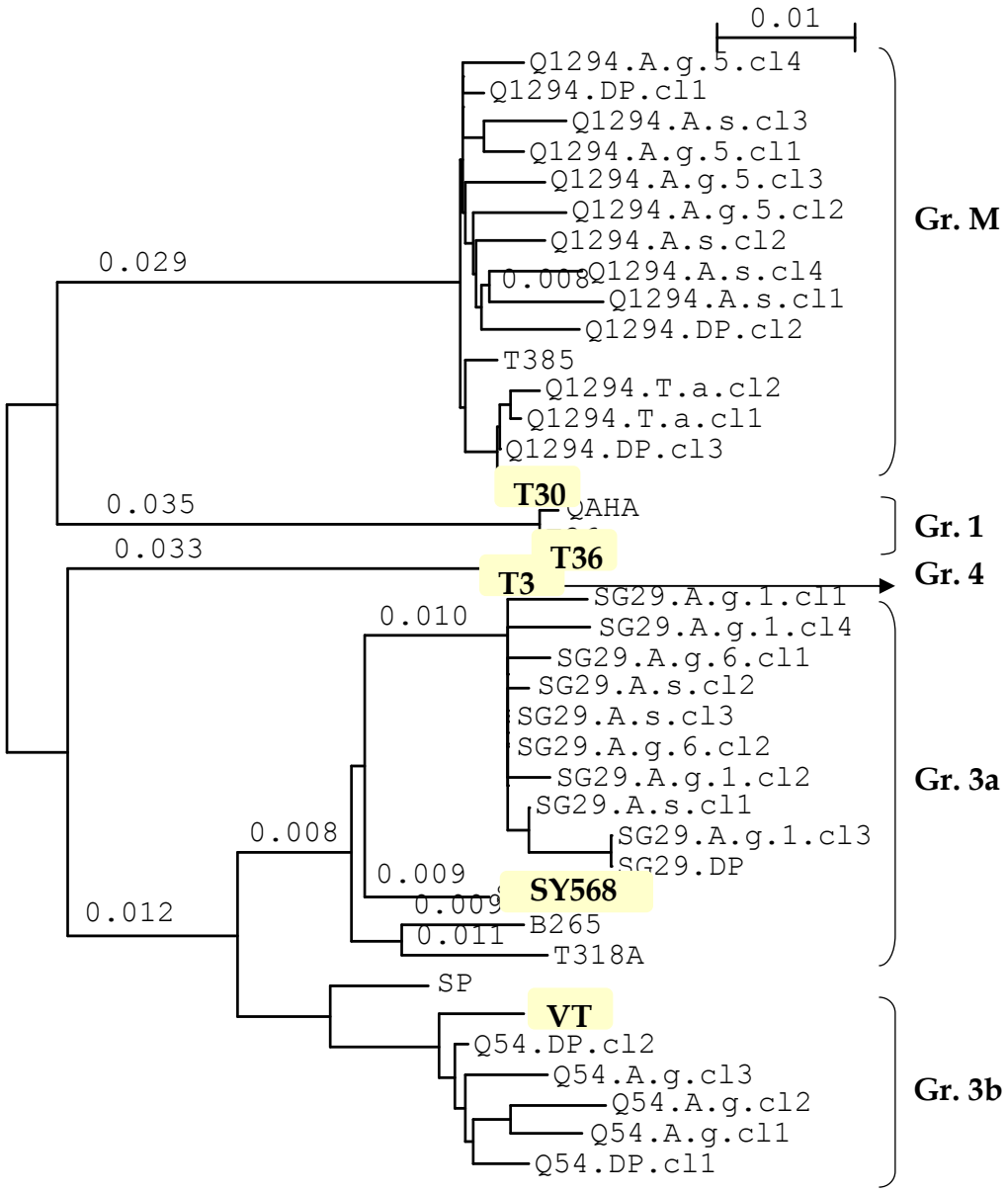
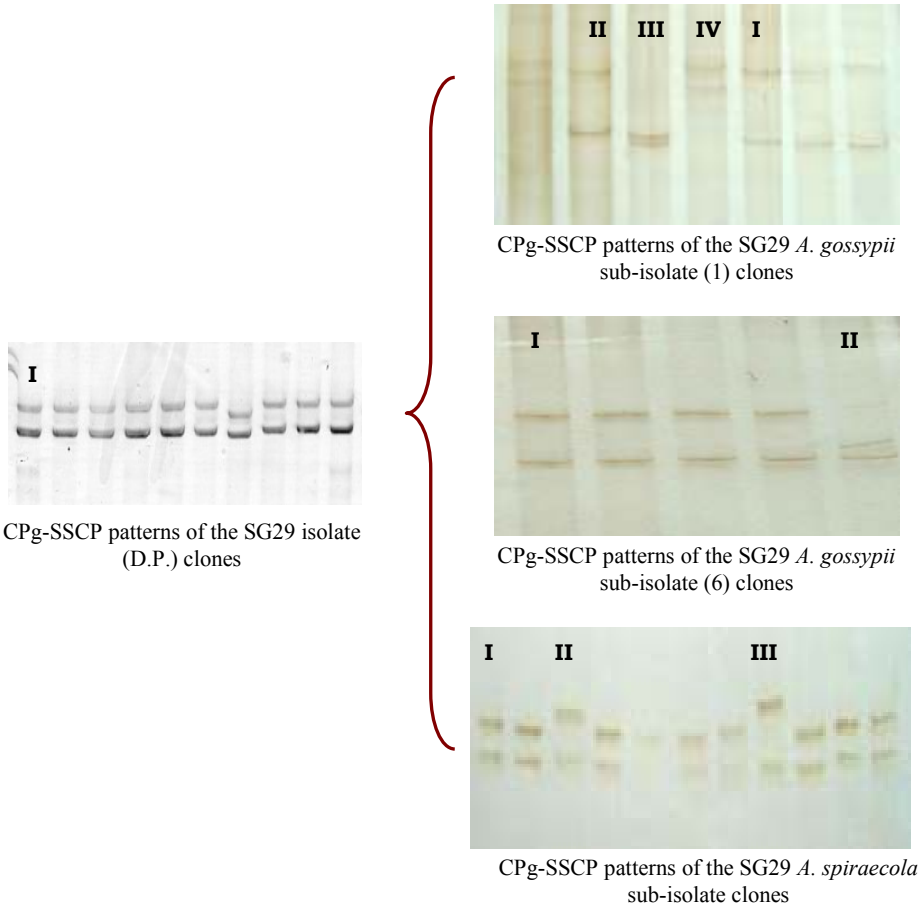


Fig 25. Phylogenetic analysis of CPg sequences yielded from the vector transmitted Mediterranean CTV isolates used before and after the aphid passage

As illustrated by the figure above, the different CPg sequences related to the MAIB_Q1294 isolate clustered together with the same phylogenetic group represented by the T30 strain-like (Florida) associated with mild symptoms (Gr M). Hence, high nucleotide sequence homology of 98% - 99% have been

exhibited either between the different sub-isolates and the donor virus source (intra-group similarity), or when confronted with both of T30 and T385 mild reference isolates

Likewise, SSCP patterns yielded from the SG29-CPg fragment showed that some distinct haplotypes have been arisen from three sub-isolates generated by both of *A. gossypii* (sub-isolate 1 and 6) and *A. spiraecola* transmission trials, with the usual predominance of the parental virus variant (Fig. 26).



CPg-SSCP patterns of the SG29 isolate (D.P.) clones

CPg-SSCP patterns of the SG29 *A. gossypii* sub-isolate (1) clones

CPg-SSCP patterns of the SG29 *A. gossypii* sub-isolate (6) clones

CPg-SSCP patterns of the SG29 *A. spiraecola* sub-isolate clones

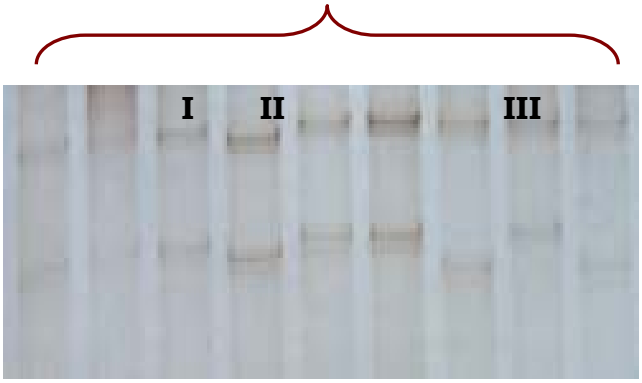
Fig 26. CPg-SSCP patterns yielded from the Italian CTV-SY isolate (SG29) sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis

Resulted sequences showing minor nucleotide variations clustered together close to the SY568 reference isolate. In addition, by extrapolation with

the APET characterization system, these sequences shared the group 3b of severe biological activities generally associated with sweet orange stem pitting. Nucleotide sequence similarities among the clones revealed from the different sub-isolates, and with comparison to the virus source (SG29. D.P) was evaluated at 98% - 99%. However, the different sub-isolate haplotypes showed a modest increase on the overall sequence nucleotide identities with respect to the original virus variant when confronted with most of the reference isolates used. Thus, both of the clone 2 from *A. gossypii* transmitted sub-isolate (6) and the clone 3 from *A. spireacola* derived one, showed 98% of nucleotide identity with SY568 isolate, with respect to 97,3% from the donor plant. As well, The latter showed 96,5% and 96,7% maximum sequence homology values with T318A and B265 reference isolates, respectively; while the same clone variants reached 97,3%-97,4% values, etc. Bearing in mind that precisely the CPg fragment of CTV is a highly conserved genomic region, any detectable variance is likely to have an important significance (Cerni *et al*., 2009).

As illustrated by the figure below (Fig. 27), new SSCP haplotypes emerged from the p25 fragment amplified from the Palestinian viral accession (MAIB_Q54) following its transmission by *A. gossypii*.

CPg-SSCP patterns of the MAIB_Q54
isolate (D.P.) clones



CPg-SSCP patterns of the
MAIB Q54 *A. gossypii* sub-isolate

Fig 27. CPg-SSCP patterns yielded from the Palestinian CTV-SY isolate MAIB_Q54 sequences before and after the aphid passage, by 10% non-denaturing polyacrylamid gel electrophoresis

By phylogenetic analysis (Fig. 28), the different clones joined the VT-strain like and clustered within the group 3b, whereby the major biological activity is associated with severe grapefruit stem pitting. The overall sequenced haplotypes showed 98% of nucleotide similarity between each other, but also with the donor plant. When compared to well known reference isolates, nucleotide sequences yielded from the *A. gossypii* derived sub-isolate exhibited minor variations with respect to the parental virus variants. As reported in the table follow, highest nucleotide identity of the virus source (MAIB_Q54 D.P) with the VT strain (Israel) ranged from 98,8% to 99,1%, while 98,2% - 98,6% have been distinguished after the aphid transmission. Similarly, maximum nucleotide

homology with the SP isolate (New Zealand) varied from 97,7% - 98% and 97,1% – 97,6% before and after the aphid transmission, respectively.

(ii) p23 gene sequencing

Despite that the CPg has been thought to be mostly involved in the CTV symptom's expression, recent investigations suggested that the phenotype appearance is associated with p23 accumulation (Sambade *et al*., 2003). Hence, in the present work, p23 rich amplicons yielded from the three aphid transmitted CTV isolates previously mentioned have been subjected to further sequence analysis. Due to the inefficient nucleotide sequence separation of the *ca*. 700 bp length fragment of concern by classical SSCP, only one *rec*DNA clone from each successfully transformed *E. coli* colony have been extracted and sequenced. Results from the phyllogenetic analysis of the *p*23 gene confirmed those obtained by the CPg fragment (Fig. 28). Thus, highest nucleotide homology values of 98% - 99% have been distinguished from the Italian SG29 variant and relative sub-isolates with the SY strain, as did the Palestinian group (MAIB_Q54) with the VT strain. While, the MAIB_Q1294 isolate and its associated variants showed *ca*. 96% as maximum nucleotide identity with T30 and T385 mild strains from Florida and Spain, respectively.

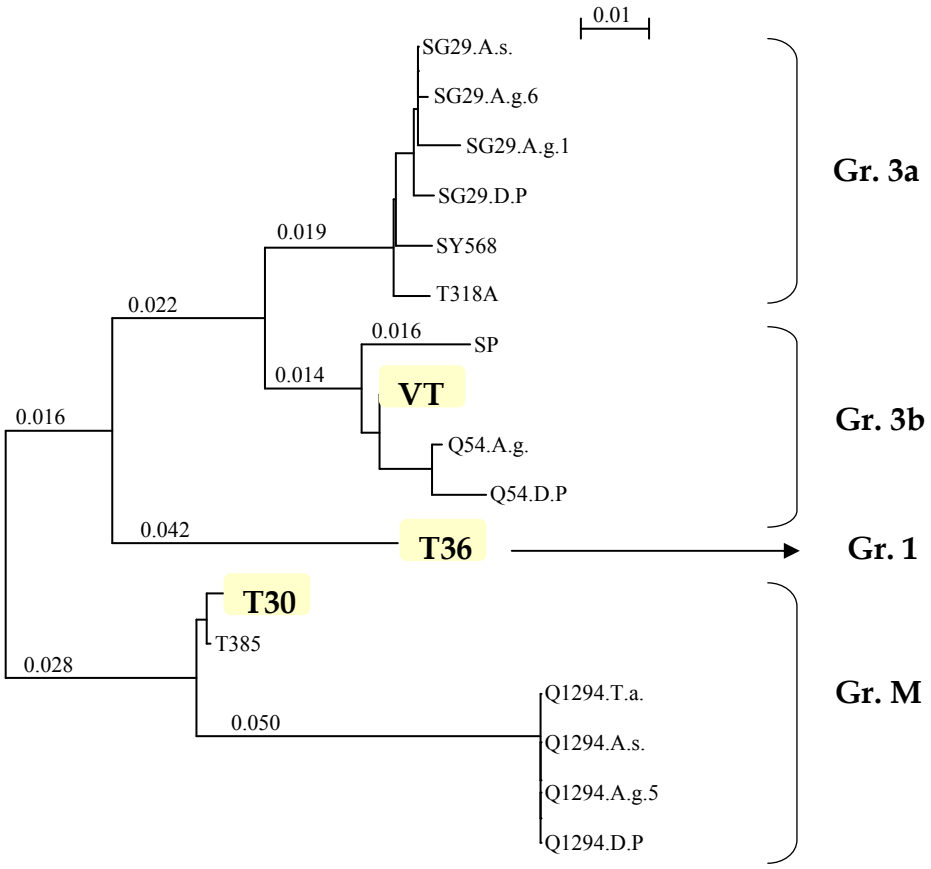


Fig 28. Phylogenetic analysis of *p*23 sequences yielded from the vector transmitted Mediterranean CTV isolates used before and after the aphid passage

5.2.2.5. Nucleotide sequence variation after aphid passage

(i) CTV CPg sequence:

When translated in amino acids, the targeted 672 bp length CPg sequence amplified from the different isolates showed some slight variations further to the transmission by different vectors.

From the Italian isolate MAIB_Q1294, unrandom Single Nucleotide Polymorphisms (SNP) have been exhibited from the *A. gossypii* sub-isolate (Q1294_T.$_{A.g}$.5), which occurred in the following nucleotide sequence positions:

3, 83, 84, 135, 140, 179, 208, 310, 338, 368, 407, 581, 555, 559, and 653 (Annex 7). Similar variations have been yielded from the *A. spiraecola* sub-isolate focusing on the following nucleotide positions: 3, 6, 52, 127, 130, 155, 156, 208, 281, 327, 435, 437, 452, 503, 634, 644, 650 and 653. Whereas, transmission by *T. aurantii* resulted in minor SNP concerning only three (60, 84 and 289) nucleotide positions. Furthermore, some nucleotide Deletion/Insertion phenomena have been shown further the transmission processes, such as the deletion of Guanidine (G) amino-acid at the 645 position from the *A. gossypii* sub-isolate, but also the Adenine (A) at 611 position and the deletion of Cytosine (C) from the 85 position in the *T. aurantii* sub-isolate.

Translation of the targeted nucleotide sequence in proteins showed that the most frequent variations have been exhibited from both of *A. spiraecola* and *A. gossypii* vector transmission. As illustrated in the Annex 8, protein changes generated by the spirea aphid have been depicted from the positions: 50, 92, 121, 143, 148, 165, 210 and 216. As well, those followed the transmission by the cotton aphid have been observed in the positions: 27, 44, 59, 111, 133, 184, 215 and 216. While, only one variation concerning the 95 protein position have been obtained from the black citrus aphid sub-isolate.

Regarding the SG29 seedling yellows-like isolate (Italy), SNPs in different amino-acidic positions were arisen from the analysed *A. gossypii* sub-isolates-1 (nucleotide positions 1, 2, 3, 4, 247, 280, 310, 347, 450 and 621) and sub-isolate-6 (209, 556) (Annex 9), resulting in minor variations in the polypeptide sequence (protein positions 1,2,116 and 207). While, only one nucleotide change have been yielded from the *A. spiraecola* sub-isolate (nucleotide position 240) which resulted in one single protein variation (polypeptide position 80) (Annex 10).

From the aphid inoculated sub-isolate deriving from the MAIB_Q54, VT-like isolate from Palestine, slight variations have been depicted from different CPg nucleotide positions (105, 157, 166, 169, 247, 319, 339 and 413) (Annex 11), resulting in very slight variations in the polypeptide sequence positions (107, 138) (Annex 12).

(ii) CTV p23 gene sequence:

In the of presence of few variations in the studied p23 CTV fragment amplified from the MAIB_Q1294 amplicons further to the aphid transmission (Annex 13), minor nucleotide variations have been occurred within both of the SG29 (Annex 14) and MAIB_Q54 isolates by the same process (Annex 15). By protein translation, the latters resulted in slight but not least polypeptide modifications within the vector inoculated sub-isolates (Annex 16).

Chapter 6. Conclusion

Citrus tristeza closterovirus is adapted to replicate on all Citrus species, and its interaction with the major tristeza sensitive sour orange rootstock gave rise to catastrophic economic losses of productive trees worldwide. CTV isolates often occur as populations of genetically related variants clustering around one or more consensus sequences governing their pathogenic characteristics. Thus, characterizing the genomic CTV populations elsewhere is of a paramount importance for identifying specific groups of isolates and contributing to early prediction of the disease impact.

In the frame of this work, the major outcome afforded through the molecular discrimination between a significant number of Mediterranean CTV isolates evidenced the great genetic diversity among the viral population structure between and within isolates of different origins, and opened new insights about occurrence of mixed infections, recombination and some spatial categorization of the isolates in the effort to understand the CTV complexity.

Therefore, consistent information gathered by the phylogenetic analysis of the CTV-CP gene sequences, which completed by the multiple molecular typing targeting the less conserved 5' co-terminal ends, showed the predominance of CTV isolates harboring a combination of genotypes.

Nevertheless, mild T30-like variants are highly distributed in the area, but occurrence of severe inducing strains is not excluded. Hence, quick decline inducing T36 isolates have been found as a stable CTV component along the Balkan Peninsula. As well, VT genotype has been revealed as the most prevalent haplotype of the CTV population within the Middle East countries.

Moreover, CPg sequence analysis from Montenegrin and Croatian isolates allowed to define an unusual CTV cluster, illustrating the high genetic variability within the East Adriatic region. this finding was in accordance with results previously postulated by Cerni *et al*., (2009), noticing the high pathogenic

potential associated with this group almost considered as “a reservoir” of severe CTV strains.

In addition, aggressive variants have been depicted from an Egyptian (MAIB_Q57) and an Italian (SG29) accessions, showing respectively highest nucleotide homology with T3 and SY568 standard strains, readily associated with severe stem pitting and seedling yellows symptoms.

Aiming to understand the mechanism of variability in the CTV population, phenomenon of differential selection pressure on the viral sequences after host change, but also after aphid transmissions have been suggested (Ayllon *et al*., 1999). This may eventually lead to the formation of new genotypes, whose combinations could cause a dramatic effect on the severity of symptoms caused by the major genotype.

From this standpoint, the successive epidemics in the Mediterranean area from Spain and Israel were turned back to the high capacity of the cotton aphid as a secondary vector to transmit severe CTV strains.

To date, the high distribution of tristeza disease, the prevalence of *A. gossypii* in citrus plantings and the new establishment of *T. citricidus* in the northern part of the area represent a high threat to the citriculture in the Mediterranean basin, and give special emphasis to the need for epidemiological investigations in order to elucidate the CTV dynamics and to prevent future outbreaks.

Experimental aphid transmission trials conducted during this work confirmed the potential of *A. gossypii* in the CTV spread by contrast to both of *A. spiraecola* and *T. aurantii* minor vectors, and evidenced the impact of establishment of an ecological adaptation between the virus population and its vector entomofauna in the CTV transmission. Thus, inefficient transmission revealed from an Albanian T36-like isolate by none of the vector species was in accordance with previous works noticing the poor aphid transmissibility of this cluster even through *T. citricidus*. This finding may reflect the higher conservation of the T36 genome sequence.

Conversely, through the isolate SG29 obtained from South Eastern Italy (Sicily Province), it has been evidenced the great impact of the *A. gossypii*-SY combination in the high transmissibility of this severe form of CTV. Additionally, using the same vector, highest transmission rate (52%) have been also displayed from the mild Italian isolate MAIB_Q1294, collected from the Ionian Coast (Apulia province). This findings firstly evidences the impact the ecological equilibrium between the virus population and its adapted vector entomofauna in the rapid CTV dissemination. Moreover, it may provide supplementary contribution for the recent outbreaks depicted from Italy (Davino *et al*., 2005), which took place in Apulia region where mild CTV isolates are occurring and *A. gossypii* was the most prevailing aphid species (Yahiaoui *et al*., 2009), but also from Sicily region, where thousand of trees were infected with the severe characterized isolate and *A. gossypii* represent 90% of the total aphid composition (Davino *et al*., 2004).

Some changes in the viral population following the aphid transmission process have been evidenced by SSCP analysis on different genomic regions, throughout the appearance of new haplotypes from the aphid derived sub-isolates, with the high frequency of the predominant haplotype profile herited from the parental CTV population.

Since mutations leading to new variants can only be detected by nucleic acid based technology, partial genome sequencing and phylogenetic analysis upon both of *p*23 and *p*25 sequences, known to be evolved on the symptoms expression and to act as powerful RNA silencing suppressors in the host cells, highlighted the occurrence of minor genomic variations more frequently on *p*25 from single infected isolates. When translated on amino-acids, the latters have been shown to be sometimes accompanied by slight variations within the correspondent polypeptide sequences of both of the proteins.

Interestingly, this research deduces that, if mixed sub-populations can be separated through the aphid transmission by *T. citricidus* (Brlansky *et al*., 2003; Moreno *et al*., 1993a; Moreno *et al*., 1993b), viral populations homogeneously

grouped around a single predominant genomic RNA variant might be less prone to get alterated after the *A. gossypii* transmission, as suggested by d'Urso *et al*. (2000). Nevertheless, minor pathogenecity variants that could be generated by aphid and/or host transmission could result in the appearance of virulent CTV isolates within the Mediterranean area.

Chapter 7. Bibliographical References

Agranovsky, A. A., Lesemann, D. E., Maiss, E., Hull, R., and Atabekov, J. G., 1995. "Rattlesnake" structure of a filamentous plant RNA virus built of two capsid proteins. *Proc. Natl. Acad. Sci.* (USA), **92**: 2470-2473.

Albiach-Martí, M.R., Grosser, J.W., Gowda, S., Mawassi, M., Satyanarayana, T., Garnsey, S.M. and Dawson, W.O. 2004. Citrus tristeza virus replicates and forms infectious virions in protoplast of resistant citrus relatives. *Molecular Breeding* **14**: 117–128.

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

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

Anonymous, 2007. Food And Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division (http://faostat.fao.org).

Anonymous, 2004. UNCTAD from FAO data: Proportion of average annual production data for 2000-2004. http://www.unctad.org/infocomm/anglais/orange/market.htm

Anonymous, 2001. Projections of World Production and Consumption of Citrus to 2010. In "CHINA/FAO Citrus, Symposium"14-17, May 2001, Beijing People's Republic of China. http://www.fao.org/docrep/003/x6732e/x6732e02.htm#N1

Aubert, B. and Vullin, G. 1998. Citrus nurseries and planting techniques. Cirad, Montpellier, France. 183 pp.

Ayllòn, 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.

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. *Journal of Virology*, **77**: 9232–9243.

Ayllon, M. A., Lopez, C., Navas-Castillo, J., Garnsey, S. M., Guerri, J., Flores, R., and Moreno, P.,2001. Polymorphism of the 5' terminal region of *Citrus tristeza virus* (CTV) RNA:

incidence of three sequence types in isolates of different origin and pathogenicity. *Archives of Virology,* **146** (1): 27-40.

Ayllon, 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. *J. Gen. Virol.* **80**: 817–821.

Ayllon, 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.

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.

Alzhanova, D.V., Napuli, A.J., Creamer, R. and Dolja, V.V. 2002. Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and HSP70 homolog. *EMBO J.* **20**: 6997-7007.

Alzhanova, D. V., Hagiwara, Y., Peremyslov, V. V., and Dolja, V. V., 2000. Genetic analysis of the cell-to-cell movement of beet yellows closterovirus. *Virology,* **268**:192-200.

Aubert, B. and Vullin, G. 1998. Citrus nurseries and planting techniques. *CIRAD-GTZ*, Montpellier Eds: 183pp.

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 Pathol*. **42**, 219–229.

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

Ben-Yehoshua, S., Goldschmidt, E.E. and Bar-Joseph, M. 1994. Citrus fruits. Pages 357-377 *In*: *Encyclopedia of Agricultural Science*, Vol. 1. C. Arentzen, ed. Academic Press, Inc., San Diego, CA.

Bar-Joseph, M., Che, X., Mawassi, M., Gowda, S., Satyanarayana, T., Ayllón, M.A., Albiach-Martí, M.R., Garnsey, S.M. and Dawson, W.O., 2002. The Continuous Challenge of *Citrus tristeza virus*. *Procceding of the 15th International Organization of Citrus Virologists IOCV,* Riverside, CA: 1-7.

Bar-Joseph, M., Marcus, R. and Lee, R.F.,1989. The continuous challenge of citrus Tristeza virus control. *Ann. Rev. Phytopathology* 27: 292-316.

Bar-Joseph, M., Roistacher, C.N. and Garnsey, S.M. 1983. The epidemiology and control of citrus tristeza diseases. Pages 61-72 *In:* Plant Virus Epidemiology. R.T. Plumb and J.M. Thresh, eds. Blackwell scientific publications, Oxford, England.

Bar-Joseph, M., Garnsey, S.M., Gonsalves, D., Moscovitz, M., Purcifull, D.E., Clark, M.F. and Loebenstein, G. 1979. The use of enzyme-linked immunosorbent assay for detection of c*itrus tristeza virus*. *Phytopathology* **69**: 190– 194.

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.

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

Bernet, G.P., Breto, M.P. and Asinis, M.J. 2004. Expressed sequence enrichment for candidate gene analysis of citrus tristeza virus resistance. *Theor. Appl. Genet.* **108**: 592–602.

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. *Eur J Plant Pathol* **120**:177–188.

Bertolini, E., Moreno, A., Capote, N., Olmos, A., de Luis, A. Vidal, E., Pérez-Panadés, J. and Cambra, M. 2007. Quantitative detection of Citrus tristeza virus in plant tissues and single aphids by real-time RT-PCR. *Eur J Plant Pathol* **120**: 177–188

Bitancourt, A.A., 1940. A podridào das radicelas dos citrus na provincial de Corrientes, Argentina. *O Biològico* **6**: 285-288, 356-364.

Bitancourt, A.A. 1937. Tristeza. Min. de Agri. De la Nacion Brasil.

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

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

Bové, J.M. and Vogel, R. 1964. Agrumes et maladies à virus dans quelques pays d'Amérique latine. I-Brasil, II-Argentine, III-Perou et Equateur. Fruits **19**-639.

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, Brlansky RH, Indsto J, 1996. Biological characterization of Australian isolates of citrus tristeza virus and separation of subisolates by single aphid transmissions. *Plant Disease* **80**, 329–33.

Broadbent, P., Bevington, K.B. and Coote, B.G. 1991. Control of stem pitting of grapefruit in Australia by mild strain protection. In "*Proceedings of the 11th Conference of the International Organization of Citrus Virologists*" (Brlansky, R.H., Lee, R.F. and Timmer, L.W., Eds.), pp. 64-70. IOCV, Riverside, California.

Bukau, B., and Horwich, A. L., 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* **92**: 351-366.

Calavan, E.C., Harjung, M.K., Blue, R.L., Roistacher, C.N., Gumpf, D.J., and Moore, P.W. 1980. Natural spread of seedling yellows and sweet orange and grapefruit stem pitting tristeza viruses at the University of California, Riverside, Pages 69-75 in: *Proc. 8th Conf. Internl. Org. Citrus Virol*., IOCV, Riverside.

Cambra, M., Gorris, M.T., Roman, M.P., Terrada, E., Garnsey, S.M., Camarasa, E., Olmos, A. and Colomer, M. 2000a. Routine detection of citrus tristeza virus by direct immunoprinting ELISA method using specific monoclonal and recombinant antibodies. *Proceeding of the 14th Conference of IOCV* (Brazil 1998): 34-41.

Cambra, M., Gorris, M.T., Marroquìn, C., Romàn, M.P., Olmos, A., Martìnez, M.C., Hermozo de Mendoza, A., Lòpez, A. and Navarro, L., 2000b. Incidence and epidemiology of citrus tristeza virus in the Valencian Community of Spain. *Virus Res*. **71**: 75-85.

Cambra, M., Olmos, A., Gorris, M.T., Marroquìn, C., Esteban, O., Garnsey, S.M., Llauger, R., Batista, L., Penà, I. and Hermozo de Mendoza, A., 2000c. Detection of citrus tristeza virus by print capture and squash capture-PCR in plant tissues and single aphids. In: Da Graça, J.K., Lee, R.F. and Yokomi, R. (Eds). Proceeding of the 14th Conference of the international Organization of Citrus Virologists, IOCV, Riverside: 42-49.

Cambra M, Camarasa E, Gorris MT, Garnsey SM, Gumpf DJ, Tsai MC, 1993. Epitope diversity of citrus tristeza virus (CTV) isolates in Spain. In: Moreno P, da Grac¸a JV, Timmer LW, eds. *Proceedings of the 12th Conference of the International Organization of Citrus Virologists*. Riverside, CA, USA: International Organization of Citrus Virologists, 33–8.

Cambra, M., Garnsey, S.M., Permar, T.A., Henderson, C.T., Gumph, D. and Vela, C.,1990. Detection of citrus tristeza virus (CTV) with a mixture of monoclonal antibodies (Abstract). *Phytopathology* **80**: 103.

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. Proc. Int. Soc. *Citriculture* 1:444-448.

Cerni, S., Skoric, D., Ruscic, J., Krajacic, M., Papic, T., Djelouah, K. and Nolasco, G. 2009. East Adriatic—a reservoir region of severe Citrus tristeza virus strains. *Eur J Plant Pathol*. 6 pp. DOI 10.1007/s10658-009-9444-0

Cerni, S., Ruscic, J., Nolasco, G., Gatin, Z., Krajàcic, M. and Skoric, D., 2008. Stem pitting and seedling yellows symptoms of citrus tristeza virus infection may be determined by minor sequence variants. *Virus Genes* **36**: 241–249.

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 (Abstract), *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. *J. Econ. Entomol.* **55**:584-590.

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.

Clark, M.F. and Bar-Joseph, M. 1984. Enzyme immunosorbent assay in plant virology. In: Maramorosh, K. and Koprowski, H. (*eds*). Methods in virology. New York, Academic Press: 51-85.

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

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

Cutrer, B. 1998. *Citrus tristeza virus*: transmission and management of aphid vectors. Texas A and M University-Kingsville Citrus Center, M.S. Thesis.

D'Onghia, A.M. 2009. Citrus certification in the Mediterranean region, In: *Citrus Tristeza Virus* and *Toxoptera citricidus*: A serious threat to the Mediterranean citrus industry Options Méditerranéennes, Série B: Studies and Research, N° B 65, - IAM-Bari "Centre International de Hautes Etudes Agronomiques Méditerranéennes" . *In press*

D'Onghia, A.M., 2005. Citrus tristeza virus and *Toxoptera citricida*: Development of management and control strategies in the Mediterranean region. Centre International des Hautes Etudes Agronomiques Méditerranéennes. *Report on the 10th Annual Meeting of the Mediterranean Research Network on Certification of Citrus* (MNCC).

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.

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.

Danielsson, R., 1985. Polyviol as mounting medium for aphids (Homoptera: Aphidoidea) and other insects. *Entomologica Scandinavica*, 15: 383-385.

Davino, M., Davino, S., Barba, M., Caruso, A., Guardo, M., D'Onghia, A.M. and Savino, V. 2004. Citrus tristeza virus (CTV). A serious threat to the Italian Citrus groves. Proc. Int. Soc. Citriculture II: 790-793.

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

Davino, S., Rubio, M. and Davino, M., 2005. Molecular analysis suggests that recent *citrus tristeza virus* outbreaks in Italy were originated by at least two independent introductions. *European Journal of Plant Pathology,* **111**: 289-293.

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

Day, E., 1996. Aphid. *Virginia cooperative extension*. 3pp http://www.ext.vt.edu/departments/entomology/factsheets/gaphids.html

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.). *Mol. Genet. Genomics*. **265**: 739–747.

Dickson, R.C., Johnson, M. McD., 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.

Dixon, A.F.G. 1998. « Aphid Ecology : An optimization Approach". 2nd Edition, Chapman and Hall, New York, USA.

Djelouah K., D'Onghia A.M., 2001a. Occurrence and spread of citrus tristeza in the Mediterranean area. Proceedings on Production and exchange of virus-free plant propagating material in the Mediterranean region. *Options Méditerranéennes* B 35, *CIHEAM publications,* 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,* Serie B33, CIHEAM publications: 108-113.

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

Dodds, J.A., Jordan, R.J., Roistacher, C.N. and Jarupat, T., 1987a. Diversity of citrus tristeza virus isolates indicated by dsRNA analysis. *Intervirology* **27**, 177–188.

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

Dolja V.V., Kreuze J.F. and Valkonen, J.P.T., 2006. Comparative and functional genomics of closteroviruses. *Virus Research* 1**17**: 38-51.

Domingo, E. and Holland, J.J. 1994. Mutation rates and rapid evolution of RNA viruses. In: *The Evolutionary Biology of Viruses* (Morse, S.S., Eds), New York: Raven Press: 161-184.

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 *p*25 coat protein gene. *Mol. Breeding*. **10**: 1–10.

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

EPPO, 1990. Citrus Tristeza Closterovirus. Specific Quarantine requirements. EPPO Technical Documents. No. 1008: <http://www.eppo.org/QUARANTINE/virus/Citrus_tristeza/CT000_ds.pdf>

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 Mol. Biol*. **66:** 153–165.

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

Fawcett, H.S. 1936. Citrus diseases and their control. 2nd Ed. McGraw-Hill Book. New York & London. 656pp. p. 440-455.

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.

Febres, V.J. 1995. Molecular characterization of citrus tristeza virus genes and their use in plant transformation. *Ph. D. Thesis. University of Florida*, Gainesville, USA. pp.

Feder, M. E., and Hofmann, G. E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu. Rev. Physiol*., **61**: 243-282.

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.

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

Frison, E.A. and Taher, M.M. , 1991. *FAO/IBPGR technical guidelines for the safe movement of citrus germoplasm*. *FAO Rome Eds,* Italy.

Garnsey, S.M., Gottwald, T.R. and Yokomi, R.K. 1998. Control strategies for Citrus Tristeza Virus. In *Plant Disease Control,* edited by Hadidi A., Khetarpal R.K. and Koganezawa H., *APS Press*: 639-658.

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

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

Garnsey, S. M., Bar-Joseph, M., and Lee, R. F. 1981. Applications of serological indexing to develop control strategies for *Citrus tristeza virus*. *Proceedings of the International Society of Citriculture* **1**: 448-452.

Garnsey, S.M., Gonsalves, D. and Purcifull, D.E. 1979. Rapid diagnosis of citrus tristeza virus by SDS-immunodiffusion procedures, *Phytopathology* **69**: 88-95.

Garnsey, S.M., Gonsalves, D. and Purcifull, D.E. 1977. Mechanical transmission of citrus tristeza virus. *Phytopathology*, **67**: 965–968.

Ghorbel, R., Lòpez, C., Fagoaga, C., Moreno, P., Navarro, L., Flores, R. and Pena, L. 2001. Transgenic citrus plants expressing the citrus tristeza virus *p*23 protein exhibit viral-like symptoms. *Mol. Plant. Pathol.,* **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.

Gonsalves, D. and Garnsey, S.M. 1989. Cross protection technique for control of plant virus diseases in the tropics. *Plant Disease* **73**: 592-597.

Gottwald, T.R. 2010. Aphid transmission and epidemiology of Citrus Tristeza Virus. In*"Citrus Tristeza virus: Complex and Tristeza disease" (Karasev, A.V. and Hilf, M.E.,* Eds*.)*, pp133-150. *The American Phytopathological Society,* Minnesota, USA.

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 Proceedings of the 15th Conference of the International Organization of Citrus Virologists (Duran-Vila, N., Milne, R.G. and da Graça, J.V., eds), pp. 83–94. Riverside, CA: IOCV.

Gottwald, T.R., Garnsey, S.M., and Barbòn, 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**: 603-608.

Gottwald, T.R., Garnsey, S.M., Cambra, M., Moreno, P., Irey, M. and Borbòn, J. 1997. Comparative effects of aphid vector species on increase and spread of *citrus tristeza virus*. *Fruits* **52** (6): 397-404.

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

Gottwald, T.R., Garnsey, S.M., Sediles-Jean, A. and Rojas-Solis, A. 1996b. Co-diffusion of serologically distinct isolates of citrus tristeza virus vectored by Toxoptera citricida in northern Costa Rica. Pages 112-119. In Proc. Conf. Int. Organ. Citrus. Virol., 13th J.V. da Graça, P. Moreno and R.K.Yokomi, eds. IOCV, Riverside, CA.

Gowda, S., Satyanarayana, T., Ayllon, M.A., Moreno, P., Flores, R. and Dawson, W.O. 2003. The conserved structures of the 5' nontranslated region of Citrus Tristeza Virus are involved in replication and virion assembly. *Virology*, **317**: 50-64.

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., 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.

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

Gumpf, D.J., J., Zheng, G.Y., Moreno, P., Diaz, J.M. 1987. Production and evaluation of specific monoclonal antibodies to citrus tristeza virus strains. *Phytophylactica* **19**: 159-1161.

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

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.

Harris, K.F., Pesic-Van Esbroeck, Z. and Duffus, J. 1995. Anatomy of a virus vector. In "Bemisia 1995: Taxonomy, Biology, Damage control and Management" pp. 289-318. Hampshire, UK.

Harris, K.F., 1989. Aphid transmission of plant viruses. In: Harris, K.F., Maramorosch, K. (Eds.), Aphids as Virus Vectors. Academic Press, New York, NY, pp. 177–204.

Harris, K.F. 1977. Aphid transmission of plant viruses. In "Aphid as Virus Vectors". (Harris, K.F. and Maramorosch, K., Eds,), pp. 177-204. Academic Press, New York.

Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. *Nature* **381**: 571-580.

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

He, X-H., Rao, A. L. N., and Creamer, R. 1997. Characterization of beet yellows closterovirus-specific RNAs in infected plants and protoplasts. *Phytopathology* **87**:347–352.

Heie, O.E. 1980. The Aphidoides (Hemiptera) of Fennoscandia and Denmark. Vol **9**: 236 pp.

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

Hermoso de Mendoza, A., Pérez, E. and Real, V. 1997. Composiciòn y evoluciòn de la fauna afìdica (Homoptera, Aphidinea) de los cìtricos valencianos. *Bol. San. Veg. Plagas* **23**: 363-375.

Hermoso de Mendoza, A., Pina, J.A., Ballaster-Olmos, J.F. and Navarro, L. 1993. Persistent transmission of citrus vein enation virus by *Aphis gossypii* and *Myzus persicae*. p. 361-363 *in* P. Moreno, J. V. da Graça, and L. W. Timmer [eds.], Proc. Conf. Intl. Org. Citrus Virol., 12th. Riverside California.

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. *Proceedings of the 10th Conference of the International Organization of Citrus Virologists IOCV,* pp: 68–70. Riverside, CA.

Hermozo de Mendoza, A., Ballester-Olmos, J.F., Pinà, 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. Pages 63-64 In: *Proc. Conf. Int. Organ. Citrus Virol., 10th* Timmer, L.W., Garnsey, S.M. and Navarro, L., eds. IOCV, Riverside, CA.

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: 9th Conf. Int. Organ. Citrus Virol.*, Riverside, CA. 68-70.

Herrera-Isidrón, L., Ochoa-Sánchez, J.C., Rivera-Bustamante, R. and Martínez-Soriano J. P. 2009. Sequence diversity on four ORFs of *citrus tristeza virus* correlates with pathogenicity. *Virology Journal*, **6**:116.

Herron, C.M., Mirkov, T.E., da Graça, J.V. and Lee, R.F. 2006. *Citrus tristeza virus* transmission by the *Toxoptera citricida* vector: *In vitro* acquisition and transmission and infectivity immunoneutralization experiments. *Journal of Virological Methods* **134**: 205–211

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

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.

Hilf, M. E., and Garnsey, S. M. 2000. Characterization and classification of *Citrus tristeza virus* isolates by amplification of multiple molecular markers. *In: 14th Conf. Int. Organ.,* Riverside, CA. *Citrus Virol.*:18-27.

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., 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.

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

Hughes, W.A. and Lister, C.A. 1953. Lime dieback in the Gold Coast, a virus disease of lime, *Citrus aurantifolia* (Christmann) Swingle. *Journal of Horticultural Sciences* **28**: 131-140.

Hughes, W.A. and Lister, C.A.1949. Lime disease in the Gold Coast. *Nature* 164: 880 *pp*.

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*. *Journal of Phytopathology*, **148**: 469–475.

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.

Ilharco, F.A. and Lemos, A. 1981. Note on a simple technique for mounting aphids on microscopic slides. *Agronomia Lusitana,* **41**: 53-57.

Irwin M.E., William, Ruesink, W.G., Isard, S.A. and Kampmeier, G.E. 2000. Mitigating epidemics caused by non-persistently transmitted aphid-borne viruses: the role of the pliant environment. *Virus Research*, **71**: 185–211.

Irwin, M.E., Ruesink, W.G., 1986. Vector intensity: a product of propensity and activity. In: *McLean, G.D., Garrett, R.G., Ruesink, W.G.* (Eds.), Plant Virus epidemics: Monitoring, modelling and Predicting Outbreaks. academic Press, Sydney, 13–33.

Jarupat, T., Dodds, J. A., and Roistacher, C. N. 1988. Effect of host passage on dsRNAs of two strains of *Citrus tristeza virus*. *In: 15th Conf. Int. Organ. Citrus Virol.*, Riverside, CA. 39-44.

Jones, R.K. 2004. Effect of cotton aphids, *Aphis gossypii* (Glover) on cotton plant development and yield components. Ph. D. Thesis submitted to the graduate faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfilment of the requirements for the degree of Master of Science in the Department of Entomology. 50 pp.

Kahlon, A.S. 2005. Molecular characterization of the population diversity of selected isolates and subisolates of *Citrus Tristeza Virus* (CTV) from Florida. PhD dissertation. University of Florida, USA. 97 *pp*.

Kano, T. and Koizumi, M. 1991. Separation of citrus tristeza virus (CTV) serotypes through aphid transmission. In: Brlansky RH, Lee RF, Timmer LW, eds. *Proceedings of the 11th Conference of the International Organization of Citrus Virologists*. Riverside, CA, USA: International Organization of Citrus Virologists, 82–5.

Karasev, A.V. and Bar-Joseph, M. 2010. Citrus Tristeza virus and the taxonomy of *Closteroviridae. In* "Citrus Tristeza virus: Complex and Tristeza disease" (Karasev, A.V. and Hilf, M.E., Eds.), pp119-132. The American Phytopathological Society, Minnesota, USA.

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

Karasev, A.V., Boyko, V.P., Gowda, S., Nikolaeva, O.V., Hilf, M.E., Koonin, E.V., Niblett, C.L., Cline, K., Gumpf, D.J., 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.

Klaassen, V. A., Mayhew, D., Fisher, D., and Falk, B. W. 1996. *In vitro* transcripts from cloned cDNAs of the lettuce infectious yellows closterovirus bipartite genomic RNAs are competent for replication in *Nicotiana benthamiana* protoplasts. *Virology* **222**, 169-175.

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.

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

Koonin, E.V., Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* **28**: 375-430.

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

Kumar, S., Tamura K., Jakobson, I.B. and Nei, M. 2001. MEGA2 Molecular Evolutionary Genetic Analysis software. *Bioinformatics* **17**: 1244-1245.

Kyriakou, A., Ioannou, N., Gavriel, J., Bar-Joseph, M., Papayiannis, Chr., Kapar-Isaia, Th. and Savva, G. 1996 Management of citrus tristeza virus in Cyprus. In: Proceedings of the 13th Conference of the International Organization of Citrus Virologists (da Graça, J.V., Moreno, P. and Yokomi, R.K., eds), pp. 172–178. Riverside, CA: IOCV.

Larsen, L.A., Jespersgaard, C. And Andersen, P.S. 2007. Single-Strand Conformation Polymorphism analysis using Capillary Assay Electrophoresis for large-scale mutation detection. *Nature protocols* **2(6)**: 1458-1466.

Lee, R. F. and Bar-Joseph, M. 2000. Tristeza. Compendium of Citrus Diseases, Second Edition L. W. Timmer, S. M. Garnsey and H. Graham (Eds.) *APS Press, St. Paul*, Minn. pp: 6*1-63.*

Lee, R. F., Pappu, H. R., Pappu, S. S., Rocha-Pena, M. A., Febres, V. J., Manjunath, K. L., Nikolaeva, O. V., Karasev, A., Cevik, B., Akbulut, M., Bencher, D., Anderson, E. J., Price, M., Ochoa-Corona, F. M., and Niblett, C. L. 1996. Progress on strain differentiation of *Citrus tristeza virus*. *Phytopathology* **14**(2): 79-87.

Lee, R.F. and Rocha-Penà, M.A. 1992. *Citrus tristeza virus. In* "Diseases of fruit crops, Plant Diseases of International Importance" (Kumar, J., Chaube, H.S., Singh, U.S. and Mukhopdhyay, A.N. Eds.), Vol. III, pp. 226-249. Prentice Hall, Englewood Cliffs, New Jersey.

Leh, V., Jacquot, E., Geldreich, A., Haas, M., Blanc, S., Keller, M., Yot, P., 2001. Interaction between Cauliflower mosaic virus ORF III product and coat protein is required for transmission of the virus by aphids. *J. Virol*. **75**, 100–106.

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

Limburg, D. D., Mauk, P. A., & Godfrey, L. D. 1997. Characteristics of beet yellows closterovirus transmission to sugar beets by *Aphis fabae*. *Phytopathology* **87**, 766–771.

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. *Journal of General Virology*, **85**: 2099– 2102.

Lòpez, C., Navas-Castillo, J., Gowda, S., Moreno, P., and Flores, R. 2000. The 23-kDa protein coded by the 39-terminal gene of citrus tristeza virus is an RNA-binding protein. *Virology*, **269**: 462-470.

Lòpez, C., Ayllon, 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.

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. *Proc. Natl Acad. Sci.* USA, 101, 15742–15747.

Lutting, M., van Vuuren, S.P., van der Vyver, J.B. 2002. Differentiation of single aphid sub-isolates from two South African Citrus Tristeza Virus isolates from grapefruit by single strand conformation polymorphism. *In* "Proceeding of the 15th conference of the International Organization of Citrus Virologists." (N. Duran-Vila, R.G. Milne and J.V. da Graça Eds.), pp. 186-196, IOCV, Riverside, CA.

Marroquin, C., Olmos, A., Gorris, M.T., Bertoloni, E., Martìnez, 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 Dis*. **48**: 131-133.

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

Mawassi, M.E., Gafny, R. and Bar-Joseph, M. 1993. The nucleotide sequence of coat protein gene of *Citrus tristeza virus:* Comparison of biologically diverse isolates collected in Israel. *Virus Genes* **7**: 265-275.

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.

Mestre, P.F., Asins, M.J., Carbonell, E.A. and Navarro, L. 1997. New gene(s) involved in the resistance of *Poncirus trifoliate* (L.) Raf. to *Citrus Tristeza Virus*. *Theor. Appl. Genet*, **95**: 691-695.

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

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

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

Moreno, P., Guerri, J., Ballester-Olmos, J. F., Fuertes-Polo, C., Albiach, R., and Martinez, M. E. 1993a. Variations in pathogenocity and double stranded (dsRNA) patterns of *Citrus tristeza virus* isolates induced by host passage. *In: 12th Conf. Int. Organ. Citrus Virol.*, Riverside, CA: 8-15.

Moreno P, Guerri J, Ballester-Olmos JF, Fuertes-Polo C, Albiach R, Martı´nez ME, 1993b. Variations in pathogenicity and double-stranded RNA (dsRNA) patterns of citrus tristeza virus isolates induced by host passage. In: Moreno P, da Grac¸a JV, Timmer LW, eds. *Proceedings of the 12th Conference of the International Organization of Citrus Virologists*. Riverside, CA, USA: International Organization of Citrus Virologists, 8–15.

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.

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: Proceedings of the 15th Conference of the International Organization of Citrus Virologists (Duran-Vila, N., Milne, R.G. and da Graça, J.V., eds), pp. 306–316. Riverside, CA: IOCV.

Navas-Castillo, J., Albiach-Martı, 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.

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.

Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G. and Tribulato, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics* **100** (8): 1155-1166.

Nolasco, G., Santos, C., Silva, G. and Fonseca, F. 2008. Development of an asymmetric PCR-ELISA typing method for citrus tristeza virus based on the coat protein gene. *Journal of Virological Methods*, **155**, 97–108.

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. *Journal of Virological 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. Entomol*. **65**: 593-594.

Norman, P.A., R.A. Sutton, and A.K. Burditt, Jr. 1968. Factors affecting transmission of tristeza virus by melon aphids. *J. Econ. Entomol*. **61**: 238-242.

Ochoa, F., Carballo, O.,Trujillo, G., Mayoral de Izaquirre, M.L. and Lee, R.F. 1993. Biological characterization and evaluation of cross-protection potential of citrus tristeza isolates in Venezuela. *In "Proceedings of the 12th Conference of the International Organization of Citrus Virologists*." (Moreno, P., da Graça, J.V. and Timmer, L.W., Eds.), pp. 1-7. IOCV, Riverside, California.

Palacios, I., Drucker, M., Blanc, S., Leite, S., Moreno, A., Fereres, A., 2002. *Cauliflower mosaic virus* is preferentially acquired from the phloem by its aphid vectors. *J. Gen. Virol.* **83**, 3163–3171.

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

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.

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.

Pearson, W. and Lipman, D. 1988. Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences* USA, **85**, 2444-2448.

Peng, C.W., Peremyslov, V.V., Mushegian, A.R., Dawson, W.O. and Dolja, VV. 2001. Functional specialization and evolution of leader preteinases in the family *Closteroviridae. J. Virol*. **75**: 12153-12160.

Peremyslov, V. V., Hagiwara, Y., and Dolja, V. V. 1999. HSP70 homolog functions in cell-to-cell movement of a plant virus. *Proc. Natl. Acad. Sci.* (USA), **96**:14771-14776.

Peremyslov, V. V., Hagiwara, Y., and Dolja, V. V. 1998. Genes required for replication of the 15.5-kilobase RNA genome of a plant closterovirus. *J. Virol.* **72**: 5870-5876.

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.

Permar, T.A. and Garnsey, S.M. 1988. A monoclonal antibody that discriminates strains of citrus Tristeza virus. *Phytopathology* **80**: 224-228.

Pirone, T.P., Blanc, S., 1996. Helper-dependent vector transmission of plant viruses. *Annu. Rev. Phytopathol.* **34**, 227–247.

Pitrat, M. & H. Lecoq, 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 Dis*. **87**:1116-1118.

Powell, C.A., Pelosi, R.R. and Cohen, M. 1992. Superinfection of orange trees containing mild isolates of *Citrus Tristeza Virus* and severe Florida isolates of *Citrus Tristeza Virus*. *Plant Disease* **76**: 141-144.

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

Raccah, B., Bar-Joseph, M. and Loesbenstein, G. 1978. The role of aphid vectors and variation in virus isolates in the epidemiology of tristeza disease. Pages 221-227 In: Plant Disease Epidemiology. P.R. Scott and A. Bainbridge, eds. Blackwell Scientific Publications, Oxford, England.

Raccah, B., Leobenstein, G., Bar-Joseph, M., Oren, Y., 1976. Transmission of tristeza by aphids prevalent on citrus and operation of the tristeza suppression program in Israel. *In*:Calavan, E.C. (Ed.), "Proceedings of the 7th Conference of the IOCV". Riverside, CA, pp. 47–49.

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

Retuerma, M.L., Price, W.C., 1972. Evidence that tristeza virus is stylet-borne. *FAO Plant Prot. Bull*. 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, 10p. <http://plantpath.ifas.ufl.edu>

Rocha-Pena, M. A., Lee, R. F., Lastra, R., Niblett, C. L., Ochoa-Corona, F. M., Garnsey, S. M. and Yokomi, R. K. 1995. *Citrus Tristeza virus* and its aphid vector *Toxopera citricida*: Threats to citrus production in the Caribbean and central and North America. *Plant Disease* **79** (5): 437-444.

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. 1991. Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. FAO Eds, Rome: 286 pp.

Roistacher C.N. and Moreno P. 1991. The worldwide threat from destructive isolates of citrus tristeza virus. In Davino S., Rubio, L. and Davino, M. 2005. Molecular analysis suggests that recent *Citrus tristeza virus* outbreaks in Italy were originated by at least independent introductions. *European Journal of Plant Pathology* **111**: 289-293.

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. *Proceedings of the 9th Conference of the International Organization of Citrus Virologists IOCV*, pp: 9–18. Riverside, CA.

Roistacher, C.N., Bar-Joseph, M., and Gumpf, D.J. 1984. Transmission of tristeza and seedling yellows tristeza by small populations of *Aphis gossypii. Plant Disease* **68**:494- 496.

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. Pages 76-82 In: *Proc. Conf. Int. Organ. Citrus Virol., 8th* Calavan, E.C., Garnsey, S.M. and Timmer L.W. eds. IOCV, Riverside, CA.

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 Dis*. **88**, 453–467.

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

Rubio, L., Ayllòn, M.A., Kong, P., Fernandez, A., Polek, M., Guerri, J., Moreno, P. And Falk, B.W. 2001. Genetic variation of citrus tristeza virus isolates from California and Spain: Evidence for mixed infections and recombination. *Journal of Virology* **75** (17): 8054-8062.

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

Rubio, L., Yeh, H.H., Tian, T. and Falk, B. W. 2000b. Heterogeneous population of defective RNAs associated with *Lettuce infectious yellows virus. Virology* **271**:205–212.

Rubio, L., Ayllòn, 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. *Annals of Applied Biology* **129**: 479-489.

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. *Archive of Virology*. **152**, 1115–1126.

Sambade, A., Lòpez, C., Rubio, L., Flores, R., Guerri, J. and Moreno, P. 2003. Polymorphism of a specific region in gene *p*23 of Citrus Tristeza Virus allows discrimination between mild and severe isolates. *Arch. Virol.*, **148**: 2325-2340.

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.

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

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

Satyanarayana, T. Bar-Joseph, M., Mawassi, M., Albiach-Marti, M.R., Ayllon, M.A., Gowda, S., Hilf, M.E., Moreno, P., Garnsey, S.M. and Dawson, W.O. 2001. Amplification of *Citrus tristeza* virus from a cDNA clone and infection of *Citrus trees*. *Virology* **280**: 87-96.

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

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. *Proceedings of the National Academy of Sciences of the United States of America*, **101**: 799–804.

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

Satyanayanana, 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.

Schwarz, R.E. 1965. Aphid-borne virus diseases of citrus and their vectors in South Africa. Flight activity of citrus aphids. *S. Afr. J. Agric. Sci*. **8**: 931-940.

Schneider, H. 1959. The anatomy of tristeza-virus-infected citrus. Pages 73-84 In “Citrus Virus Diseases. J.M. Wallace, eds.” University of California, Div. Agric. Sci. Berkeley, CA.

Selma, G.Z., Norma, C., Liliana, S. and Oscar, G. 1999. Sequence variability in p27 gene of Citrus Tristeza Virus (CTV) revealed by SSCP analysis. *Plant Biotechnology* **2**: 9 pp.

Sentandreu, V., Castro, J.A., Ayllón, M.A., Rubio, L., Guerri, J., González-Candelas, F., Moreno, P. and Moya, A. 2006. Evolutionary analysis of genetic variation observed in *citrus tristeza virus* (CTV) after host passage. *Archive of Virology*. **151**, 875–894.

Sharma, S. R. 1989. Factors affecting vector transmission of citrus tristeza virus in South Africa. *Zentralblatt für Mikrobiologie* **144**: 283-294.

Sieburth, P.J., Nolan, K.G., Hilf, M.E., Lee, R.F., Moreno, P. and Garnsey, S.M. 2005. Discrimination of stem-pitting from other isolates of *citrus tristeza virus*. *Proceeding of the 16th Conference of International Organization of Citrus Virologists IOCV*, pp. 1–10. Riverside, CA.

Stoezel, M.b. 1994. Aphids (Homoptera: Aphididae) of potential importance on citrus in the United States with illustrated keys to species. *Proceedings of the Entomological Society of Washington,* **96**: 74-90.

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. Virol.,* **80**: 1111-1117.

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

Tolkowsky, S. 1938. Hesperides. A history of the Culture and Use of Citrus Fruit. Vale and Curnow, London.

Toxopeus, H.J. 1937. Stock-scion incompatibility in citrus and its cause. *J. Pom. Hort. Sci.* **14**: 360-364.

Valiela, M.F.V. 1948. Informe preliminary acerca de la etiologia de la "Podredumbre de las raicillas" del naranjo agrio injertado. *Rev. Invest. Agric*. **2**: 139-146.

Van den Heuvel, J.F.J.M., Bruyere, A., Hogenhout, S.A., Ziegler-Graff, V., Brault, V., Verbeek, M., Van Der Wilk, F. and Richards, K. 1997. The N-terminal region of luteovirus readthrough domain determines binding to *Buchnera* GroEL and is essential for virus persistence in the aphid. *J. Virol* **71**: 7258-7265.

Vun Vuuren, S.P., Collins, R.P., and da Graça, J.V. 1991. The performance of exotic *Citrus tristeza virus* isolates as preimmunizing agents for sweet orange on sour orange rootstock under natural disease pressure in South Africa. In "*Proceedings of the 11th Conference of the International Organization of Citrus Virologists*" (Brlansky, R.H., Lee, R.F. and Timmer, L.W. Eds.), pp. 60-63. IOCV, Riverside, California.

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.

Wallace, J.M. 1956. Tristeza disease of citrus, with special reference to its situation in the United States. F*AO Plant Prot. Bul*l **4**: 77-94.

Webber, H. J. 1948. History and development of the citrus industry. *The Citrus Industry*-Volume I, *University of California Press, Riverside,* pp: 1-25.

Webber, H.J. 1943. The "Tristeza" disease of sour orange rootstock. *Proc. Amer. Soc. for Hort. Sci.* **43**: 160-168.

Woolston, C.J., Czaplewski, L.G., Markham, P.G., Goad, A.S., Hull, R., Davies, J.W., 1987. Location and sequence of a region of cauliflower mosaic virus gene II responsible for aphid transmissibility. *Virology* **160**, 246–251.

Wunderlich, L. 2000. Aphid identification 101: Yield and Cost Comparison of Standard and IPM Lettuce Pest Management Field Assistant Job Announcement. *Valley Views: The Newsletter of the Central Coast Vegetable IPM Project*. 8pp.

Yahiaoui D., Addante R. and Djelouah K'. 2009. Preliminary *Citrus tristeza virus* (CTV) vectors monitoring in Apulia region In: *Citrus Tristeza Virus* and *Toxoptera citricidus*: A serious threat to the Mediterranean citrus industry Options Méditerranéennes, Série B: Studies and Research, N° B 65, IAM-Bari "Centre International de Hautes Etudes Agronomiques Méditerranéennes" . *In press.*

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 Physiol*. **131**: 482–492.

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.

Yokomi, R.K., Polek, M. and Gumpf, D.J. 2010. Transmission and Spread of Citrus Tristeza Virus in Central California. *In "Citrus Tristeza virus: Complex and Tristeza disease" (Karasev, A.V. and Hilf, M.E.,* Eds.*)*, pp151-165. *The American Phytopathological Society,* Minnesota, USA.

Yokomi, R.K., Lastra, R., Stoetzel, M.B., Damgstreet, V.D., Lee, R.F., Garnsey, S.M., Rocha-Pena, 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. Entomol.* **87**: 1078-1085.

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" pp. 194-198. CATIE-

University of Florida-INIFAP/SAH-Universidad de Central de Venezuela-USDA, Maracay, Venezuela.

Yokomi, R. K., and Damsteegt, V. C. 1991. Comparison of citrus tristeza virus transmission efficacy by *Toxoptera citricidus* and *Aphis gossypii*. *In* "Proceedings, Aphid–Plant Interactions: Populations to Molecules" (D. C. Peters, J. A. Webster, and C. S. Choubler, Eds.), pp. 229–241. Stillwater, UK.

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

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" (Lastra, R., Ochoa,, F.L., Lee, R.F., Niblett, C.L., Rocha-Penà, M.A., Garnsey, S.M. and Yokomi, R.K. Eds), pp. 194-198. CATIE-University of Florida-INIFAP/SAH-Universidad de Central Venezuela-USDA, Maracay, Venezuela.

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.

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

Zeman, V. 1930. Una enfermada nueva en los naranjales de Corrientes. *Physis*. **19**, 410-411.

Zemzami, M., Soares, C. M., Bailey, A. M., Niblett, C. L., & Nolasco, G. 2002. Molecular characterization and classification of Morrocan isolates of Citrus tristeza closterovirus. *In* J. V. da Graça, N. Duran & R. G. Milne (Eds.), Proceedings of the 15th Conference of the International Organization of Citrus Virologists (IOCV) (pp. 8–12). Riverside.

Zemzami, M., Daden, M., Djelouah, K., Frasheri, D., Soulaimani, A. and D'Onghia, A.M. 2007. Serological characterization of a collection of Mediterranean citrus tristeza virus (CTV) isolates. *In Proceeding for the Mediterranean Network on Certification of Citrus*, *Options Méditerraéennes,* Serie B, CIHEAM publications: *In Press*.

ANNEX 1. 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 (1 M) 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 2. Buffers and gels used for electrophoresis

Buffer	Material	Quantity	Note
TAE 20x (stock solution: 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 (stock solution: 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-TBE gel	Distilled water Acryl-bis 40% TAE 3x 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 3x 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 de-ionized water
Nitric acid solution	Nitric acid pure	1ml	- Bring volume to 100 ml using de-ionized water
Silver equilibrium solution	$AgNO_3$ Formaldehyde 37%	100 mg 150 µl	- Bring volume to 100 ml using de-ionized 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 3. Bacterial media and solutions used for cloning and plasmid DNA extraction

Solutions	Components	Quantities	Notes
STET (100 ml) pH 8.0	NaCl Tris-HCl EDTA Saccarose Triton X-100	0.1 M 2 mM 50 Mm 8% 0,5%	
Lysozyme		20 mg/ml	- Stored at -20°C
Ammonium acetate pH 5.5	CH_3CO_2NH4	5 M	- Sterilize by autoclaving
PEG-NaCl	PEG 8000 NaCl	20% 2,5%	- Sterilize by autoclaving
Luria-Bertani (LB) Liquid	Tryptone Yeast extract NaCl	1% 0,5% 0,5%	- Sterilize by autoclaving
Luria-Bertani (LB) Solid	Tryptone Yeast extract NaCl Agar	1% 0,5% 0,5% 0,3%	- Sterilize by autoclaving
TE Buffer (pH 8)	Tris EDTA	1 mM 1 mM	- Sterilize by autoclaving

ANNEX 4. 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 5. 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 three-quarters black. Siphunculi slightly swollen over distal two-thirds. Cauda with a constriction.	**I**
	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*

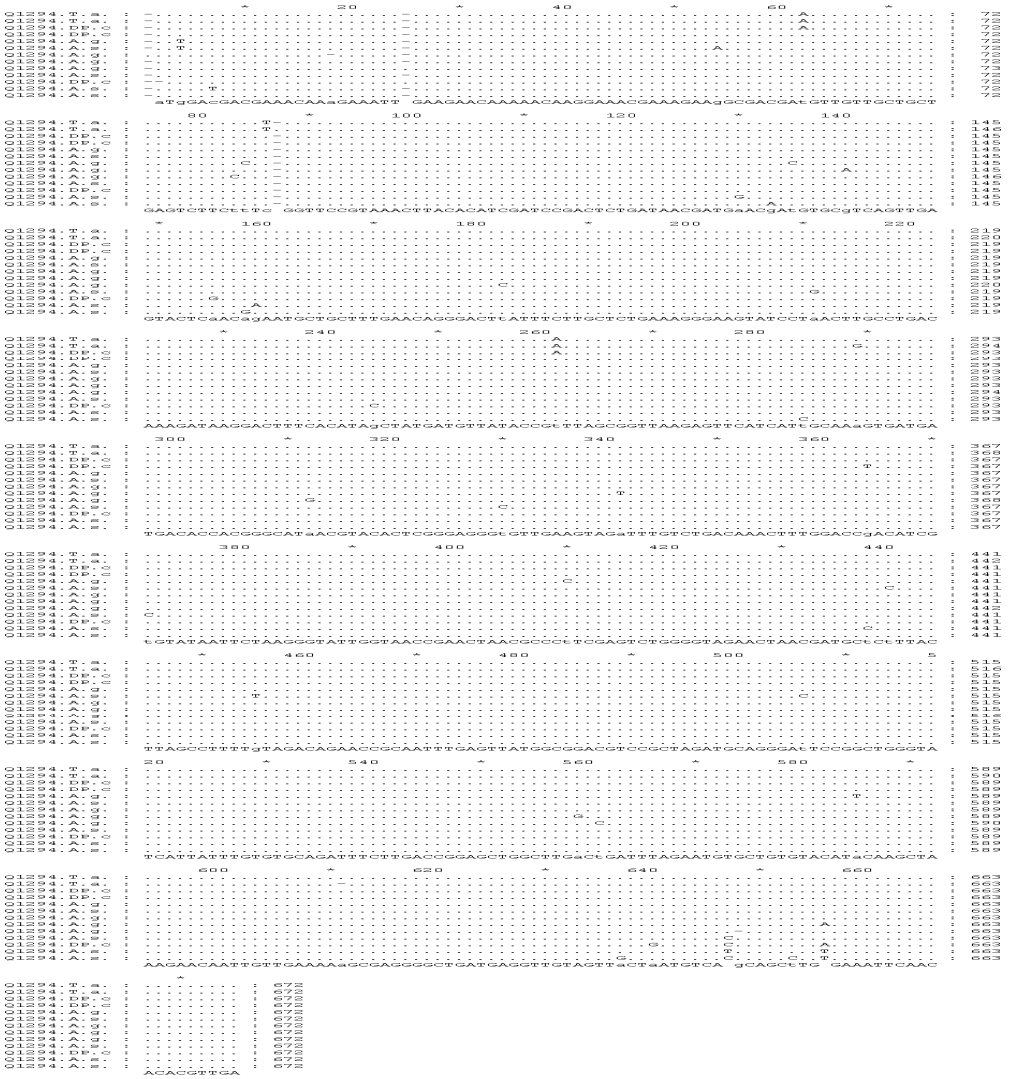
ANNEX 6. Buffers used in serological assays: DTBIA and ELISA

(Clark and Bar-Joseph, 1984)

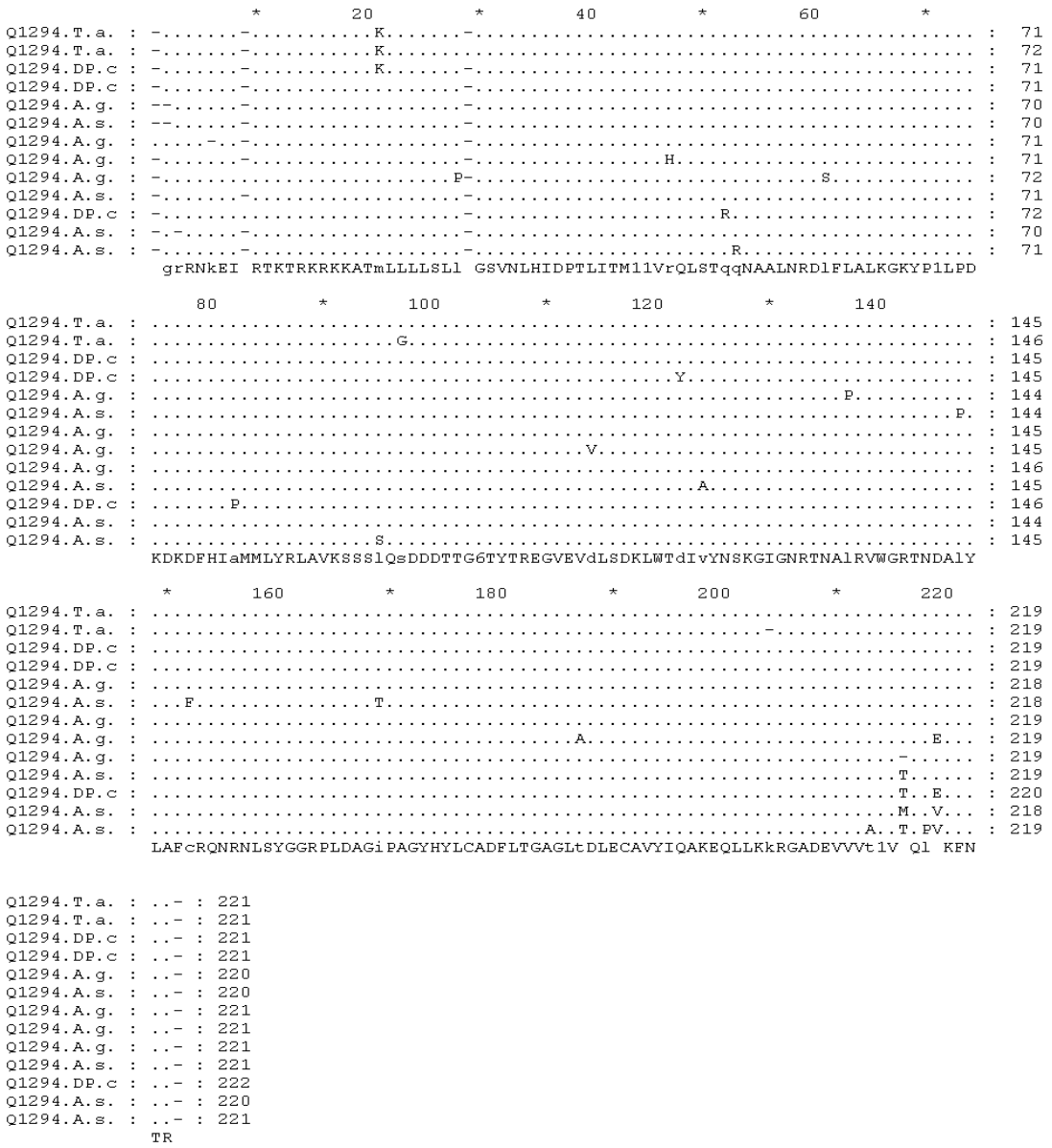
Buffer	Components	Proportions	Notes
Phosphate buffer saline (PBS) 1X; pH 7.4	NaCl KH_2PO_4 Na_2HPO_4 NaN_3 KCl	8,0 g 0,20 g 1,15 g 0,20 g 0,20 g	Dissolution in 1l distillated water.
Washing buffer (W.B); pH 7.4	PBS (1X) Tween-20	1l 0,5 ml	
Extraction buffer (E.B); pH 7.4	Polyvinilpirolidine Tween-20	20.0 g 0,5 ml	Dissolution in 1lof 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 0,5 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 7. CTV p25 nucleotide sequence of the MAIB_Q1294 isolate and its aphid derived sub-isolates

D.P: donor plant, A.g., A.s. and T.a.: A. gossypii, A. spiraecola and T. aurantii sub-isolates, respectively.



Annex 8. Predicted p25 amino-acid sequence of the CTV MAIB_Q1294 isolate and its aphid derived sub-isolates



* *D.P: donor plant, A.g., A.s. and T.a.: A. gossypii, A. spiraecola and T. aurantii sub-isolates, respectively.*

Annex 9. CTV p25 nucleotide sequence of the SG29 isolate and its aphid derived sub-isolates

```
                  380         *       400         *       420         *       440
SG29.A.g.6 : ..................................................................... : 444
SG29.A.s.c : ..................................................................... : 444
SG29.A.g.1 : ..................................................................... : 444
SG29.A.g.1 : ..................................................................... : 444
SG29.A.s.c : ..................................................................... : 444
SG29.A.g.6 : ..................................................................... : 444
SG29.A.g.1 : ..................................................................... : 444
SG29.DP    : .....................................G.....................C............ : 444
SG29.A.g.1 : .....................................G.....................C............ : 444
SG29.A.s.c : ..................................................................C.... : 444
             TTAATTCTAAGGGTATCGGTAACCGTACTAACGCCCTTCGaGTCTGGGGTAGAAGTAACGA GCCCTTTATTTA

                  *       460         *       480         *       500         *       5
SG29.A.g.6 : ..................................................................... : 518
SG29.A.s.c : ..................................................................... : 518
SG29.A.g.1 : ..................................................................... : 518
SG29.A.g.1 : .....C............................................................... : 518
SG29.A.s.c : ..................................................................... : 518
SG29.A.g.6 : ..................................................................... : 518
SG29.A.g.1 : ..................................................................... : 518
SG29.DP    : ..................................................................... : 518
SG29.A.g.1 : ..................................................................... : 518
SG29.A.s.c : ..................................................................... : 518
             GCGTTtTGTAGACAGAATCGCAATTTGAGTTATGGCGGACGTCCGCTAGATGCAGGGATTCCGGCCGGGTATCA

             20          *       540         *       560         *       580         *
SG29.A.g.6 : ..................................................................... : 592
SG29.A.s.c : ..................................................................... : 592
SG29.A.g.1 : ..................................................................... : 592
SG29.A.g.1 : ..................................................................... : 592
SG29.A.s.c : ..................................................................... : 592
SG29.A.g.6 : .....................................G............................... : 592
SG29.A.g.1 : ..................................................................... : 592
SG29.DP    : ..................................................................... : 592
SG29.A.g.1 : ..................................................................... : 592
SG29.A.s.c : ..................................................................... : 592
             TTACCTGTGTGCAGATTTCTTGACCGGAGCTGGCTTGaCTGATTTAGAATGTGCTGTGTACATACAAGCTAAAG

                  600         *       620         *       640         *       660
SG29.A.g.6 : ..................................................................... : 666
SG29.A.s.c : ..................................................................... : 666
SG29.A.g.1 : ..................................................................... : 666
SG29.A.g.1 : ............................A.......................................... : 666
SG29.A.s.c : ..................................................................... : 666
SG29.A.g.6 : ..................................................................... : 666
SG29.A.g.1 : ..................................................................... : 666
SG29.DP    : ...................G.............................................. : 666
SG29.A.g.1 : ...................G.............................................. : 666
SG29.A.s.c : ..................................................................... : 666
             AACAATTGTTGAAGAAGCGaGGGGCTGAtGAAATCGTAGTTACCAATGTCAGGCAGCTTGGGAAATTCAACACA

               *
SG29.A.g.6 : ..... : 672
SG29.A.s.c : ..... : 672
SG29.A.g.1 : ..... : 672
SG29.A.g.1 : ..... : 672
SG29.A.s.c : ..... : 672
SG29.A.g.6 : ..... : 672
SG29.A.g.1 : ..... : 672
SG29.DP    : ..... : 672
SG29.A.g.1 : ..... : 672
SG29.A.s.c : ..... : 672
             CGTTGA
```

** D.P: donor plant, A.g., A.s.: A. gossypii and A. spiraecola sub-isolates, respectively.*

Annex 10. Predicted p25 amino-acid sequence of the CTV SG29 isolate and its aphid derived sub-isolates

```
                    *       20        *       40        *       60        *
SG29.A.g.6 : ..................................................................... :  74
SG29.A.s.c : ..................................................................... :  74
SG29.A.g.1 : DY................................................................... :  74
SG29.A.g.1 : ..................................................................... :  74
SG29.A.s.c : ..................................................................... :  74
SG29.A.g.6 : .....................................................................S.... :  74
SG29.A.g.1 : ..................................................................... :  74
SG29.DP    : ..................................................................... :  74
SG29.A.g.1 : ..................................................................... :  74
SG29.A.s.c : ..................................................................... :  74
             mdDETKKLKNKNKEAKEGDDVVAAESSFGSLNFHIDPTLIAMNDVRQLSTQQNAALNRDLFLTLKGKYPnLSDK

                 80        *       100        *       120        *       140
SG29.A.g.6 : ..................................................................... : 148
SG29.A.s.c : ..................................................................... : 148
SG29.A.g.1 : ..................................................................... : 148
SG29.A.g.1 : ..................................................................... : 148
SG29.A.s.c : .....F............................................................... : 148
SG29.A.g.6 : ..................................................................... : 148
SG29.A.g.1 : ....................................G............................. : 148
SG29.DP    : ..................................................................... : 148
SG29.A.g.1 : ..................................................................... : 148
SG29.A.s.c : ..................................................................... : 148
             DKDFHlAM6LYRLAVKSSSLQSDDDTTGI3YTREGVEVDLSdKLWTDVVFNSKGIGNRTNALRVWGRSNDALYL

                  *       160        *       180        *       200        *       220
SG29.A.g.6 : ..................................................................... : 222
SG29.A.s.c : ..................................................................... : 222
SG29.A.g.1 : ..................................................................... : 222
SG29.A.g.1 : ..............................................................E...... : 222
SG29.A.s.c : ..................................................................... : 222
SG29.A.g.6 : ....................................A............................... : 222
SG29.A.g.1 : ..................................................................... : 222
SG29.DP    : ..................................................................... : 222
SG29.A.g.1 : ..................................................................... : 222
SG29.A.s.c : ..................................................................... : 222
             AFCRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLtDLECAVYIQAKEQLLKKRGAdEIVVTNVRQLGKFNT


SG29.A.g.6 : .- : 223
SG29.A.s.c : .- : 223
SG29.A.g.1 : .- : 223
SG29.A.g.1 : .- : 223
SG29.A.s.c : .- : 223
SG29.A.g.6 : .- : 223
SG29.A.g.1 : .- : 223
SG29.DP    : .- : 223
SG29.A.g.1 : .- : 223
SG29.A.s.c : .- : 223
             R
```

* *D.P: donor plant, A.g., A.s: A. gossypii and A. spiraecola sub-isolates, respectively*

Annex 11. CTV p25 nucleotide sequence of the MAIB_Q54 isolate and its aphid derived sub-isolates

```
                    *        20         *        40         *        60         *
Q54.DP.cl2 : ..................................................................... :  74
Q54.A.g.cl : ..................................................................... :  74
Q54.DP.cl1 : ..................................................................... :  74
Q54.A.g.cl : GATT................................................................. :  74
Q54.A.g.cl : CGAT................................................................. :  74
             ACGACGAAACAAAGAAATTGAAGAACAAAAACAAGGAAACGAAAGAAGGCGACAATGTTGTTGCAGCGGA

                  80         *       100         *       120         *       140
Q54.DP.cl2 : ..................................................................... : 148
Q54.A.g.cl : ..................................................................... : 148
Q54.DP.cl1 : ..................................G.................................... : 148
Q54.A.g.cl : ..................................................................... : 148
Q54.A.g.cl : ..............................T...................................... : 148
             GTCTTCTTTCGGTTCTGTAAACTTACACATcGATCCGaCTCTGATAGCGATGAACGATGTGCGTCGGTTGGGTA

                   *       160         *       180         *       200         *       220
Q54.DP.cl2 : ..................................................................... : 222
Q54.A.g.cl : ........G..........................................................G..... : 222
Q54.DP.cl1 : .........G...........T..............................................G..... : 222
Q54.A.g.cl : ................C.....................................................G..... : 222
Q54.A.g.cl : .........G.........G..................................................... : 222
             CCCAACAGa TGCCGCTtTGaAcAGAGATTTGTTTCTTACTTTGAAAGAGAAGTATCCTAATTTGTCTGATAAA

                    *       240         *       260         *       280         *
Q54.DP.cl2 : ..................................................................... : 296
Q54.A.g.cl : ..................................................................... : 296
Q54.DP.cl1 : ..................................................................... : 296
Q54.A.g.cl : ........................G........................................G..... : 296
Q54.A.g.cl : ..................................................................... : 296
             GATAAGGACTTTCACTTAGCTATGaTGTTGTATCGTTTAGCGGTTAAGAGTTCATCATTGCAAAGCGATGACGA

                   300         *       320         *       340         *       360         *
Q54.DP.cl2 : ..................................................................C..... : 370
Q54.A.g.cl : ........................T.....................C..........................G..... : 370
Q54.DP.cl1 : ..................................................................... : 370
Q54.A.g.cl : ..................................................................... : 370
Q54.A.g.cl : ..................................................................... : 370
             CACTACGGGTATAACGTACACTcGGGAGGGCGTCGAAGTGGAtTTGTCTGACAAACTTTGGACtGACGTCGTGT

                    380         *       400         *       420         *       440
Q54.DP.cl2 : ..................................................................... : 444
Q54.A.g.cl : ..........................................A.......................G..... : 444
Q54.DP.cl1 : ..................................................................... : 444
Q54.A.g.cl : ..................................................................... : 444
Q54.A.g.cl : ..................................................................... : 444
             TTAATTCTAAGGGTATCGGTAACCGTACTAACGCCCTTCGAGtCTGGGGTAGGACTAACGATGCCCTTTATTTA

                  *       460         *       480         *       500         *       5
Q54.DP.cl2 : ..................................................................... : 518
Q54.A.g.cl : ..................................................................... : 518
Q54.DP.cl1 : ..................................................................... : 518
Q54.A.g.cl : ..................................................................... : 518
Q54.A.g.cl : ..................................................................... : 518
             GCCTTTTGTAGACAGAATCGCAATTTGAGTTATGGCGGACGTCCGCTAGATGCAGGGATTCCGGCCGGGTATCA

             20         *       540         *       560         *       580         *
Q54.DP.cl2 : ..................................................................... : 592
Q54.A.g.cl : ..................................................................... : 592
Q54.DP.cl1 : ..................................................................... : 592
Q54.A.g.cl : ..................................................................... : 592
Q54.A.g.cl : ..................................................................... : 592
             TTACCTGTGTGCAGATTTCTTGACCGGAGCTGGCTTGACTGATTTAGAGTGTGCTGTGTACATACAAGCTAAAG

                  600         *       620         *       640         *       660
Q54.DP.cl2 : ..................................................................... : 666
Q54.A.g.cl : ..................................................................... : 666
Q54.DP.cl1 : ..................................................................... : 666
Q54.A.g.cl : ..................................................................... : 666
Q54.A.g.cl : ..................................................................... : 666
             AACAATTGTTGAAGAAGCGAGGGGCTGATGAAGTCGTAGTTACCAATGTCAGGCAGCTTGGGAAATTCAACACA

               *
Q54.DP.cl2 : ...... : 672
Q54.A.g.cl : ...... : 672
Q54.DP.cl1 : ...... : 672
Q54.A.g.cl : ...... : 672
Q54.A.g.cl : ...... : 672
             CGTTGA
```

** D.P: donor plant; A.g: A. gossypii sub-isolates.*

Annex 12. Predicted p25 amino-acid sequence of the CTV MAIB_Q54 isolate and its aphid derived sub-isolates

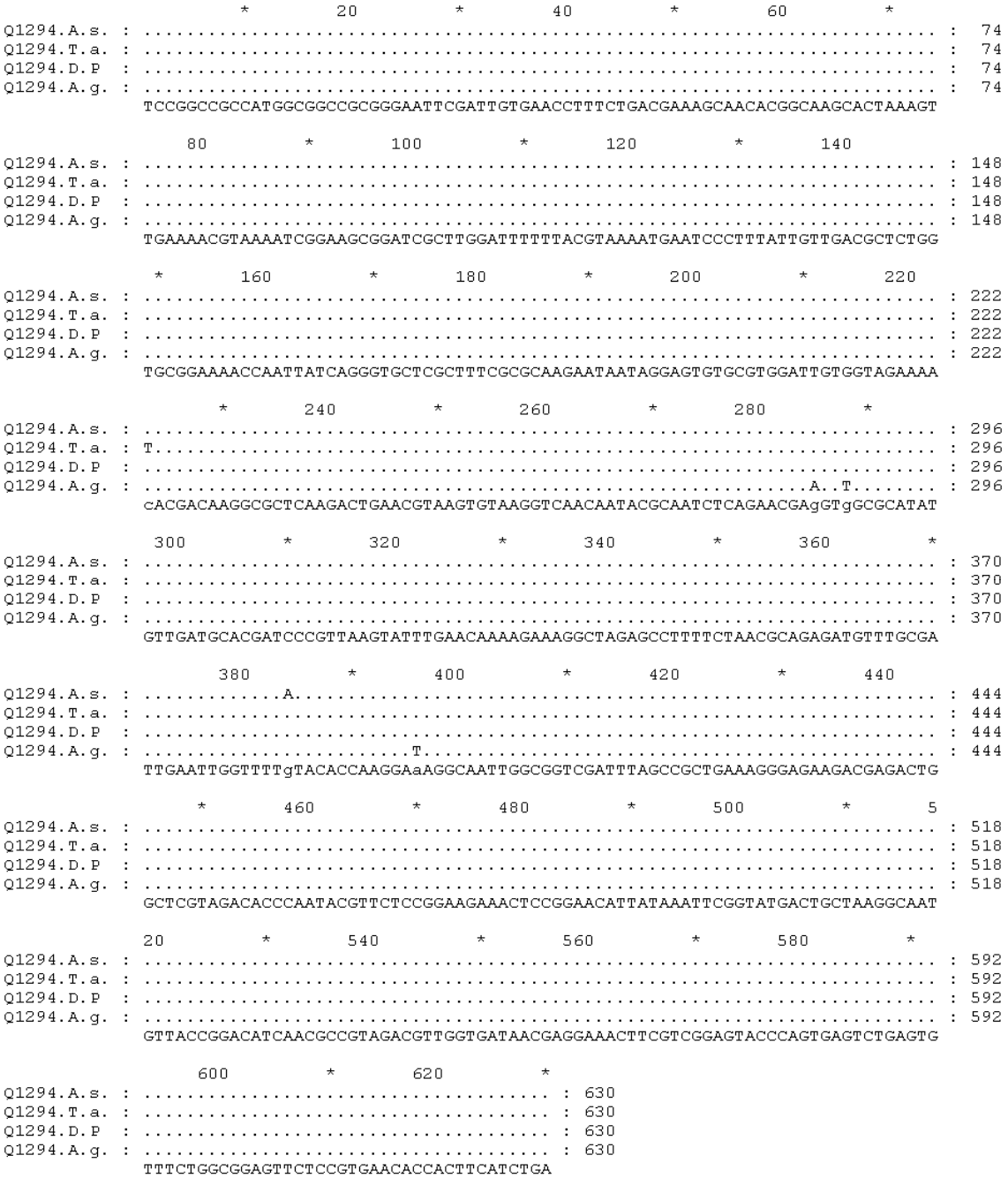
```
                    *        20         *        40         *        60         *
Q54.DP.cl2 : ..................................................................... :  75
Q54.A.g.cl : ..................................................................... :  75
Q54.DP.cl1 : ..................................A.............S..................... :  75
Q54.A.g.cl : DY................................................................... :  75
Q54.A.g.cl : RY..............................................S..................... :  75
               DETKKLKNKNKETKEGDNVVAAESSFGSVNLHIDPtLIAMNDVRRLGTQQ AAL1RDLFLTLKEKYPNLSDKD

                 80         *       100         *       120         *       140         *
Q54.DP.cl2 : ..................................................................... : 150
Q54.A.g.cl : ..............................W.............................D............ : 150
Q54.DP.cl1 : ..................................................................... : 150
Q54.A.g.cl : ..................................................................... : 150
Q54.A.g.cl : ..................................................................... : 150
             KDFHLAM6LYRLAVKSSSLQSDDDTTGITYTrEGVEVDLSDKLWTDVVFNSKGIGNRTNALRvWGRTNDALYLAF

                    160         *       180         *       200         *       220
Q54.DP.cl2 : .....................................................................- : 223
Q54.A.g.cl : .....................................................................- : 223
Q54.DP.cl1 : .....................................................................- : 223
Q54.A.g.cl : .....................................................................- : 223
Q54.A.g.cl : .....................................................................- : 223
             CRQNRNLSYGGRPLDAGIPAGYHYLCADFLTGAGLTDLECAVYIQAKEQLLKKRGADEVVVTNVRQLGKFNTR
```

* *D.P: donor plant, A.g.: A. gossypii sub-isolates.*

Annex 13. CTV p23 nucleotide sequence of the MAIB_Q1294 isolate and its aphid derived sub-isolates



* *D.P: donor plant, A.g., A.s. and T.a.: A. gossypii, A. spiraecola and T. aurantii sub-isolates, respectively.*

Annex 14. CTV p23 nucleotide sequence of the SG29 isolate and its aphid derived sub-isolates

```
                  *       20        *       40        *       60        *
SG29.A.g.6 : ..................................................................... :  74
SG29.A.s.  : ..................................................................... :  74
SG29.D.P   : ....G................................................................ :  74
SG29.A.g.1 : ...................................................................G.. :  74
             ATGGaCGATACTAGCGGACAAACTTTTATTTCTGTGAACCTTTCTGACGAGAGCAACACAGCTAGTACTGAaAT

                 80        *       100       *       120       *       140
SG29.A.g.6 : ..................................................................... : 148
SG29.A.s.  : ..................................................................... : 148
SG29.D.P   : ..................................................................... : 148
SG29.A.g.1 : ..................................................................... : 148
             CAAAGCCGTAAGTTCGGAAGCGGATCGCTTGGAATTTTTACGGAAAATGAATCCCTTCATTATCGACGCTTTGA

              *       160       *       180       *       200       *       220
SG29.A.g.6 : ................G.................................................... : 222
SG29.A.s.  : ..................................................................... : 222
SG29.D.P   : ..................................................................... : 222
SG29.A.g.1 : ................................................A.................... : 222
             TACGGAAAAATAGTTATCaAGGCGCTCGCTTTCGCGCGAGAATAATAGGAgTGTGCGTGGATTGTGGTAGAAAA

                    *       240       *       260       *       280       *
SG29.A.g.6 : ..................................................................... : 296
SG29.A.s.  : ..................................................................... : 296
SG29.D.P   : ................A.................................................... : 296
SG29.A.g.1 : ..................................................................... : 296
             CACGATAAGGCATCGAGgACTGAACGTAAGTGTAAGGTCAACAATACGCAGTCTCAGAACGAGGTGGCGCATAT

              300       *       320       *       340       *       360       *
SG29.A.g.6 : ..................................................................... : 370
SG29.A.s.  : ..................................................................... : 370
SG29.D.P   : ..................................................................... : 370
SG29.A.g.1 : ..................................................................... : 370
             GTTAATGCACGATCCCGTAAAATATTTAAATAAAAGAAAAGCTAGAGCCTTTTCTAACGCAGAGATGTTTGCGA

                    380       *       400       *       420       *       440
SG29.A.g.6 : ..................................................................... : 444
SG29.A.s.  : ..................................................................... : 444
SG29.D.P   : ..................................................................... : 444
SG29.A.g.1 : ..................................................................... : 444
             TCGATTTGGTTATGCACACCAAAGAAAGGCAATTAGCGGTTGATTTGGCCGCTGAAAGGGAGAAGACGAGATTG

                 *       460       *       480       *       500       *       5
SG29.A.g.6 : ..................................................................... : 518
SG29.A.s.  : ..................................................................... : 518
SG29.D.P   : ..................................................................... : 518
SG29.A.g.1 : ..................................................................... : 518
             GCTCGTAGACACCCGATGCGTTCTCCGGAAGAGACTCCGGAACATTATAAATTCGGTATGACTGCTAAGGCAAT

             20        *       540       *       560       *       580       *
SG29.A.g.6 : ..................................................................... : 592
SG29.A.s.  : ..................................................................... : 592
SG29.D.P   : ..................................................................... : 592
SG29.A.g.1 : ................................................................C.... : 592
             GTTACCGGACATCAACGCTGTAGACGTTGGTGATAACGAAGACACCTCGTCGGAGtACCCAGTAAGTCTGAGTG

                 600       *       620       *
SG29.A.g.6 : ..................................... : 630
SG29.A.s.  : ..................................... : 630
SG29.D.P   : ..................................... : 630
SG29.A.g.1 : ..............................T..... : 630
             TTTCCGGCGGAGTTCTCCGTGAACACCACTTCaTCTGA
```

* *D.P: donor plant, A.g., A.s.: A. gossypii and A. spiraecola sub-isolates, respectively.*

Annex 15. CTV p23 nucleotide sequence of the MAIB_Q54 isolate and its aphid derived sub-isolates

```
                 *        20         *        40         *        60         *
Q54.D.P  : ..................................................................... :  76
Q54.A.g. : ..................................................................... :  76
           ATGGACGATACTAGCGGACAAACTTTCATTTCTGTGAACCTTTCTGACGAAAGCAACACAGCTAGCACTGAAGTCA

             80         *       100         *       120         *       140         *
Q54.D.P  : .G................................................................... : 152
Q54.A.g. : ..................................................................... : 152
           A ACCGTAAGTTCGGAAGCGGATCGCTTGGAATTTTTACGGAAAATGAATCCCTTTATTATTGACGCTTTGATACG

                160         *       180         *       200         *       220
Q54.D.P  : ..................................................................... : 228
Q54.A.g. : ..............................G..................T..................... : 228
           GAAAACCAATTATCAGGGTGCTCGCTTTCGC CGAGAATAATAGGAGTG GCGTGGATTGTGGTAGGAAACACGAT

            *       240         *       260         *       280         *       300
Q54.D.P  : ..................................................................... : 304
Q54.A.g. : ..............T.................T.................................... : 304
           AAGGCATCGAGGAC GAACGTAAGTGTAAAG CAACAACACACAATCTCAGAACGAGGTGGCGCACATGTTAATGC

                *       320         *       340         *       360         *       380
Q54.D.P  : ..................................................................... : 380
Q54.A.g. : ..................................................................... : 380
           ACGATCCCGTGAAATATTTAAATAAAAGAAAAGCTAGAGCCTTTTCTAACGCAGAGATGTTTGCGATCGATTTGGT

                   *       400         *       420         *       440         *
Q54.D.P  : ..................................................................... : 456
Q54.A.g. : ..................................................................... : 456
           TATGCATACCAAAGAAAGGCAATTAGCGGTTGATTTGGCCGCTGAAAGGGAGAAGACGAGATTGGCTCGTAGACAC

            460         *       480         *       500         *       520         *
Q54.D.P  : ..................................................................... : 532
Q54.A.g. : ..................................................................... : 532
           CCGATGCGTTCTCCGGAGGAAACTCCGGAACATTATAAGTTCGGTATGACTGCTAAGGCAATGTTACCGAACATCA

                540         *       560         *       580         *       600
Q54.D.P  : ................................T..................................... : 608
Q54.A.g. : ..................................................................... : 608
           ACGCTATAGACGTTGGTGATAACGAAGACACT CGTCGGAATACCCAGTGAGTCTGAGTGTTTCAGGCGGAGTTCT

            *       620         *
Q54.D.P  : ..................... : 630
Q54.A.g. : ..................... : 630
           CCGTGAACACCACTTCATCTGA
```

* *D.P: donor plant; A.g: A. gossypii sub-isolates.*

Annex 16. Predicted p23 amino-acid sequence of CTV MAIB_Q1294, SG29 and MAIB_Q54 isolates and their aphid derived sub-isolates, respectively

```
                    *       20        *       40        *       60        *
Q1294.A.s. : ..................................................................... :  75
Q1294.T.a. : .....................................................................Y :  75
Q1294.D.P  : ..................................................................... :  75
Q1294.A.g. : ..................................................................... :  75
             SGRHGGRGNSIVNLSDESNTASTKVENVKSEADRLDFLRKMNPFIVDALVRKTNYQGARFRARIIGVCVDCGRKh

                80        *       100       *       120       *       140       *
Q1294.A.s. : ..................................................................... : 150
Q1294.T.a. : ..................................................................... : 150
Q1294.D.P  : ..................................................................... : 150
Q1294.A.g. : ...................................................D.................. : 150
             DKALKTERKCKVNNTQSQNEVAHMLMHDPVKYLNKRKARAFSNAEMFAIELVLYTKeRQLAVDLAAEREKTRLAR

                      160       *       180       *       200       *
Q1294.A.s. : ...................................................- : 209
Q1294.T.a. : ...................................................- : 209
Q1294.D.P  : ...................................................- : 209
Q1294.A.g. : ...................................................- : 209
             RHPIRSPEETPEHYKFGMTAKAMLPDINAVDVGDNEETSSEYPVSLSVSGGVLREHHFI


                     *       20        *       40        *       60        *
SG29.A.g.6 : ...................................................R.................. :  75
SG29.A.s.  : ..................................................................... :  75
SG29.D.P   : .G................................................................... :  75
SG29.A.g.1 : ..................................................................... :  75
             MdDTSGQTFISVNLSDESNTASTEIKAVSSEADRLEFLRKMNPFIIDALIRKNSYqGARFRARIIG6CVDCGRKH

                80        *       100       *       120       *       140       *
SG29.A.g.6 : ..................................................................... : 150
SG29.A.s.  : ..................................................................... : 150
SG29.D.P   : ..................................................................... : 150
SG29.A.g.1 : ..................................................................... : 150
             DKASRTERKCKVNNTQSQNEVAHMLMHDPVKYLNKRKARAFSNAEMFAIDLVMHTKERQLAVDLAAEREKTRLAR

                      160       *       180       *       200       *
SG29.A.g.6 : ...................................................- : 209
SG29.A.s.  : ...................................................- : 209
SG29.D.P   : ...................................................- : 209
SG29.A.g.1 : ..........................................H................F- : 209
             RHPMRSPEETPEHYKFGMTAKAMLPDINAVDVGDNEDTSSEyPVSLSVSGGVLREHHFi


                    *       20        *       40        *       60        *
Q54.D.P  : ....................................................T.....R......... :  77
Q54.A.g. : ..................................................................... :  77
           MDDTSGQTFISVNLSDESNTASTEVKTVSSEADRLEFLRKMNPFIIDALIRKTNYQGARFR RIIGV VDCGRKHDK

             80        *       100       *       120       *       140       *
Q54.D.P  : ..................................................................... : 154
Q54.A.g. : .........V....................................................... : 154
           ASRTERKCK NNTQSQNEVAHMLMHDPVKYLNKRKARAFSNAEMFAIDLVMHTKERQLAVDLAAEREKTRLARRHPM

               160       *       180       *       200       *
Q54.D.P  : ................................S.....................- : 209
Q54.A.g. : ...................................................- : 209
           RSPEETPEHYKFGMTAKAMLPNINAIDVGDNEDT SEYPVSLSVSGGVLREHHFI
```

** D.P: donor plant, A.g., A.s. and T.a.: A. gossypii, A. spiraecola and T. aurantii sub-isolates, respectively.*