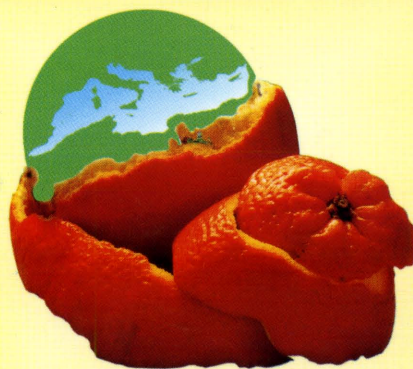


***Citrus tristeza virus and Toxoptera citricidus:
a serious threat to the Mediterranean citrus industry***

Edited by:

Anna Maria D'Onghia, Khaled Djelouah, Chester N. Roistacher



Mediterranean Network on Certification of Citrus

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Centre International de Hautes Etudes
Agronomiques Méditerranéennes

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Président / President: Abdelaziz MOUGOU
Secretariat General / General Secretariat: Bertrand HERVIEU

11, rue Newton 75116 Paris, France
Tél.: +33 (0) 1 53 23 91 00 - Fax: +33 (0) 1 53 23 91 01 et 02
secretariat@ciheam.org
www.ciheam.org

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IAM Instituts Agronomiques Méditerranéens Mediterranean Agronomic Institutes Bari - Chania - Montpellier - Zaragoza

IAM-Bari

Dir.: Cosimo LACIRIGNOLA
Via Ceglie 9
70010 Valenzano, Bari, Italy
Tel. (39) (080) 4606 111 - Fax: (39) (080) 4606 206
iamdir@iamb.it
www.iamb.it

IAM-Chania

Dir.: Alkinoos NIKOLAIDIS
P.O. Box 85
GR . 73100 Chania, Crete, Greece
Tel. (30) 28210 35000 - Fax: (30) 28210 35001
alkinoos@maich.gr
www.maich.gr

IAM-Montpellier

Dir.: Vincent DOLLÉ
3191, Route de Mende
34093 Montpellier Cedex 5, France
Tel. (33) (0)4 67 04 60 00 Fax: (33) (0)4 67 54 25 27
dolle@iamm.fr et/and sciuto@iamm.fr
www.iamm.fr

IAM-Zaragoza

Dir.: Luis ESTERUELAS
Av. Montañana 1005
50059 Zaragoza, Spain
Tel. (34) 976 716000 - Fax (34) 976 716001
iamz@iamz.ciheam.org
www.iamz.ciheam.org

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Scientific Editors: Anna Maria D'Onghia,
Khaled Djelouah, Chester N. Roistacher

Compilation: Giuseppe Santoro, Stefania Gualano

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Foreword

More than other fruit trees, many factors are known to limit the production and processing of citrus. Apart from management inefficiencies and environmental challenges, the major constraints involve numerous pests and diseases which can seriously wipe out the citrus industry. At present, the most striking challenge that the Mediterranean citriculture is facing is the threat of *Citrus tristeza virus* (CTV) with the advent of *Toxoptera citricidus*, the brown citrus aphid (BrCA), which was found in northern Portugal and Spain. Based on the devastating experience of other countries, it is predictable that this aphid will be able to efficiently spread the severe strains of CTV with an enormous social, economic and environmental impact. In addition to this, the global climate change has extended the risk of introducing emerging diseases from neighboring hot regions, such as the destructive citrus huanglongbing (HLB) and relative vector, the new major enemy for citrus growing countries for which no cure is currently possible.

The establishment of a free trade area of agricultural products in the Mediterranean, as envisaged in the 1995 Barcelona Declaration, can highly increase these threats if strict quarantine measures and healthy citrus propagating materials are not promptly adopted. It is notorious and experienced that these measures are economically and environmentally more efficient than trying to eliminate, usually without any success, the disease outbreaks from which the infection can further be propagated by the vectors. The time scale needed by the research sector to find reliable solutions is insufficient and not supported by the political will.

Within this context, the mission of the Mediterranean Research Network on the Certification of Citrus (MNCC) is primarily the establishment of close collaborations between groups of experts and citrus stakeholders for the development of a regional plan able to contrast present and forthcoming citrus phytosanitary threats. This publication is therefore designed to analyze the evolution in the Mediterranean area of citrus tristeza virus and its vectors, primarily the brown citrus aphid, to report the state of the art on the virus presence in the region and set up to highlight the advances in virus detection, characterization and control.

The network multidisciplinary approach can raise awareness and suggest possible solutions, but it is up to the growers to take the future of the sector in hand pushing governments to adopt measures for preserving the Mediterranean citrus industry from social, economic and environmental consequences. Whether the Mediterranean governments can do something to save our citrus industry is still to be seen and to hope.

Cosimo Lacirignola
CIHEAM-MAIB Director

Introductory session

The CIHEAM-Mediterranean Research Network on Certification of Citrus (MNCC): the regional programme for the control of *Citrus tristeza virus* and its major vector *Toxoptera citricidus* in the Mediterranean

D'Onghia A.M.

Coordinator of the CIHEAM-Mediterranean Research Network on Certification of Citrus
CIHEAM – Mediterranean Agronomic Institute, Valenzano (Ba), Italy

Citrus tristeza virus (CTV) is present in most of the Mediterranean countries, usually inducing the 'quick decline' of all citrus spp. when grafted onto the sour orange rootstock, which is highly susceptible. Other exotic virus strains, CTV-stem pitting (CTV-SP) and CTV-seedling yellows (CTV-SY), are apparently not spread yet in the region. Apart from the use of CTV-infected material, this virus is transmitted in the Mediterranean by different aphid species, mainly by *Aphis gossypii*. In 2002 and 2003, *T. citricidus*, the most efficient virus vector, known as the brown citrus aphid (BrCA), was reported in Northern Spain and Portugal, respectively. It was the first finding of BrCA in a Mediterranean country, which was officially reported in 2005.

The presence in Northern Portugal and Spain of *T. citricidus* represents a serious threat to the Mediterranean citrus industry since the virus inoculum is widely distributed in the whole region and the susceptible sour orange is the predominant rootstock. *T. citricidus* can tremendously enhance virus dissemination up to 100%, selecting the severe exotic virus strains (CTV-SP and CTV-SY), which have apparently a very limited presence in the region. These strains can not be controlled by the use of tolerant/resistant rootstocks (trifoliate orange and its hybrids), which are effective with the CTV-quick decline strain (CTV-QD).

Considering the socio-economic and environmental importance of citrus in the Mediterranean region, preventive measures should soon be applied (strict quarantine measures, virus and vector monitoring, elimination of infected plants, certification of propagating material), while the elimination of the severe virus strains and, eventually, the pre-immunization of nursery plants should be considered in case of failure. To this aim all Mediterranean countries must make efforts in spreading information and raising awareness on CTV and its main vector for a rapid and urgent application of all possible control measures. It is important to outline that the use of 'healthy' certified citrus propagating material is surely the first action for preventive or delaying virus/vector entrance and dissemination.

I – CIHEAM initiative

This situation prompted CIHEAM/Mediterranean Agronomic Institute of Bari to urgently promote a harmonized common action for the control of the virus and BrCA in the whole region in the framework of the activities of the Mediterranean Research Network on Certification of Citrus (MNCC). The research network was established in 1995 aimed at:

- raising awareness on the sanitary problems affecting citrus for preventing dissemination of harmful pathogens across the Mediterranean area;

- setting up, standardization, validation of technical protocols for pest monitoring and sanitary controls in certification before their application on a large scale in a specific country;
- updating information on the sanitary status of citrus trees in the Mediterranean countries;
- characterizing new citrus pathogens of Mediterranean importance;
- promoting the use of healthy citrus propagating material as an essential preventive measure for pathogen and pest control;
- disseminating information on EU regulations for achieving quality requirements through the certification programmes;
- supporting harmonized legislative instruments for the application of phytosanitary rules.

II – MNCC thematic workshops

The network promoted 3 thematic workshops on '*Citrus tristeza virus* and *Toxoptera citricidus*: development of management and control strategies in the Mediterranean region', which were organized with the collaboration of the Plant Protection institutions of the hosting country and FAO.

Apart from the representatives from CIHEAM and other international organizations (FAO, EPPO, GTZ), participants mostly from Phytosanitary or Plant Protection Services, scientific institutions of 22 countries mainly of the Mediterranean region attended the workshops; moreover, experts from the Mediterranean region and from USA highly contributed giving their technical support (Fig. 1,2).

The first thematic workshop was organized at Faro, Portugal (7-8 April, 2005), the country where BrCA was firstly reported and where control actions had to be urgently taken. In this workshop, a previous MNCC project proposal for the control of CTV in the Mediterranean region was revised on the basis of a new scenario given by the entrance of *T. citricidus* in the area.

After the workshop, a technical trip to Northern Portugal was organized for a small group of experts to visit the sites infested by BrCA. The area is too cold for commercial citrus growing; however, it is characterized by the abundance of backyard citrus in many of the houses in the cities and countryside.

Following Faro recommendations, a second MNCC thematic workshop was organized in the same year in Cairo, Egypt (22-23 September, 2005). A regional programme proposal was presented based on the most recent developed technical protocols for virus and vector monitoring and aimed at providing Mediterranean countries with harmonized regulations and procedures to be shortly adopted. This workshop was also devoted to spread information and raise awareness on these topics in Egypt as well as in the Near Eastern citrus-growing countries.

In the last thematic workshop in Adana, Turkey (8-10 June, 2006) the MNCC proposal of the regional programme for the mandatory control in the Mediterranean Region of CTV and its major vector *T. citricidus*, was finalized by MNCC representatives of 11 Mediterranean countries in addition to Sudan, under the coordination of CIHEAM/Mediterranean Agronomic Institute of Bari.

III – MNCC proposal of the regional programme for the mandatory control of *Citrus tristeza virus* and its major vector *Toxoptera citricidus* in the Mediterranean Region

The regional proposal developed and endorsed by MNCC country representatives will be presented to their governments in order to submit it to different donors as a priority issue for the Region. The proposal is structured as follows.

- an introductory part on the (i) importance of citriculture in the Mediterranean by the agricultural, economic, social and environmental point of view; (ii) severe citrus deterioration caused by CTV; (iii) the state of the art on CTV and its vectors; and (iv) the risk of a disaster by the dissemination of *T. citricidus* in the area;
- a justification that CTV is present as isolated foci or outbreaks in all Mediterranean citrus-growing countries and *T. citricidus* is now present in Portugal and Spain, thus seriously threatening the whole area;
- the objectives which are to provide the region with harmonized tools (useful information, technical protocols and regulations) for controlling CTV and preventing the introduction and dissemination of *T. citricidus*; the project will involve the national, regional and international organizations and institutions;
- the beneficiaries who are represented by all citrus stakeholders, primarily citrus farmers, nurserymen and Ministerial Plant Protection and Quarantine Services;
- the coordination of CIHEAM jointly with MNCC partners and with the collaboration of other organizations and donors;
- the facilities and equipments necessary to the implementation of the programme;
- the workplan which is focusing on the (i)enhancement of harmonized phytosanitary control measures and regulations (quarantine and certification); (ii)CTV monitoring and characterization; (iii)elimination of CTV-infected plants; (iv)monitoring aphid population and implementing efficient control measures; (v)raising awareness; (vi)strengthening of harmonized research and know-how; (vii)technology transfer and information exchange;
- the expected results.

In the meantime, posters and brochures in Arabic were also produced as information sheets for the identification of tristeza disease and its vectors. This material includes photos on the disease and on the vectors as well as on the control strategies based on virus and vector monitoring, elimination of virus foci and the mandatory use of certified propagating material mainly on tolerant rootstocks. The material produced has been widely distributed in different Mediterranean countries to support the extension programme. In Greece, Malta and Turkey these booklets and posters were also translated in the local language.



Figure 1. Participants to MNCC workshops.

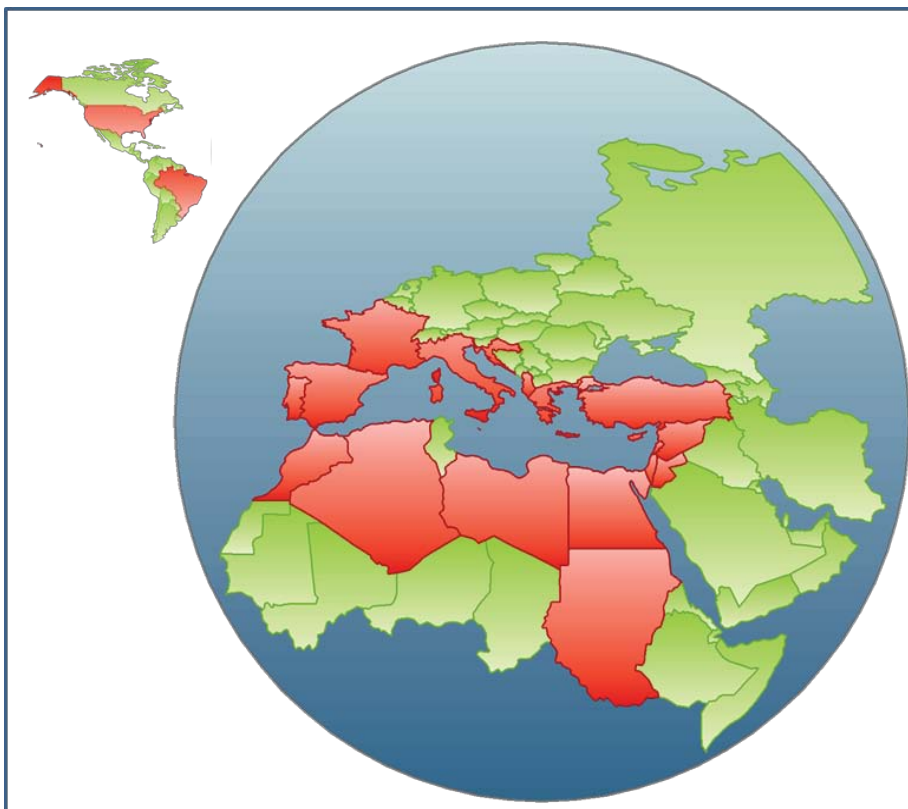


Figure 2. Represented countries at the MNCC workshops.

The Mediterranean citriculture: productions and perspectives

Lacirignola C., D'Onghia A.M.

CIHEAM – Mediterranean Agronomic Institute, Valenzano (Ba), Italy

I – The history

Citrus fruits were native to China and other oriental regions like Malaysia, India and Thailand. They have accompanied the history of mankind, from the earliest to the latest times. They conquered all continents and penetrated any society and culture, due to the beauty of the plant and the flower, called Zagara, and also to the precious qualities of the fruit. Historians today believe that the ancestor of the citrus trees in Europe and Middle East was *Citrus medica* L. (citron), which was introduced by Alexander the Great from India into Greece, Turkey, and North Africa in the late 4th century BC (Malcolm, 2006).

The term citrus fruits includes different types of fruits and products. Although oranges are the major fruit in the citrus fruits group, accounting for about 70% of citrus output, the group also includes small citrus fruits (such as tangerines, mandarins, clementines and satsumas), lemons, limes and grapefruits.

It is not known how, where, or when the exceptional present-day varieties of citrus trees developed, but a general consensus of opinions believes that all these citrus developments and improvements were obtained by natural and artificial selection and natural evolution.

II – The world citrus industry

From an economic point of view, citrus fruits rank first in terms of world fruit production and international trade value. Citrus production is estimated at around 100 million tons produced annually. Citrus fruits are cultivated in many countries around the world (140 countries according to FAOSTAT, 2007) although production shows geographical concentration in certain areas, mainly in the Northern Hemisphere, accounting for about 70% of total citrus production. The main citrus fruit-producing countries are Brazil, China, the United States although the whole Mediterranean region (CLAM countries) ranks first worldwide. These countries represent more than two thirds of the global citrus fruit production (CLAM, 2007) (Fig. 1).

Citrus is marketed throughout the world as a beneficial healthy fruit that contains Vitamin C and numerous other vitamins and minerals. Because of their nutritional and organoleptic qualities, citrus fruits contribute to nutritional balance for both Northern and Southern populations.

There are two clearly differentiated markets in the citrus sector: fresh citrus fruits market, with a predominance of oranges, and processed citrus products market, mainly orange juice. Over the last two decades, a major breakthrough was the growth in trade of small citrus fruits (tangerines, clementines, mandarins and satsumas) at the expense of fresh oranges. This is due to the evolution of consumer preferences more oriented to small-sized, easy-peeler and seedless fruits. Consumption of citrus fruit juices has also increased, thanks to product convenience and healthiness, quality improvements, price competitiveness, promotional activity and technological advances in processing, storage and packaging. Among the major citrus varieties, only grapefruit has a level of processed utilization comparable to oranges.

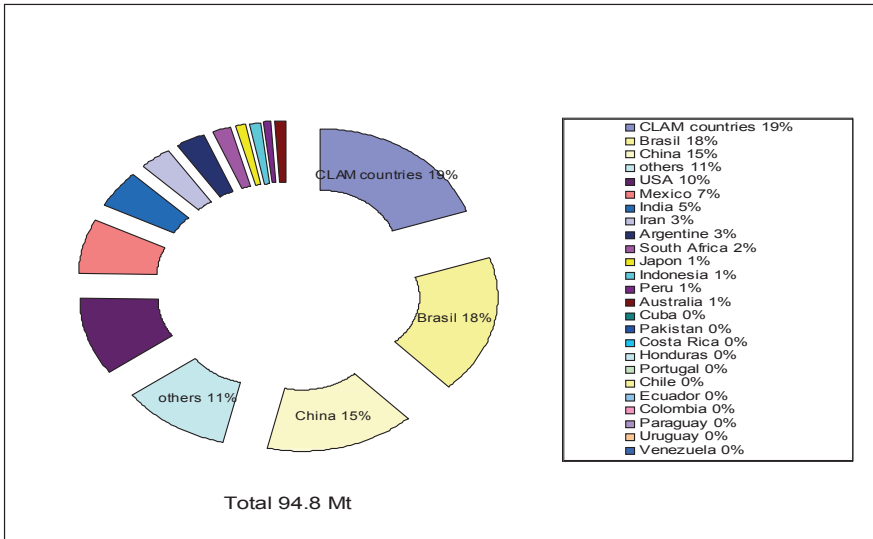


Figure 1. Distribution of worldwide citrus production.
(source data: CLAM, 2007).

III – Citriculture in the Mediterranean Basin

According to CLAM data, the Mediterranean Basin accounts for about 20% of the world citrus production and about 60% of the world fresh citrus trade (Fig. 1, 2). In this region, citrus fruits are produced mainly for fresh fruit consumption. Spain is the leading producing country, whereas Italy and Egypt rank second and third, respectively (Fig. 3).

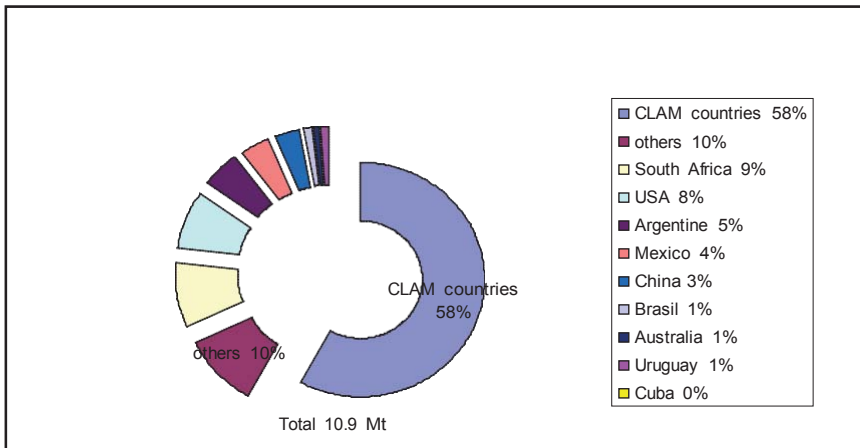


Figure 2. Worldwide citrus exportations.
(source data: CLAM, 2007).

The production is mainly composed of sweet oranges and easy peeler fruits (mandarins and mandarins-like) although other species, such as lemons and limes, are of high interest for specific

areas: lemons are widely cultivated in the Euro-Mediterranean Region, whereas lime in the Near East Region. Grapefruit production is restricted to Israel which is the first exporting country in the Mediterranean and the fourth worldwide. A new entrant to the fresh grapefruit production is Turkey (Imbert, 2007).

The wide range of varieties, with their staggered maturities, allows the fresh fruit market to be supplied throughout most months of the year.

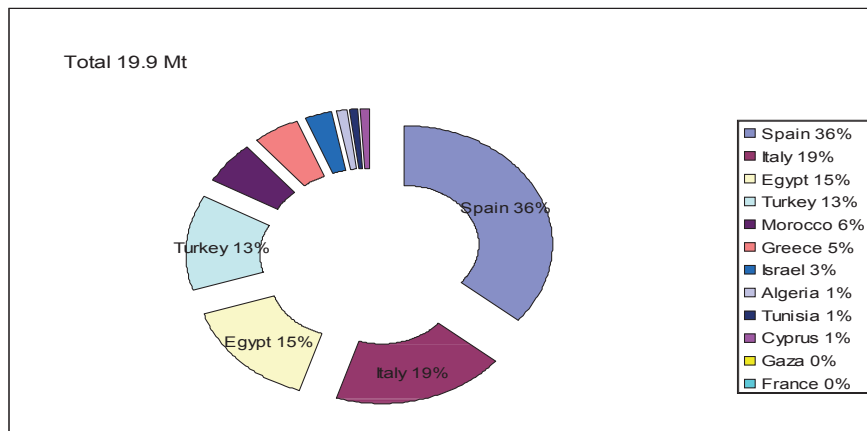


Figure 3. Distribution of citrus production in the Mediterranean basin.
(source data: CLAM, 2007).

On the contrary about the general trend over the last 10 years, production increased in the Mediterranean region exceeding for the first time 20 million tons during the 2006-2007 season. Production records were reached for all species except for grapefruit (Imbert, 2007).

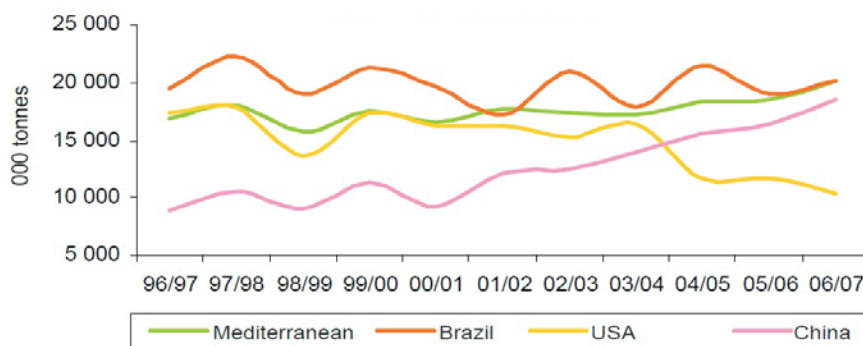


Figure 4. Citrus production trend in the period 1996/1997-2006/2007.
(source data: CLAM, 2007).

Citrus is a major segment in the Mediterranean agricultural industry; developing countries account for almost 40% of the production and citriculture represents a major source of income to a significant number of farmers. According to data provided by the Euro Mediterranean Citrus Network (2007), the Citrus farm size varies from less than 1 ha to a few 100 ha. Farms larger than

10 ha account for 80% of the production and are usually technologically advanced. Most of the fruit is harvested by hand although harvesting machines have been developed. It is a source of employment at various levels of the chain (production, processing trade and farming consumable suppliers). Hence, citrus plays an invaluable role as a driving force to the economy of the entire Mediterranean region.

The Mediterranean has been an important diversification zone for the three most important economic species (oranges, mandarins, lemons); numerous are the germplasm banks in the region, usually under the responsibility of governmental institutions; they represent a valuable reservoir of genetic resources for breeding programmes, but also for commercial purposes. Most of the native genotypes are still widely grown, but their productions are mainly oriented to the local market. The strong replacement of these genotypes with international varieties, highly demanded by the market, is a risk for their possible extinction. Their enhancement through clonal and sanitary selection programmes can surely increase their cultivation and characterize their products for a better economic competition in the market. The market is in fact assisting to a change in consumption patterns, particularly in the form of an increasing focus on the quality and the value-added aspects of the product. European countries are posing particular attention to the quality standards and the traceability of products which have a native origin such as the Italian red orange in Sicily, the Nadorcot/Afourer mandarin in Morocco, the clementine in Corsica and many others.

All together, the Mediterranean countries are the most important exporting regions in the world. Most of the Mediterranean citrus production is used for internal consumption as fresh fruit and for exportation, which represent 42 % and 34%, respectively (Fig. 4).

Based on the species, the Mediterranean basin accounts for 75% of the worldwide exportation of easy peeler fruits (mandarin and mandarin-like fruits) (Tab. 1).

Table 1. The Mediterranean and world trade share per group of varieties in 2006-2007.
(source data: CLAM, 2007).

	World trade	Mediterranean share	Main exporting countries	
Orange	5,370,000	58%	Spain	1,450,000
			South Africa	900,000
			Egypt	760,000
			United States	546,000
			Morocco	264,000
Easy peelers	3,300,000	75%	Spain	1,656,000
			China	367,000
			Morocco	317,000
			Turkey	312,000
			South Africa	100,000
Lemon & lime	2,000,000	45%	Spain	497,000
			Mexico	387,000
			Argentina	355,000
			Turkey	328,000
			United States	384,000
Grapefruit	1,000,000	28%	United States	215,000
			South Africa	215,000
			Turkey	135,000
			Israel	78,000
			Spain	37,000
Total Citrus	11,670,000	58%	Spain	3,640,000
			South Africa	1,215,100
			United States	1,046,021
			Egypt	793,800
			Turkey	775,174

According to CLAM (2007), the current trend in exports of the different Citrus groups of varieties is a leading example of agriculture guided by the market in a constant effort of meeting the consumer's demand, and always aiming at increasing the value added to production. Nevertheless, Mediterranean producers possess a major advantage. Their unique varietal range of easy peelers and orange means that they can generate growth by lengthening the marketing season and facilitating access to the numerous potential markets (e.g. eastern Europe and North America today; Asia and Latin America in the future).

IV – Phytosanitary challenges to the Mediterranean Citrus Industry

The Mediterranean citriculture is characterized by the high prevalence of sour orange (*Citrus aurantium* L.), a rootstock well adapted to the dominant calcareous soils of the region. Sour orange is considered a universal rootstock being also tolerant to *Phytophthora gummosis*, salinity and drought, which are considered the prevalent biotic and abiotic stresses of citrus in the region. However, sour orange is highly susceptible to citrus Tristeza disease, which is the most severe virus disease affecting these species; it is present in most of the countries in the region and is transmitted by different aphid species. The risk of contamination by tristeza has spurred the use of tolerant rootstocks, Poncirus hybrids (Citrange, Citrumelo) to replace sour orange. The recent introductions in Northern Portugal and Spain of the main virus vector, *Toxoptera citricidus*, is a serious risk to the Mediterranean citriculture, due to its efficiency in spreading the severe virus strains. The situation experienced in other regions of the world after the establishment of this aphid is marked by a massive destruction, irrespective of rootstocks. In the Mediterranean, *Citrus tristeza virus* (CTV) is widely present, but currently, only Spain and Israel have experienced tristeza epidemics and have adapted their citrus to live with the disease, whereas other countries are only recently facing the disease outbreaks. The situation in the region is still lucky, due to the absence of diseases which are even more destructive than tristeza, among which huanglongbing (HLB) is for sure the most serious disease of sweet orange, mandarin and grapefruit trees.

V – Conclusions

The citrus fruit sector is evolving in a context of highly competitive global markets. Despite the global crisis, the Mediterranean citriculture is at present the leading citrus-producing region in the world, thanks to the production and exportations of easy-peeler fruits; in addition to this, unremitting attention is paid to the quality of citrus fruits as well as to the peculiar characteristics of the native products. This success is in contrast with the stagnation or even with the definitive production slump of the world top juice industries, Brasil and Florida, which are fighting against a broad range of diseases, primarily the citrus hurricane named 'huanglongbing disease induced by *Candidatus liberibacter* spp'. The crisis in these two countries will profoundly change the world scenario of the citrus sector. It can turn into an opportunity for the Mediterranean citrus industry, if adequate and efficient quarantine measures are jointly adopted by the countries in the region to contrast the entrance and spread of this and other destructive citrus diseases.

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Citrus tristeza virus

Yokomi R.K.

United States Department of Agriculture - Agricultural Research Service (USDA-ARS),
Parlier, CA 93648, USA

I – Identity

1. Preferred scientific name

Citrus tristeza virus (CTV)

2. Taxonomic position

CTV is a member of the closterovirus group which have thread-like, flexuous virions, insect vectors, cause characteristic cytopathological structures (inclusion bodies) in infected phloem tissues, and have a positive sense, single-stranded RNA genome of up to 20 kb. Based on molecular characterization of CTV and other members of the closterovirus group, enough genetic diversity occurs among members to propose that the closteroviruses belong to the family Closteroviridae which contains homologues of cellular heat-shock proteins (Dolja *et al.*, 1994). The Closteroviridae are composed of three genera: Citrivirus (CTV = type member) which has one 19.3 kb genome component and 12 open reading frames (ORFs); Closterovirus (beet yellows virus = type member) which has one 15.4 kb genome and 9 ORFs; and Biclavirus (lettuce infectious yellows = type member) which has two genome components of 8.1 and 7.1 kb and 9 ORFs.

3. Internationally used common names

Tristeza

Stem pitting (SP)

Hassaku dwarf

Podredumbre de las raicillas

4. Other common names

Citrus quick decline (QD)

Grapefruit stem pitting

Sweet orange stem pitting

Seedling yellows (SY) Lime

dieback disease Pummelo

yellow dwarf (China)

5. Notes on taxonomy and nomenclature

Failure of some citrus selections to graft propagate successfully on sour orange rootstocks was observed in different areas prior to 1900, but an association of this condition with a disease was only gradually established through observations in South Africa and Java (Roistacher, 1995; Wallace, 1978). The disease appeared in Argentina in 1931 and in southern Brazil in 1937 after importation of infected plants from South Africa and Moreira (1942) referred to it as tristeza, which in Portuguese and Spanish means melancholy or sadness. The probable viral

etiology of CTV was demonstrated when Meneghini (1946) showed that the causal agent was transmissible by the oriental citrus aphid (=brown citrus aphid), *Aphis citricida* (= *Toxoptera citricida* (Kirkaldy)). This was quickly followed by reports in California and South Africa that a quick decline of sour orange rooted citrus was bud transmissible. A lime dieback disease in Africa was eventually associated with CTV infection (Wallace, 1978; Bar-Joseph *et al.*, 1989). Some isolates of CTV induce a seedling yellows (SY) reaction in inoculated sour orange and grapefruit plants. SY was once thought to be associated with a separate virus (Fraser, 1959; McClean, 1974). Generally, the most severe strains of CTV cause stem pitting in various cultivars regardless of rootstock and also develops on seedling trees. Severe stem pitting in susceptible cultivars leads to a loss in tree vigor and a decrease in fruit quality and quantity. Extensive diversity among CTV isolates has been well established and molecular characterization studies now in progress suggest that CTV may well be complex of related viruses.

II – Hosts

Natural hosts for CTV include nearly all citrus species, interspecific hybrids, some citrus relatives and some intergeneric hybrids. The only natural noncitrus host that has been reported is *Passiflora* (Kitajima *et al.*, 1974; Müller *et al.*, 1974)). Some of the more important economic hosts are: sweet orange [*Citrus sinensis* (L.) Osb.], grapefruit [*C. paradisi* Macf.], mandarins [*C. reticulata* Blanco], limes [*C. aurantifolia* (Christm.) Swing.] and *C. latifolia* Tan.], lemons [*C. limon* (L.) Burm. f.], pumelo [*C. grandis* (L.) Osb.], tangelos [*C. reticulata* x *C. paradisi*], tangors [*C. reticulata* x *C. sinensis*], calamondin [*C. madurensis* Lour.], and kumquat [*Fortunella margarita* (Lour.) Swing.].

Sweet lime [*C. limettoides* Tan], citron, [*C. medica* L.], and combava (*C. hystrix* (DC.), Swing.) are also infected, as are commonly used rootstock varieties such as Rangpur lime [*C. limonia* Osb.], rough lemon [*C. jambhiri* Lush.], sour orange [*C. aurantium* L.], volkamer lemon [*C. volkameriana* Ten. and Pasq.], and alemow [*C. macrophylla* Wester].

1. Affected plant stages

The virus is phloem limited and can be detected in leaves, stems, fruits, and roots. Greatest concentrations of virions are found in young growth under relatively mild temperature conditions.

2. Notes on host range

Many natural hosts of CTV remain essentially symptomless when infected by most CTV isolates. Mandarins, sweet oranges and rough lemon are among common tolerant hosts. Some citrus species show a selective susceptibility and are readily infected by some CTV isolates and not by others (Garnsey *et al.*, 1996c). *Poncirus trifoliata* (L.) Raf., a citrus relative commonly used as a rootstock, is highly resistant to nearly all isolates of CTV and this resistance is also found in some trifoliolate orange hybrids.

Other hosts which have been experimentally infected either by a phid, graft, or mechanical inoculation include: *Aegle marmelos* (L.) Corr. Se rr., *Aeglopsis chevalieri* Swingle, *Afraegle paniculata* (Schum.) Engl., *Citropsis gilletiana* Swingle and M. Kellerman, *Microcitrus australis* (Planch.) Swingle, *Pamburus missionis* (Wight) Swingle (Müller and Garnsey, 1984); *Passiflora gracilis* Jacq., *P. caerulea* L., *P. incense*, and *P. incarnata* (Roistacher and Bar-Joseph, 1987). CTV can replicate in protoplasts of *Nicotiana benthamiana* (Navas-Castillo *et al.*, 1995).

III – Identification

1. Virus morphology and characteristics

CTV particles are flexuous rods 10-11 nm in diameter and 2,000 nm long. The particles are easily sheared and extracts from partially purified preparations typically contain many broken particles of various lengths. The capsid protein subunits are helically arranged along the particle with a basic pitch of 3.7 nm and ten subunits in each turn of the helix (Bar-Joseph *et al.*, 1972). The nucleic acid is single-stranded positive sense RNA with a molecular weight of about 6.5×10^6 which is composed of 19,296 nucleotides (nt) in the isolate T36 from Florida (Karasev *et al.*, 1995) or 19226 nt in the isolate VT from Israel (Mawassi *et al.*, 1996). These encode 12 open reading frames (ORF) potentially coding for at least 17 protein products. Nine 3' co-terminal subgenomic RNAs have been found in infected citrus tissue (Hilf *et al.*, 1995). One ORF encodes the capsid protein of approximately 25,000 daltons, another (p27) encodes a divergent coat protein which has been shown to coat one end of the virion forming a “rattlesnake” structure, and a third encodes a homologue of HSP70 heat shock protein which is also found in other closteroviruses. Functions of other ORFs have been inferred from sequence comparisons with other viruses, but not confirmed (Karasev *et al.*, 1995). Sequence comparisons between strains are in progress and indicate a relatively high level of conservation toward the 3' end and divergence up to 40 % toward the 5' terminal (Mawassi *et al.*, 1996).

Many CTV-infected plants contain defective RNAs which are composed of portions of the 5' and 3' terminal sequences of the CTV genome (Mawassi *et al.*, 1995). Some of the defective RNAs have been found to contain small portions of non-virus encoded RNA linking the 5' and 3' terminal portions. Defective RNAs are often detected as prominent bands in purified extracts of double-stranded RNA from infected tissues. The significance of defective RNAs on symptom expression has not been determined (Mawassi *et al.*, 1995).

2. Symptoms

Virulence is affected by the CTV isolate and environmental conditions. Since there are hundreds of citrus species, hybrids, and citrus relatives, an isolate's virulence should be defined in terms of specific hosts. Being phloem-limited, most CTV symptoms are associated with viral disruption of phloem and its function. Some isolates cause few symptoms, even in plants that are normally reactive such as Mexican lime (Bové *et al.*, 1988). Most CTV strains cause vein flecking, leaf cupping, a transient leaf epinasty in young leaves, and some stem pitting on CTV-sensitive plants such as Mexican lime, *C. macrophylla*, or *C. hystrix*. These vary from mild to severe and impact fruit quality when severe.

Some isolates of CTV cause a decline when field trees of sweet orange, mandarins, or grapefruit grafted on sour orange rootstocks become infected. This decline is associated with a virus-induced phloem necrosis at the budunion which blocks normal translocation of carbohydrates to the root system. As the root system deteriorates, trees begin to decline. Symptoms appear a year or more after infection and may occur gradually over several years or very suddenly (QD). Canopy symptoms are wilting, chlorosis and an abnormal crop of small fruit which may persist after tree death in trees affected by QD. Clinical symptoms can often be seen by removing a patch of bark across the budunion. Trees which decline slowly usually will have thicker bark immediately below the union and the face of the bark surface below the union will have many small conical pits (honeycombing) corresponding to bristle-like protuberances from the wood (Schneider, 1954). Trees affected by QD lack honeycombing, but will frequently show a yellow brown stain at the budunion. If budwood from scions infected with decline strains of CTV are propagated on sour orange seedlings, the budlings may be stunted and chlorotic, but rarely collapse and die (Bransky *et al.*, 1986).

Other CTV strains cause stem pitting in commercial cultivars of grapefruit, lime, and sweet orange. Stem pitting does not kill trees, but affected trees may have thin canopies and produce fewer fruit of reduced size and quality (Marais *et al.*, 1996). Dieback occurs in severely affected limes. Chronically infected trees sometimes show a bumpy or ropy appearance of trunks and limbs on larger trees. The twigs and limbs are brittle and easily broken. Early stages of stem pitting are detectable only upon removal of bark. These pits consist of depressions in the outer wood with corresponding pegs or projections on the inner bark face. Stem pitting severity can range from very few small pits to many pits to a more general disruption of the cambium which results in many fine, sandpaper-like pits and abnormally thickened bark. CTV strains which cause stem pitting in grapefruit do not necessarily cause stem pitting in sweet orange, and vice versa. CTV strains have also been reported which cause stem pitting in mandarin, rough lemon, and Gou Tou rootstocks.

Some strains of CTV also induce a seedling yellows (SY) reaction (a dwarfing and general chlorosis) in inoculated grapefruit, lemon, and sour orange seedlings. The SY reaction usually, but not always, accompanies the presence of decline on sour orange and/or stem pitting strains of CTV.

3. Similarities to other diseases

Tristeza-induced decline can be confused with other decline diseases affecting citrus including foot rot and blight. Association of decline with trees only on sour orange rootstocks in the absence of external lesions in the bark of the trunk or major roots is often an indication of CTV (Whiteside *et al.*, 1988). Extensive root damage caused by root weevils, nematodes, gophers, or waterlogging may, however, cause tree decline in trees grafted on sour orange rootstock. Citrus blight, an infectious decline disease of unknown etiology, can also affect trees on sour orange rootstock. This decline can usually be differentiated in the field from CTV-induced decline on sour orange rootstock because blight is associated with a xylem dysfunction. There are no budunion symptoms of blight, blighted trees have a delayed flush rather than a precocious flush and deterioration of the root system follows development of decline but does not cause it (Garsey and Young, 1975).

CTV is the most frequent cause of stem pitting in citrus, but other virus and viruslike agents can also cause stem pitting. Cristacortis can cause large pits in limbs and trunks of some cultivars. Cachexia, a citrus viroid, can also produce stem pits with gumming on susceptible rootstocks, such as *C. macrophylla*, and on some mandarins.

Other phloem pathogens of citrus, such as greening, can induce visual canopy effects similar to those caused by severe stem pitting isolates of CTV, especially in grapefruit. Co-infection of greening and CTV is common in Asian citrus areas. Greening alone does not cause stem pitting and CTV alone does not induce leaf mottling and rarely causes stunting or chlorosis in mandarins (Whiteside *et al.*, 1988).

4. Diagnostic methods

The traditional method to detect CTV is to graft-inoculate *Citrus aurantifolia* (Christm.) Swing. (Key lime or Mexican lime) plants (Roistacher, 1991). This host reacts to most isolates by developing clearing or flecks in the veins of new leaves formed after inoculation. In some cases the severity of reaction in limes is indicative of the isolate's virulence in other hosts, but the correlation is not exact. To determine CTV-decline effects, sweet orange plants grafted on sour orange rootstock are inoculated and monitored for symptoms of dwarfing and chlorosis over 6-12 months. However, some isolates that cause decline of field trees grafted on sour orange are symptomless in the same condition under glasshouse or screenhouse conditions (Ballester-Olmos *et al.*, 1993). Stem pitting is determined by inoculating seedlings of an appropriate or susceptible the cultivar, usually sweet orange or grapefruit, and monitoring growth over 12-15 months. The main stem

of the indicator plants is stripped of bark and examined for stem pitting. To test an isolate for SY reaction, the isolate is grafted to a seedling of sour orange, lemon, or grapefruit and observed for 6-12 months in the greenhouse for stunting and chlorosis symptoms. This is often considered a presumptive indication for a severe isolate of CTV, but some virulent stem pitting isolates do not cause SY and vice versa. Some isolates induce SY, but do not cause stem pitting in grapefruit or sweet orange.

Serological methods, such as ELISA in microplates (Garnsey and Cambra, 1991) or in tissue prints (Garnsey *et al.*, 1993), are routinely used for CTV detection (Garnsey and Cambra, 1991). Polyclonal antisera have been produced to a number of different isolates and these detect nearly all isolates. Monoclonal antibodies (Mabs) have also been produced (Permar *et al.*, 1990; Tsai and Su, 1991; Vela *et al.*, 1986). Some react to epitopes which are widely conserved among diverse CTV isolates and these also provide nearly universal detection, especially if two are used in combination (Cambra *et al.*, 1993). Other Mabs are more isolate specific, and one (MCA13) has been used to differentiate mild isolates from those that cause decline and stem pitting in Florida (Permar *et al.*, 1990). No monoclonal antibody has been developed which reacts specifically to only decline or stem pitting CTV isolates. A variety of other serological tests have also been utilized to detect CTV (Cambra *et al.*, 1991; Rocha-Peña and Lee, 1991).

Electron microscopy (EM) also has been used for CTV detection. Immunospecific EM, is very sensitive and specific for diagnosis (Garnsey *et al.*, 1980). Light microscopy has been used for the detection of characteristic CTV-induced inclusion bodies in the phloem of infected plants (Edwardson and Christie, 1978; Garnsey *et al.*, 1980; Brlansky *et al.*, 1988).

Analysis of double-stranded RNAs in infected tissues can result in characteristic patterns that are diagnostic for CTV (Dodds and Bar-Joseph, 1983) and may discriminate between some isolates (Dodds *et al.*, 1987; Moreno *et al.*, 1990; Guerri *et al.*, 1991).

With the recent completion of the sequencing of the T36 CTV isolate, assays have been developed utilizing reverse transcriptase polymerase chain reaction (RT-PCR) and/ or hybridization with specific probes. Use of these assays allow testing of homology at discrete areas of the viral genome. Development of these assay provide highly sensitive detection methods which can differentiate CTV strains having different biological activities (Albiach *et al.*, 1995; Gillings *et al.*, 1993).

IV – Geographic distribution

Tristeza apparently originated in Asia and existed there for many years in tolerant cultivars which were either propagated vegetatively as cuttings or by seed. New areas of citriculture in other continents were first established from seed and were free of CTV infection. Subsequently, CTV has been introduced into nearly all citrus-growing areas via virus-infected budwood or plants. In many areas infections have become wide spread due to propagation and secondary spread by aphids. In some areas little or no secondary spread has occurred from the few existing infected trees.

CTV is very common in commercial citrus in southeast Asia, Australia, southern Africa, India, Japan, South America, and most Pacific Islands, and where the brown citrus aphid is present nearly all field-grown trees are infected. CTV is widespread in parts of Spain, Florida, parts of California, portions of Central America and most of the Caribbean islands. CTV is present, but not widespread in parts of the Mediterranean region and Mexico (Tab. 1).

Table 1. Geographic distribution of CTV

COUNTRY	STATUS	REFERENCES
ASIA		
-Brunei Darussalam	P	Büchen-Osmond <i>et al.</i> 1996
-China	W	Wallace 1978
-India	W	Wallace 1978
-Indonesia	W	Wallace 1978
-Iran	P	Büchen-Osmond <i>et al.</i> 1996
-Israel	L,W	Wallace 1978
-Japan	W	Wallace 1978
-Korea, Rep.	P	Büchen-Osmond <i>et al.</i> 1996
-Malaysia	W	Wallace 1978
-Nepal	W	Büchen-Osmond <i>et al.</i> 1996
-Pakistan	W	Büchen-Osmond <i>et al.</i> 1996
-Philippines	W	Wallace 1978
-Saudia Arabia	P	Büchen-Osmond <i>et al.</i> 1996
-Sri Lanka	W	Büchen-Osmond <i>et al.</i> 1996
-Taiwan	W	Wallace 1978
-Thailand	W	Büchen-Osmond <i>et al.</i> 1996
-Turkey	P	Büchen-Osmond <i>et al.</i> 1996
-Vietnam	W	Büchen-Osmond <i>et al.</i> 1996
AFRICA		
-Algeria	P	Büchen-Osmond <i>et al.</i> 1996
-Benin	P	Bové and Vogel 1981
-Cameroon	P	Büchen-Osmond <i>et al.</i> 1996
-Comores	P	Bové and Vogel 1981
-Central African Republic	P	Büchen-Osmond <i>et al.</i> 1996
-Chad	P	Büchen-Osmond <i>et al.</i> 1996
-Egypt	P	Wallace 1978
-Ethiopia	P	Büchen-Osmond <i>et al.</i> 1996
-Gabon	P	Büchen-Osmond <i>et al.</i> 1996
-Ghana	P	Wallace 1978
-Ivory Coast	P	Bové and Vogel 1981
-Kenya	P	Büchen-Osmond <i>et al.</i> 1996
-Madagascar	P	Bové and Vogel 1981
-Mauritius	W	Bové and Vogel 1981
-Morocco	P	Wallace 1978
-Mozambique	P	Büchen-Osmond <i>et al.</i> 1996
-Nigeria	P	Büchen-Osmond <i>et al.</i> 1996
-Reunion	W	Bové and Vogel 1981
-South Africa	W	Wallace 1978
-Tanzania	P	Büchen-Osmond <i>et al.</i> 1996
-Uganda	P	Büchen-Osmond <i>et al.</i> 1996
-Zaire	P	Büchen-Osmond <i>et al.</i> 1996
-Zambia	P	Büchen-Osmond <i>et al.</i> 1996
-Zimbabwe	W	Wallace 1978
NORTH and CENTRAL AMERICA		
-Antigua and Barbuda	P	Büchen-Osmond <i>et al.</i> 1996
-Belize	L	Yokomi <i>et al.</i> 1994
-Bermuda	P	Büchen-Osmond <i>et al.</i> 1996
-Costa Rica	W	Yokomi <i>et al.</i> 1994
-Cuba	L	Lee <i>et al.</i> 1995
-Dominican Republic	W	Yokomi <i>et al.</i> 1994
-El Salvador	P	Yokomi <i>et al.</i> 1994
-Honduras	P	Yokomi <i>et al.</i> 1994
-Guatemala	P	Yokomi <i>et al.</i> 1994

COUNTRY	STATUS	REFERENCES
-Jamaica	W	Yokomi <i>et al.</i> 1994
-Mexico	P	Yokomi <i>et al.</i> 1994
-Netherlands Antilles	P	Büchen-Osmond <i>et al.</i> 1996
-Nicaragua	P	Yokomi <i>et al.</i> 1994
-Panama	W	Yokomi <i>et al.</i> 1994
-Puerto Rico	W	Yokomi <i>et al.</i> 1994
-Trinidad and Tobago	W	Yokomi <i>et al.</i> 1994
-USA		
Arizona	P	Wallace 1978
California	L	Wallace 1978
Florida	W	Wallace 1978
Hawaii	W	Garnsey <i>et al.</i> 1991
Texas	P	Wallace 1978
SOUTH AMERICA		
-Argentina	W	Wallace 1978
-Bolivia	W	Büchen-Osmond <i>et al.</i> 1996
-Brazil	W	Wallace 1978
-Chile	W	Wallace 1978
-Colombia	W	Büchen-Osmond <i>et al.</i> 1996
-Ecuador	W	Büchen-Osmond <i>et al.</i> 1996
-Guyana	P	Wallace 1978
-Paraguay	W	Büchen-Osmond <i>et al.</i> 1996
-Peru	W	Büchen-Osmond <i>et al.</i> 1996
-Surinam	W	Wallace 1978
-Uruguay	W	Wallace 1978
-Venezuela	W	Wallace 1978
EUROPE		
-Italy	P	Wallace 1978
-Madeira	W	Lee <i>et al.</i> 1995
-Portugal	P	Büchen-Osmond <i>et al.</i> 1996
-Spain	W	Wallace 1978
-Former Yugoslavia	P	Bové and Vogel 1981
OCEANIA		
-American Samoa	P	Büchen-Osmond <i>et al.</i> 1996
-Australia	W	Wallace 1978
New South Wales	W	Lee <i>et al.</i> 1995
Queensland	W	Lee <i>et al.</i> 1995
-Fiji	W	Wallace 1978
-French Polynesia	W	Büchen-Osmond <i>et al.</i> 1996
-New Caledonia	W	Büchen-Osmond <i>et al.</i> 1996
-New Zealand	W	Büchen-Osmond <i>et al.</i> 1996
-Tonga	W	Bové and Vogel 1981
-Western Samoa	W	Büchen-Osmond <i>et al.</i> 1996

L: Low spread; P: Present; W: Widespread.

V – Biology and ecology

1. Transmission

Natural spread of CTV is primarily through propagation of infected budwood and by aphids. CTV is not seed-borne. Propagation of new plants from buds from infected plants is responsible for long-distance spread of CTV and extensive bud propagation from a single plant can rapidly increase foci of inoculum. Topworking old citrus plantings to new scion varieties using infected buds has been a common way of CTV spread in some countries. Tree to tree spread is by aphids.

CTV is semipersistently transmitted by several aphid species (Bar-Joseph and Lee, 1989). Aphid vectors acquire the virus from an infected tree with feeding times ranging from 5 min. to hours, however, not by brief probes. The transmission efficiency of the vector increases as the acquisition and feeding times are increased up to 24 hours. There is no latent period, and the virus does not multiply or circulate in the aphid. The time required to inoculate a plant is the same as for acquisition. Aphids remain viruliferous for 24-48 hours after feeding on infected plants. Many aphids species that feed on an infected citrus tree can acquire CTV, as detected by ELISA (Cambra *et al.*, 1981), but only a few species can transmit it to new plants. *T. citricida* is the most efficient vector, and where it exists, is often the most abundant aphid on citrus (Yokomi *et al.*, 1994). Most isolates of CTV, including severe stem pitting isolates, are effectively vectored by *T. citricida*, but a few isolates are vectored less efficiently. *Aphis gossypii* Glover (melon or cotton aphid) can transmit some isolates efficiently and is the most important CTV vector in regions where *T. citricida* is not present (Hermoso de Mendoza *et al.*, 1984; Roistacher *et al.*, 1984; Yokomi *et al.*, 1989; Ballester-Olmos *et al.*, 1993). In contrast to *T. citricida*, it has a wide host range and only occasionally colonizes citrus. *Toxoptera aurantii* (Boyer de Fonscolombe) can transmit some CTV isolates but is less efficient than brown citrus aphid or melon aphid (Hermoso de Mendoza *et al.*, 1984). It also has a wide host range and occasionally colonizes citrus. *A. spiraeicola* Patch (spirea aphid) is an inefficient vector of CTV under experimental conditions, but is very common on citrus worldwide and also is extremely polyphagous (Hermoso de Mendoza *et al.*, 1984; Yokomi and Garnsey, 1987). These aphids, except *T. citricida*, are distributed worldwide. Several other aphids have been shown to transmit CTV experimentally, but are not likely to be significant.

2. Notes on transmission

CTV is readily graft transmissible if a union is formed between the phloem of the donor and receptor host. A variety of graft-inoculation methods are used to experimentally transmit the virus (Roistacher, 1991). Buds, sections of leaves that include veins, and stem pieces can all be used as inoculum. Mechanical transmission of CTV is difficult and has only been done experimentally by slash-inoculation of the stems of receptor plants with concentrated extracts from CTV-infected plants. CTV can also be transmitted experimentally by dodder.

3. Epidemiology

Primary infections of CTV are usually established via propagation of infected plants. Epidemics of CTV decline observed in many countries began with importation and propagation of infected plants in areas heavily planted with CTV-free trees on sour orange. When efficient vectors were present epidemics of decline often followed. Although CTV epidemiology is significantly affected by the citrus cultivar and horticultural practices, the most important factors are the CTV isolate and the aphid vector. When *T. citricida* is present, temporal and spatial spread of CTV spread is increased (Garnsey *et al.*, 1996b). This aphid has a narrow host range and migrants move from citrus to citrus to start new aphid colonies and, in this process, can transmit CTV if they are viruliferous. High aphid populations also coincide with new flush which is favorable for virus acquisition and inoculation. The other vectors are much less efficient than *T. citricida*, and also have a wider host range. Migrants may originate in other crops prior to feeding on citrus and may feed on a different plant species after leaving citrus. Therefore, aphid host range and feeding behavior likely affect pattern and rate of spread (Gottwald *et al.*, 1996b). It is assumed that aphid population levels may be correlated with rates of spread, but threshold levels for minimum and maximum levels of transmission have not been established. Natural spread is generally slow in desert regions where natural thermotherapy may keep inoculum at lower levels in plants and may vary seasonally in temperate areas as well. CTV spread rate in sweet oranges is generally higher than that observed in grapefruit (Moreno *et al.*, 1988; Gottwald *et al.*, 1996a). CTV isolates in Meyer lemon and some mandarins have not spread appreciably from these hosts unless *T. citricida* is present. The latent periods between inoculation and systemic infection

and between infection and symptom expression also affect evaluation of disease development. Presence of other strains may also influence rate of virus movement (Hermoso de Mendoza *et al.*, 1984).

VI – Pest significance

1. Economic impact

CTV is the most economically important virus pathogen of citrus worldwide. Millions of citrus trees on sour orange have been killed by CTV decline epidemics in Argentina, Brazil, Venezuela, Peru, Florida, California, Israel, Spain, and other locations. It is estimated that world wide there are over 200 million trees on sour orange rootstock which are at risk to this disease. Sour orange is popular because it produces a vigorous tree with high quality fruit, is adaptable to many soil conditions including high lime and salt content and has tolerance to many other viruses, viroids, and virus-like pathogens and Phytophthora. Use of tristeza-tolerant rootstocks often risks losses from other factors. In addition to decline, many severe CTV isolates cause stem pitting diseases of susceptible scion cultivars and these occur even when tolerant rootstocks are used. Stem pitting weakens trees and eventually reduces fruit size, quality, and quantity (Marais *et al.*, 1996). Grapefruit and lime are very sensitive to stem pitting. Sweet orange is more tolerant but can be severely affected by some isolates.

2. Phytosanitary Risk

The phytosanitary risk for CTV is associated with importation of infected plants or budwood for propagation in a new citrus-growing area. The risk associated with dry tissue or fresh fruit is negligible.

VII – Control

Control strategies for CTV differ according to the incidence and severity of the CTV isolates in an area and with the cultivars and rootstocks used. No single control strategy is applicable in all situations (Garnsey *et al.*, 1996a; Lee *et al.*, 1994).

1. Exclusion and Quarantine

When CTV is absent or rare, preventive efforts should be made to avoid introduction of CTV into the growing area by having quarantines on importation of live citrus tissue. A practical and safe method to legally introduce cultivars from other regions and to free these of infection is necessary and reduces industry pressure to illegally introduce new cultivars or germplasm resources. Procedures for safe international movement of citrus germplasm have been devised (Frison and Taher, 1991).

2. Certification Programs

Careful control of propagating material remains the single most effective means to avoid rapid and extensive CTV epidemics. Most commercial citrus are clonally propagated by using buds from a selected scion cultivar to a nucellar seedling as a rootstock. Budwood is usually taken from a mature, vigorous tree and used directly or increased in a nursery block to produce thousands of buds from a single source. Thus, propagation of CTV-infected trees can be prevented by using virus-free scion trees protected from natural infection by isolation or use of insect-free screenhouses or by shoot tip grafting (Navarro, 1993). Rapid indexing tests are available to verify freedom from CTV infection.

3. Eradication and suppression

If a few trees become infected in a CTV-free area and indigenous aphids are poor vectors, natural spread can be slowed appreciably by a vigilant eradication and suppression program. However, an effective survey program is essential and when CTV is detected, infected trees must be removed immediately and surveillance maintained (Garnsey *et al.*, 1996a). Eradication is rarely effective once infections are well established, especially in the presence of favorable vector conditions.

4. Resistant/tolerant rootstocks

Numerous rootstocks are tolerant or resistant to CTV decline and use of these is essential for economic production of citrus in many areas. Some examples are Cleopatra and Sunki mandarins, rough lemon, Rangpur lime, trifoliate orange, and trifoliate orange hybrids such as Troyer and Carrizo citranges and Swingle citrumelo. CTV resistant/tolerant rootstocks are often susceptible to other problems such as citrus blight, viroids, nematodes, or poor soil conditions.

5. Tolerant Scions

Most mandarins are generally tolerant to CTV, although some hybrids, such as some tangelos, are seriously affected by stem pitting. In most areas in Asia where CTV isolates are severe, mandarins are the principal varieties produced due, in part, to their tolerance to stem pitting. There are no CTV-tolerant limes although Persian limes are more tolerant than small acid limes. All grapefruits are susceptible to grapefruit stem pitting isolates of CTV. Sweet oranges vary in susceptibility to sweet orange stem pitting, but none are truly tolerant. Pera orange, a major variety in Brazil, is very susceptible while Valencia is one of the more tolerant cultivars.

6. Cross protection

Infection with a mild isolate of CTV may protect a tree from becoming infected with or showing symptoms of a more virulent strain CTV (Gonsalves and Garnsey, 1989). This is a strategy for control of stem pitting in areas where severe isolates of CTV and the brown citrus aphid are endemic. Cross protection is for production of grapefruit in South Africa (Van Vuuren *et al.*, 1993) and Australia (Broadbent *et al.*, 1991), and Pera sweet orange and Galego lime in Brazil (Müller and Costa, 1972; Costa and Müller, 1980). In these countries, protective isolates have been selected from vigorous trees that remained in areas destroyed by the disease and their protective capacity confirmed in controlled experiments. Protection often is effective only between certain isolates and many mild isolates show little protective effect (Roistacher *et al.*, 1993). Furthermore, mild protective isolates are often effective only in the specific cultivar in which they were selected. Effective long-term cross protection against decline of trees grafted on sour orange rootstock has not been demonstrated, though significant delay of symptom onset has been observed with some mild isolates (Yokomi *et al.*, 1991; Moreno *et al.*, 1993c).

Cross protection is an empirical practice and the basis for the strain interaction involved is not understood. One of the difficulties to implement an effective cross protection is that many CTV isolates contain a mixture of strains that differ at molecular level and in symptom expression (Moreno *et al.*, 1993a). The balance of strains in a CTV isolate may change depending on the host and other factors (Moreno *et al.*, 1993b) and, hence, its specific interactions with other isolates. Therefore, until additional understanding on the molecular mechanism involved in this interaction will be available, cross protection has to be considered as a practical procedure to delay or reduce damage caused by severe isolates in some citrus cultivars grown in specific areas.

New technologies for citrus transformation (Moore *et al.*, 1992; Peña *et al.*, 1995) and the increasing knowledge on CTV genome (Karasev *et al.*, 1995) may allow production of transgenic citrus plants with resistance mediated by viral sequences.

7. Vector control

Vector suppression is an unproven strategy for CTV control. In the case of semipersistently transmitted viruses, viruliferous winged aphids may inoculate citrus trees several kilometers from the donor tree. It is not clear what level of vector control is necessary to reduce spread of CTV. However, vector control may have potential to reduce secondary spread (Gourmet *et al.*, 1994). Biological controls to restrict build up of citrus aphids, especially *T. citricida*, may be feasible (Tang and Yokomi, 1996). Although insecticides may not act quickly enough to prevent primary infection by viruliferous aphids, they could reduce local aphid populations and decrease rate of secondary spread. Insecticidal control of vector populations may have use in specific situations such as in a citrus nursery or to protect budwood sources. A long residual systemic insecticide with minimum impact on biological control agents is preferred. CTV titer is highest when trees are forming new shoots in spring and fall. Aphid flights also peak at this time and, hence, these periods should be targeted for control actions.

8. Integrated disease management

Incidence and spread of CTV is a complex process that involves interaction of the plant, pathogen and vector. Conventional approaches have been directed at one or several components for CTV control. An integrated disease management (IDM) strategy should incorporate as many elements as possible based on our fundamental knowledge of the disease (Garnsey *et al.*, 1996a).

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The brown citrus aphid, *Toxoptera citricida*

Yokomi R. K.

United States Department of Agriculture – Agricultural Research Service (USDA-ARS),
Parlier, CA 93648, USA

I – Identity

1. Preferred scientific name

Toxoptera citricida (Kirkaldy)

2. Taxonomic position

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Homoptera (Hemiptera)
Suborder:	Sternorrhyncha (Homoptera)
Superfamily:	Aphidoidea
Family:	Aphididae
Subfamily:	Aphidinae
Tribe:	Aphidini

3. Synonyms

Aphis aeglis Shinji
Aphis citricidus (Kirkaldy)
Aphis citricola van der Goot
Aphis nigricans van der Goot
Aphis tavaresi Del Guercio
Myzus citricidus Kirkaldy
Paratoxoptera argentiniensis EE Blanchard
Toxoptera aphoides van der Goot
Toxoptera citricidus (Kirkaldy)

4. Common names

Brown citrus aphid (English)
Oriental citrus aphid (English)
Tropical citrus aphid (English)
Black citrus aphid (English)
Abura mushi (Japanese)
Dà jú yá (Chinese)

5. Notes on taxonomy and nomenclature

The aphid was first described as *Myzus citricidus* and was noted to be similar to *Myzus cerasi*, common on citrus throughout Hawaii, and a likely an introduction from China (Kirkaldy, 1907). The species name, *citricidus*, was derived as a Latin adjective of the noun meaning “citrus killer” and had a masculine ending to agree with *Myzus*. Since *Toxoptera* Koch is the correct genus for the aphid and is feminine, it is necessary that its nomenclature be feminine (e.g., *Toxoptera citricida*), as apposed to the feminine/masculine combination (e.g. *Toxoptera citricidus*) (Stoetzel, 1994b).

II – Hosts

1. Affected plant stages

T. citricida colonizes young leaves, stems, blossoms, and growing points of host plants. Established colonies may be able to complete their life history on hardening shoots but these will not support new colonies.

2. List of hosts

Major hosts (citrus scion varieties): sweet orange [*Citrus sinensis* (L.) Osbeck.], grapefruit [*C. paradisi* Macfad.], mandarin [*C. reticulata* Blanco], lime [*C. aurantifolia* (Christm.) Swing.], lemon [*C. limon* (L.) Burm. f.], pummelo [*C. maxima* (Burm.) Merr.], satsuma [*C. unshiu* (Mack.) Marc.], tangelo [*C. x tangelo* J. Ingram and H.E. Moore], tangor [*C. x nobilis* Lour.].

3. Notes on host range

Primary hosts of *T. citricida* are citrus and citrus relatives (Rutaceae) (Order Geraniales, Suborder Geraniineae, mostly in the Subfamily Aurantioideae, Tribe Citreae). Typically, Aurantioideae are trees or shrubs with evergreen leaves. Flowers are usually white and often fragrant. Many genera bear subglobose fruit with a green, yellow, or orange peel with numerous oil glands that result in a nice aroma when handled. Most commercial citrus varieties and rootstocks are good hosts of *T. citricida*. In addition, relatives such as calamondin [*X Citrofortunella microcarpa* (Bunge) Wijnands] and orange jessamine [*Murraya paniculata* (L.) Jack.] can support *T. citricida*. There are reports that *T. citricida* has been collected on many non-citrus plants (Essig, 1949), however, there is no verification that these are reproductive hosts capable of sustaining a population of the aphid. These reports may have resulted from misidentification of aphids. In addition, the aphid may be able to survive on some nonrutaceous hosts temporarily as they migrate away from a crowded food source (Yokomi *et al.*, 1994).

III – Geographic distribution

T. citricida is believed to be native to Asia where citrus originated. Since the first half of the twentieth century, the aphid has been known to be widely distributed on citrus in Asia, India, New Zealand, Australia, Pacific Islands (including Hawaii), Africa south of the Sahara, Madagascar, Indian Ocean Islands, and South America. This distribution is attributed to movement of infested leaves or propagations. Areas where citrus was established by seed or aphid-free propagations have remained uninfested (e.g. North and Central America, Caribbean Basin) until recently (Yokomi *et al.*, 1994). The Mediterranean region except for North-West Spain and northern Portugal remains free of the aphid. Because ancestral citrus (old line) is known to contain many virus and virus-like agents, many countries prevent entry of citrus propagations from abroad. This, undoubtedly, has restricted the aphid’s hitchhiking potential. On the other hand, there is now

more intercontinental movement of people and commerce than ever before and the threat of introduction by this route remains at its highest level (Tab. 1).

Although the aphid's origin is tropical/subtropical, presence of a sexual stage and overwintering as eggs in Japan (Komazaki, 1982) suggests that *T. citricida* can adapt to different climates. Due to the aphid's restricted host range to citrus and its relatives, the most favorable citrus environs for *T. citricida* occur when weather is warm and humid which result in frequent stimulation of new growth cycles. Similarly, desert/semi-arid and cooler regions provides conditions favorable for *T. citricida* only seasonally. Populations typically increase rapidly following colony initiation and results crowding, in a decline in host suitability, and production of winged (alate) aphids. Winged morph production could also be triggered by the physiology of the host. A key requirement for spread of *T. citricida*, however, is that the alata must alight on citrus with new shoot growth to successfully establish a new colony.

Table 1. Geographic distribution of *Toxoptera citricida*.

COUNTRY	STATUS	REFERENCES
ASIA		
-Cambodia	W	Blackman and Eastop 1984
-China	W	Essig 1949
-Hong Kong	W	Blackman and Eastop 1984
-India	W	Prunthi and Mani 1945
-Indonesia	W	Blackman and Eastop 1984
-Japan	W	Essig 1949
-Malaysia	W	Essig 1949
-Nepal	W	Knorr and Shah 1971
-Philippines	W	Blackman and Eastop 1984
-Singapore	W	Blackman and Eastop 1984
-Taiwan	W	Essig 1949
-Viet Nam	W	Essig 1949
-Thailand (SE Asia)	W	Blackman and Eastop 1984
AFRICA		
-Kenya	P	Essig 1949
-Ivory Coast	P	Thouvenel and Fauquet 1977
-Ethiopia	P	Abate 1988
-Burundi	P	Seco <i>et al.</i> 1992
-Mauritius	W	Essig 1949
-Reunion	W	Etienne and Vilardebo 1978
-South Africa	W	Essig 1949
-Swaziland	W	Essig 1949
-Belgium Congo	P	Essig 1949
-British East Africa	P	Essig 1949
-Cameroon	P	Essig 1949
-French West Africa	P	Essig 1949
-Mozambique	P	Essig 1949
-Nairobi	P	Essig 1949
-Rhodesia	P	Essig 1949
-Uganda	P	Essig 1949
-Angola	P	Van Harten and Ilharco 1975
NORTH AND CENTRAL AMERICA		
-Cayman Islands	P	Halbert 1996b.
-Costa Rica	W	Lastra <i>et al.</i> 1991
-Cuba	W	Yokomi <i>et al.</i> 1994
-Dominica	W	Aubert <i>et al.</i> 1992
-Dominican Republic	W	Aubert <i>et al.</i> 1992
-Guadeloupe	W	Aubert <i>et al.</i> 1992

COUNTRY	STATUS	REFERENCES
-Haiti	W	Yokomi <i>et al.</i> 1994
-Jamaica	W	Yokomi <i>et al.</i> 1994
-Martinique	W	Aubert <i>et al.</i> 1992
-Mexico	W	Michaud and Alvarez 2000
-Nicaragua	W	Yokomi <i>et al.</i> 1994
-Panama	W	Yokomi <i>et al.</i> 1994
-Puerto Rico	W	Yokomi <i>et al.</i> 1994
-St. Kitts and Nevis	W	Yokomi <i>et al.</i> 1994
-St. Lucia	W	Yokomi <i>et al.</i> 1994
-Trinidad and Tobago	W	Essig 1949
United States		
-Florida	P	Halbert 1996a
SOUTH AMERICA		
-Argentina	W	Essig 1949
-Bolivia	P	Smith and Cermeli 1979
-Brazil	W	Essig 1949
-Chile	W	Essig 1949
-Colombia	W	Smith and Cermeli 1979
-Ecuador (So. America)	W	Stoetzel 1994a
-Paraguay	P	Smith and Cermeli 1979
-Peru	W	Essig 1949
-Surinam	P	Smith and Cermeli 1979
-Uruguay (So. America)	W	Stoetzel 1994a
-Venezuela	W	Smith and Cermeli 1979
EUROPE		
-Madeira and NW Portugal	W	Aguiar <i>et al.</i> 1994, D'Onghia, 2005
-NW Spain		D'Onghia, 2005
OCEANA		
-Australia	W	Essig 1949
-Cook Islands (Pacific Islands)	W	Blackman and Eastop 1984
-New Zealand	P	Essig 1949
-Tonga	P	Carver <i>et al.</i> 1993
-Fiji	P	Essig 1949
-Samoa	P	Essig 1949

W: Widespread; P: Present.

IV – Biology and ecology

T. citricida is anholocyclic and thelytokous throughout most of its range, preferring warm climates. It can, however, tolerate colder areas such as southern Japan by developing a holocyclic stage and overwintering as eggs (Komazaki, 1993). Development time is temperature dependent. At 20°C, *T. citricida* has a nymphal development time is 6-8 d with an average pre-reproductive period of 8.1 d, longevity is 28.4 d. Fecundity is 58.5 offspring/female with an intrinsic rate of natural increase (r_m) of 0.36, net reproductive rate of 56.2, mean generation time of 11.2 d. Its thermal threshold is 8.4°C and required 125 degree days for development (Komazaki, 1982). Takanashi (1989) reported slightly longer generation time under similar conditions and differentiated between alata and aptera development time. Winged morphs develop when populations become crowded and/or food source declines in quality and disperse in search of new hosts to begin new colonies. A spring and a fall flight peak of *T. citricida* occur in South Africa (Schwartz, 1965), Australia (Carver, 1978), and Brazil (Nickel *et al.*, 1984). In Japan, *T. citricida* populations peak 3 times per year but can be found on citrus in all seasons, except when overwintering (Komazaki, 1993). Because the host range of the aphid is restricted to citrus and its relatives (all relatively non-cold hardy), it is unlikely that the aphid can exist outside citrus growing areas or climates.

T. citricida's major impact is due to its efficient transmission of *Citrus tristeza virus* (CTV) (Costa and Grant; 1951, Yokomi *et al.*, 1994), a phloem-limited closterovirus (Bar-Joseph and Lee, 1989). Two types of CTV strains are economically important: 1) those that cause decline of citrus budded onto sour orange (*Citrus aurantium* L.) rootstock; and 2) those that cause stem pitting of grapefruit and sweet orange regardless of rootstock. Both are readily transmissible by *T. citricida*.

CTV is semipersistently transmitted by citrus aphids (Racah *et al.*, 1976). Aphids acquire virus from an infected tree with feeding times as short as 5-10 min. but transmission efficiency increase with feeding times up to 24 h. There is no latent period and the virus does not multiply or circulate in the aphid. The time required to inoculate a plant is the same as for acquisition. The aphid is capable of spreading the virus for 24-48 hours without reacquisition (Meneghini, 1948). *T. citricida* also transmits citrus vein enation (woody gall) virus, a probable luteovirus (da Graça and Maharaj, 1991). Migrating populations of *T. citricida* are also associated with the spread of certain nonpersistently-transmitted viruses such as chili veinal mottle virus (Blackman and Eastop, 1984) and soybean mosaic virus in China (Halbert *et al.*, 1986).

Symptoms. New, tender shoots are vulnerable to *T. citricida* colonization and support rapid population buildup. Aphids are external feeders and extract plant sap from the host by penetrating their stylets into phloem. Excess plant sap is excreted as honeydew which supports sooty mold growth. Heavy infestation by *T. citricida* is noted when growing points of citrus are covered by the dark-colored aphid and the flush bends under the physical weight of the colony. Aphid-tending ants are often present with *T. citricida* and collect honeydew. When disturbed, *T. citricida* populations sway rapidly in unison, making stridulatory movements with their hind legs presumably to fend off their enemies. Flowers are not a preferred host tissue. Mature leaves, stems, and fruit can not sustain *T. citricida* population.

V – Natural enemies

Most of the reported natural enemies of *T. citricida* are predators. If predators are present in or adjacent to the citrus grove when *T. citricida* colonies are forming, they can be effective even when aphid levels are low. Recently in Puerto Rico, predators, especially coccinellids, were observed decimating small *T. citricida* colonies and eliminating or reducing and/or delaying winged aphid production (JP Michaud, personal communication).

The principal primary parasitoids of *T. citricida* are solitary endophagous Hymenoptera in the families Aphidiidae and Aphelinidae. The aphidiids are wasplike in appearance and, at pupation, produce a mummy with a typical crusty golden, swollen appearance. They range in adult size from one to several mm. Aphelinids are usually less than 1 mm in adult size, possess reduced wing venation and an abdomen which appears broadly attached to thorax. They turn an aphid into a black mummy. Female adult aphelinids also feed on aphid haemolymph, a behavior that is essential for completion of oogenesis. In Taiwan, *T. citricida* is parasitized by *Lipolexis gracilis* and *Lipolexis scutellaris* (Tao and Chiu, 1971), whereas in Australia, it is parasitized by *Aphelinus gossypii* (Carver, 1978). In Japan, *Lysiphlebia japonica* is the principal parasitoid of *T. citricida* (Kato, 1970; Takanashi, 1990).

Entomopathogenic fungi attack *T. citricida* and can decimate a population with dramatic speed. A critical requirement for efficacy of such fungi is high humidity. *Verticillium lecanii* has been reported to attack *T. citricida* in Venezuela (Rondón *et al.*, 1980) and other fungi has been observed associated with the aphid in South Africa (Samways, 1984) (Tab. 2).

Table 2. List of the natural enemies of *Toxoptera citricida*

NAME	TYPE	STAGES	COUNTRIES
Aphelinidae			
<i>Aphelinus gossypii</i> Timberlake	Parasitoid	All	So. China, Australia
<i>Aphelinus spiraeocolae</i> Evans and Schauff	Parasitoid	All	So. China
Aphidiidae			
<i>Aphidius colemani</i> Viereck (<i>A. platensis</i> Brethes)	Parasitoid	All	Argentina, Australia
<i>Aphidius matricariae</i> Haliday	Parasitoid	All	Peru
<i>Lipolexis gracilis</i> Forster	Parasitoid	All	So. China, Taiwan
<i>Lipolexis scutellaris</i> Mackauer	Parasitoid	All	Taiwan, India
<i>Lysiphlebia japonica</i> (Ashmead)	Parasitoid	All	Japan
<i>Lysiphlebus testaceipes</i> (Cresson)	Parasitoid	All	Peru, Puerto Rico, Venezuela, USA, Cuba, Europe
<i>Trioxys indicus</i> Subba Rao and Sharma (<i>Binodoxys indicus</i>)	Parasitoid	All	India
Coccinellidae			
<i>Chiomenes sexmaculata</i> (Fabricius)	Predator	All	So. China
<i>Ceothera</i> sp.	Predator	All	Brazil
<i>Coccinella octopunctata</i> (Fabricius)	Predator	All	Taiwan, Japan
<i>Coccinella repanda</i> Thunberg	Predator	All	Australia, Taiwan, Japan
<i>Coccinella septempunctata</i> Mulsant	Predator	All	Taiwan
<i>Cycloneda sanguinea</i> (Linnaeus)	Predator	All	Brazil, Venezuela
<i>Diomus</i> sp.	Predator	All	Brazil
<i>Exoplectra</i> sp.	Predator	All	Brazil
<i>Harmonia conformis</i> (Boisdural)	Predator	All	Australia
<i>Leis axyridis</i> (Pallas)	Predator	All	So. China
<i>Leis conformis</i> (Boisd.)	Predator	All	So. Australia
<i>Leis dimidiata</i> Fabricius	Predator	All	So. China, Taiwan
<i>Lemnia biplagiata</i> Schwartz	Predator	All	Taiwan
<i>Lemnia saucia</i> (Mulsant)	Predator	All	So. China, Japan
<i>Lemnia swinhoi</i> Crotch	Predator	All	Taiwan
<i>Menochilus sexmaculatus</i> (Fabricius)	Predator	All	Taiwan
<i>Pentilia</i> sp.	Predator	All	Brazil
<i>Propylea japonica</i> (Thunberg)	Predator	All	Taiwan
<i>Pseudaspidimerus japonensis</i> Nakune and Araki	Predator	All	Taiwan
<i>Scymnus frontalis quadripustulatus</i> Herbst	Predator	All	Taiwan
<i>Scymnus hoffmanni</i> Weise	Predator	All	So. China, Taiwan
Syrphidae			
<i>Epistrophe balteata</i> (De Geer)	Predator	All	So. China
<i>Ischiodon scutellaris</i> Fabricius	Predator	All	So. China
<i>Melangyna viridiceps</i> (Macquart)	Predator	All	Australia
<i>Ocyrtamus gastrostactus</i> (Wiedemann)	Predator	All	Venezuela
<i>Simosyrphus grandicornis</i> (Macquart)	Predator	All	Australia
<i>Syrphus serrarius</i> Wiedemann	Predator	All	S. China, Japan
Chrysopidae			
<i>Chrysopa boninensis</i> Okamoto	Predator	All	Taiwan, So. China
<i>Chrysopa formosana</i> Matsumura	Predator	All	Taiwan, Japan
<i>Chrysopa septempunctata</i> Wesmael	Predator	All	So. China, Japan
<i>Chrysopa signata</i> Schneider	Predator	All	Australia
Chamaemyiidae			
	Predator	All	So. China

VI – Pest significance (economic impact)

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

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

VII – Identification

1. Morphology

Of the 16 to 20 aphid species reported to feed on citrus, 5 species are most commonly encountered: *T. citricida*; *Aphis spiraecola* Patch; *Aphis gossypii* Glover; *Toxoptera aurantii* (Boyer de Fonscolombe); and *Aphis craccivora* Koch (latter not common). Adult *T. citricida* are shiny black and nymphs are grey or reddish brown, but color alone is not distinctive because other aphids on citrus have dark coloration.

Winged adult female (alata): 1.1-2.6 mm in length; antennae six segmented with I, II, and III heavy black and other segments banded at joints, secondary rhinaria 7-20 on III and 0-4 on IV, setae on ant. III subequal to or exceeding diameter of segment; siphunculi black, elongate; cauda black, elongate with 25-40 setae; stridulatory apparatus on abdomen present; forewing with pterostigma light brown and media usually twice-branched.

Wingless adult female (aptera): 1.5-2.8 mm in length; oval; antennae six segmented with no secondary rhinaria; segments not banded, but segments I and II black, segments III and IV pale and slightly swollen, and segments V and VI dark at least at joints, setae on antennal III at least as long as the diameter of the segment; siphunculi black, elongate, and only slightly longer than cauda; cauda black and elongate with about 30 setae; "knees" of all three pairs of legs very dark; stridulatory apparatus present.

2. Similarities to other pests

T. citricida can be confused with *T. aurantii*, the black citrus aphid, because of its presence on citrus, dark brown-black coloration, size, and presence of stridulatory apparatus on the abdomen. However, alata of these aphids can be readily differentiated using a hand lens. *T. citricida* has

antennae III entirely black, forewing pterostigma light brown and media vein twice branched; *T. aurantii* has antennae III, IV, V, and VI banded at joints, forewing pterostigma conspicuously dark blackish-brown and media vein once-branched. Wingless adults and nymphs are more difficult to distinguish. The easiest character on apterae is the antennae. *T. aurantii* antennae have several banded joints; whereas *T. citricida* antennae have one prominent band near the middle. Setal length and patterns can be used to differentiate the aphids but require higher magnification. The cauda of *T. citricida* is bushy with 25-40 setae; whereas that of *T. aurantii* is less bushy with 8-19 setae. Another black aphid that occur on citrus is *Aphis craccivora* Koch, cowpea aphid. It can be distinguished by its strikingly white legs (knees of hind leg may be dark) and 7 caudal setae. Full descriptions and citrus aphid keys are reported in more details by Stroyan (1961), Stoezel (1994a) and Halbert and Brown (1996).

3. Detection and inspection methods

Field infestations of *T. citricida* can best be detected by periodic visual inspection of new shoot growth of citrus. Winged forms can be monitored by yellow traps or suction traps.

VIII – Control

1. Cultural Control

Efforts to manage virus inoculum are the most important control strategy (Garnsey *et al.*, 1996) because spread of severe strains of CTV is the major problem associated with *T. citricida*. The first factor to consider is the prevalence of CTV and its strains in your area. If virulent stem pitting strains and *T. citricida* are endemic, citrus scion varieties tolerant to CTV should be planted. These include mandarins, pummelos, tangelos, and tangor. Only CTV-tolerant or resistant rootstock should be used. Avoid planting grapefruit or Pera sweet orange unless they have been preinfected with a cross-protecting CTV strain. If CTV strains are less virulent than the previous scenario, sweet oranges and grapefruit, preferably preinoculated with a mild CTV isolate, can be grown with consideration for the market targeted (e.g. fresh fruit, domestic, export, juice, etc.). When CTV problems are anticipated, closer plant spacing should be considered to maximize land use during the grove's early years. Trees that decline or become stunted can either be replaced or simply removed and neighboring trees allowed to fill in.

Close plant spacing is becoming a common practice in the United States in new groves. Tree size is managed by mechanical hedgers that trim the sides and tops of trees. This practice produces conditions excellent for CTV spread and allows tree canopies to touch in the direction of the row. Pruning induces new shoot growth in which CTV multiplication is optimal as long as temperature and moisture are favorable. Citrus aphid migration, including that of *T. citricida*, peak in spring and fall (Carver, 1978; Schwartz, 1965). Hence, the uniform growth that results from pruning maximizes opportunities for CTV acquisition and inoculation.

If CTV incidence is undetectable or mild and *T. citricida* is not established in your region, citrus trees grafted on sour orange rootstock may still be acceptable (Garnsey *et al.*, 1996). This decision depends on the risk of losses due to CTV versus the advantages gained by the use of sour orange (e.g. salinity, cold hardiness, phytophthora, high soil pH, poor drainage). Several areas have managed CTV by eradication of infected trees (e.g., Israel, California). This program is cost effective if virus incidence is low and spread is slow (Garnsey *et al.*, 1996).

Regardless of the present CTV/aphid vector situation, a citrus budwood certification program is essential for a good citrus industry. CTV and all other citrus virus and viruslike agents are readily graft transmissible. Diagnostic methods are available for testing and detection of citrus pathogens in budwood sources. Recent developments in serology and molecular biology allow some rapid

evaluation of pathogen virulence. Thermotherapy and shoot tip grafting are now standard methods to eliminate pathogens from budwood. If a cross-protective CTV isolates are available, they can be incorporated into the budwood certification program.

2. Biological control

Although natural enemies are important in regulating aphid populations, they alone may not be satisfactory for controlling plant virus diseases. Aphid populations on citrus are often too variable to provide sufficient natural enemies for effective vector control. One concept is to direct biological control activities to reduce migrant vector populations before they spread through susceptible crops (Mackauer, 1976). Given that alternate prey are available, natural enemies could reduce *T. citricida* populations to mitigate secondary spread of CTV (tree to tree within a field), especially if conservation and augmentation efforts are used. In Japan, *L. japonica* is the most important parasitoid of *T. citricida* (Takanashi, 1990). In South America, various natural enemies have been observed attacking *T. citricida* but none have been used for augmentation in a biological control program.

Lysiphlebus testaceipes (Cresson) was found attacking *T. citricida* in Puerto Rico (Yokomi and Tang, 1996) but parasitism rate was low as was previously observed in Australia (Carver, 1984). Murakami *et al.*, (1984) did not find effective parasitoids of *T. citricida* in the Cerrados region of Brazil and suggested that *L. japonica* be imported and released against *T. citricida*. *T. citricida* was introduced in south Florida in the later half of 1995 and spread throughout the state on citrus in a few years. Assuming that biological agents colonize new areas slower than their hosts, multiple augmentative releases of mass-reared parasitoids at various sites should be conducted (Wellings, 1994). A classical biological control effort has been undertaken using this strategy in Florida with the release of *L. japonica* and *Aphelinus spiraecolae* (Tang *et al.*, 1996). Both these and other parasitoids have not become established. However, *L. testaceipes* now effectively parasitizes *T. citricida* (Persad and Hoy, 2003) This suggests that this parasitoid has adapted to the aphid. *L. testaceipes* is a robust species and should be a good prospect for introduction to new areas where the aphid becomes established.

Since most predators are generalist feeders, presence of alternate prey provides stability to their contribution as a biological control agent. The spirea aphid, *Aphis spiraecola* Patch is a cosmopolitan species with a wide host range and is quick to colonize new citrus shoots (Cole 1925; Miller, 1929). This aphid also develops large populations rapidly on citrus which attracts predators. If *T. citricida* arrive when these predators are present, they readily attack *T. citricida*. However, if predators discover *T. citricida* population shortly after colonization, their probability of establishment is low without alternate prey as another food source (JP Michaud, personal communication).

3. Host plant resistance

No information is available on the experimental range of *T. citricida* on rutaceous or nonrutaceous plants.

4. Chemical control

Insecticidal control of *T. citricida* to slow spread of CTV is an unproven strategy. Although insecticides may not act quickly enough to prevent primary infection by viruliferous aphids, reduction of aphid populations would decrease secondary spread. Its effectiveness depends on longevity of suppression and extent of the treated area in relation to inoculum reservoir and migratory activity of the aphid (Knapp *et al.*, 1996). It should be cautioned that use of foliar insecticides can interfere with biological control agents and, ultimately, their use to protect citrus, a perennial crop, is temporary. In the continental United States, most CTV spread occurs during spring and fall when temperatures are mild. This is concomitant with when CTV titer (virus

replication) in infected citrus trees are highest and when shoot growth and migration of *T. citricida* peak. Therefore, this time frame should be targeted if chemical control is attempted.

CTV is transmitted only by vectors that colonize citrus because it is phloem-limited. Thus, its epidemiology resembles persistently transmitted viruses more in this regard than nonpersistently transmitted viruses. Since vector control has been shown to limit spread of some luteoviruses (Gourmet *et al.*, 1994) it could be expected to have some impact on CTV spread. Please note, however, that no data exists to recommend chemical control for CTV control.

5. Integrated pest management

It is not clear what level of vector control is necessary to reduce spread of CTV. The typical integrated pest management (IPM) approaches do not apply for CTV control. Economic thresholds are contingent both on *T. citricida* population and CTV inoculum pressure. Host plant resistance to the aphid is not available. A unique management strategy must be practiced for CTV in the presence of *T. citricida*. A strong regulatory component covering both propagation and inoculum control (detection and removal of wild and possibly urban reservoirs of CTV) (Garnsey *et al.*, 1996; Halbert and Brown, 1996). Management (conservation and/or augmentation) of biological control agents is feasible. Insecticidal control of vector populations may have use in specific situations such as in a citrus nursery or to protect budwood sources. Some value may result by use of selective insecticides working in tandem with natural enemies. In the final analysis, vector management should be one component of a disease management strategy which also other available elements including: mild strain cross-protection; tolerant rootstocks; above mentioned regulatory measures, isolation or protection of nursery stock; and citrus scions with tolerance or resistance to CTV (Garnsey *et al.*, 1996).

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First part

CTV and its vectors in the Mediterranean basin

Historical review of *Citrus tristeza virus* in Portugal

Nolasco G.

Centre for Biodiversity, Functional and Integrative Genomics, Universidade do Algarve
Campus de Gambelas, Faro, Portugal

Abstract. Most of the citrus production in Portugal is located in the Algarve region, in the south, which accounts for almost 70% of the national production. A boom of citrus planting occurred in the late 1980s with the introduction of modern varieties. This was accompanied by the introduction of CTV in the plant propagation material. Surveys for the virus and vectors started also at that time accompanied by the eradication of several *foci* of the disease. In parallel, works aiming at the molecular characterization of the virus were pursued. Up to the year 2000 most of the virus variants found in the orchards were similar to the Spanish predominant variant. Later the appearance of different variants became frequent. In 2005 new *foci* were detected in the north western part of the country in places where *T. citricidus* was present. With these new virus variants, the complete set of strains existing at worldwide level is now present in Portugal mainland.

Keywords. Citrus – Portugal – Rootstocks – Tristeza – Virus.

Aperçu historique du virus de la tristeza des agrumes au Portugal

Résumé. Au Portugal, la production des agrumes est essentiellement concentrée dans la région de l'Algarve, dans le sud du pays, qui représente presque 70 % du total national. Au cours des années 80, le verger agrumicole a connu un nouvel essor à la suite de l'introduction des variétés modernes. Parallèlement, le CTV a été introduit à travers le matériel de multiplication. Des enquêtes ont donc été entreprises pour la détection du virus et de ses vecteurs, accompagnées de l'éradication de plusieurs foyers de la maladie. En même temps, la caractérisation moléculaire du virus a été complétée. Jusqu'à l'an 2000, la grande partie des variantes du virus décelées dans les vergers étaient similaires à la variante prédominante en Espagne. Successivement, diverses autres variantes ont été observées. En 2005, de nouveaux foyers ont été identifiés dans le nord-ouest du pays, dans des régions où le *T. citricidus* était présent. Vu la mise en évidence de ces nouvelles variantes, il est possible de conclure que la gamme complète des souches de CTV connues à l'échelle mondiale est actuellement représentée au Portugal.

Mots-clés. Agrumes – Portugal – Porte greffes – Tristeza – Virus.

I – Introduction

Citrus trees are grown all over mainland Portugal, covering an area of 26,200 Ha (Anonymous, 2007). The Southern region of Portugal, Algarve, has the most dynamic and intensive citriculture and accounts for 17,860 Ha. In the remaining areas, citrus is grown in small scattered orchards or as backyard trees. The Algarve region accounts for 60% of the national sweet orange production, 85% of mandarin production and 40% of lemon production.

The first boom of citrus planting in Algarve occurred in the period 1960-1970 as a result of the implementation of new irrigation schemes. The citrus area was raised from 1560 Ha in 1950 to 5500 ha in 1970. At that time most of the production was based on sweet orange (Washington navel and local varieties with reduced commercial value) grafted onto sour orange rootstock. Symptoms attributable to graft-transmissible diseases could be easily observed in the field e.g. conspicuous symptoms of concave gum and psorosis.

A second boom of citrus planting occurred in the late 1980s as a consequence of the income of specific funds from the EU for the development of Portuguese agriculture and of the increasing demand for citrus fruits. In 1989 the citriculture in Algarve reached about 15,000 Ha. Since then the curve of the citrus-growing area has been flattening. This boom was accompanied by a strong desire of the farmers to obtain modern varieties with high market value. A significant influx of illegally introduced budwood occurred in that period in parallel with authorized limited importations of certified plants from Spain. Starting at that time, most of the new plantations were grafted on tolerant rootstocks, namely Troyer and Carrizo, which now account for 70% of the plantations. The introduced varieties that are nowadays the most representative are for the sweet oranges: Newhall, Navelina, Lanelate and Frost Valencia; for the clementines and hybrids: Fina, Nules, Marisol, Hernandina, Encore, Ortanique, Nova and Fortuna.

II – Historical review of CTV in Portugal

Before the late 1980s boom of citriculture in Algarve, no systematic survey for CTV had been done. Declining trees could be sporadically observed in the orchards but its cause was usually attributed to root damages by rats.

1986. In a collaborative action between the University of Algarve and the Regional Services of Agriculture, Nolasco (unpublished results) performed a prospective ELISA survey using the 3DF1 monoclonal antibodies provided by IVIA, targeting old declining trees. No positive results were found.

1988. A first systematic survey was done in part of Algarve based on aerial photography maps (scale 1/15000) by Nolasco and Faustino (Faustino, 1989). The survey was done by ELISA (Ingenasa MCA, 3DF1) on 1/1000 of the trees, corresponding to 236 Ha. No virus was detected in the field.

In an effort to modernise the citriculture sector, a special authorization for the limited importation of citrus plants from certified sources was issued. The material was imported from two certified citrus nurseries from Valencia region in Spain and re-tested in Portugal by ELISA. Surprisingly, some batches were infected, reaching 6% incidence. Confirmation of the positive results was done by ISEM by Dr. Diamantina Louro at the Central Plant Protection Services in Lisbon. Two of these isolates were preserved and later their coat protein genes sequenced (Sequeira and Nolasco, 2002) and deposited in the GenBank (haplotypes 19-121 and 25-120, accessions AF184114 and AF184114, respectively).

1989 – 1994. The prohibition to further import new varieties led several farmers to illegally import budwood from Spain for top-working old orchards. This situation pushed the governmental services to start surveys in nurseries and in suspicious top-worked orchards. CTV was detected occasionally in locations all over the country, being in principle, eradicated. This enabled Portugal to be considered as a Protected Zone for CTV during this period and subsequent years. However, the definite destruction of the infected material occurred sometimes several months after the first detection. Some old citrus introductions present in the germplasm collections were also surveyed. CTV was detected in an infected line of symptomless Genoa lemon in a germplasm collection of a deactivated citrus research station in Setubal (near Lisbon). Later molecular characterization of this isolate showed that it was made up of a single haplotype, 28C, which was deposited in the GenBank (accession number AF184118), (Nolasco, unpublished results). Surveys in the surroundings of infected foci always produced negative results, suggesting the absence of natural aphid vectoring.

During this period the first legislation envisaging citrus certification was issued, (Decreto lei 277/91 and Portaria 416/94), but except for CAC material, it was not mandatory.

1995 - 2000. Two types of ELISA surveys were designed: targeted sampling (nurseries, top-worked or chards and their surroundings) in which 5% to 10% of the plants were tested and random sampling of orchards at a rate of 1 plant per 1.5 Ha (approximately 0.1% to 0.24% of the plants) was done. However, due to logistic limitations, in the random sampling survey only a small part of Algarve region could be tested every year, averaging 775 ha per year. The targeted survey detected 3 foci in nurseries and 15 in top-worked orchards with illegally introduced material. This resulted in the destruction of about 31 thousand trees. No infected plants were detected in the surroundings of hot spots. In the random sampling assay 3 foci were detected which corresponded also to top-worked orchards. The results obtained during these years characterize a situation in which CTV is starting to become endemic and which is mainly disseminated by low quality agricultural practice.

Collaboration of the University of Algarve with National and Regional Plant Protection Services has allowed the molecular characterization of the infected material detected since 1989. Most of the coat protein gene sequences obtained from these isolates were very closed related (less than 1% difference at nucleotide level) to the sequence of the haplotype 25-120 (introduced from Spain in 1988) and to the mild T30 isolate from Florida. Partial biological characterization on Mexican Lime, Madam Vinous and Sour Orange as well as the absence of reaction with monoclonal antibody MCA13, showed that these isolates were of mild type (Nolasco, 2000). On the contrary, the isolate obtained in Setubal had a haplotype which differed by less than 2% from the VT haplotype obtained from a severe isolate from Israel. The biological and serological characterization confirmed that this is a severe isolate although not inducing stem pitting on sweet orange. Other samples collected in the central part of Portugal depicted a coat protein gene sequence very similar to the haplotype 28C obtained in Setubal.

Surveys for CTV vectors were done almost every year. The most abundant species captured in Moericke traps was *Aphis spiraecola*, followed by *A. gossypii* and *Toxoptera aurantii*. The prevalence of these two was variable from place to place. *A. craccivora* was rare and *T. citricidus* was not found (Ramos *et al.*, 2000). Except for *T. citricidus*, the ability of these aphids to acquire the virus from infected plants was demonstrated in the laboratory by RT-PCR (Reis *et al.*, 2000).

2001 - 2002. These years mark, probably, the starting of the natural transmission of CTV in the Algarve region. Eighteen new foci were found, including old orchards in the surroundings of newly top-worked orchards. Additionally, an orchard which was previously found negative became infected. In this period 13,800 trees were marked for eradication. It should be noticed that since the start of the random sampling systematic surveys from 1995 to 2002 only 7,000 Ha, i.e., only 40% of the total citrus area has been surveyed in Algarve.

2003 - 2005. Due to economic constraints, the governmental services decreased significantly the intensity of the systematic surveys. The molecular characterization of novel isolates based on the coat protein gene was carried out. Besides haplotypes molecularly close to the 25-120 (Spanish origin), new haplotypes appeared which were never found before in Portugal. These new types were geographically found close to the region of Silves, Algarve, and had a close relationship with haplotypes found in very severe CTV isolates e.g. SY568 from the USA (GenBank Accession AF001623) or 13C from Madeira Island (GenBank Accession AF184118). These findings suggest a new source of CTV, whose origin can be traced back to the budwood illegally introduced from Spain, which is usually molecularly close to the 25-120 haplotypes.

During this period *T. citricidus* was found in the north western part of Portugal (Ilharco and Sousa-Silva, Pers com.). A few scattered small orchards in that region were surveyed for CTV in the fall of 2005. CTV was found in infested trees. Molecular characterization of CTV from 2 trees about 30 Km apart showed that these harbored a mixture of haplotypes which include some newer ones in Portugal (close to T36 from Florida, T3 from Florida and B249 from Venezuela). The structure of the CTV population found in both trees was not significantly different (Nolasco *et al.*, 2007).

These findings suggest another new source of introduction and the existence of natural spreading of CTV by *T. citricidus* in the region.

Taking into account all the coat protein gene haplotypes that have been detected, Portugal has now all the haplotypes that can be found elsewhere in the world.

III – Conclusions

This history illustrates a few critical points on the introduction of CTV in a country. To become competitive, the farmers feel the pressure to obtain new varieties. In the absence of an efficient national programme for the development of citriculture, the illegal introduction of budwood starts. In practice the Plant Protection Services can not control all the budwood movement. Hence, an efficient survey is of paramount importance to be able to evaluate the evolution of the situation in the field. This needs a sampling rate much higher than that applied in Portugal. A few calculations show that with the sampling rate used, if the disease incidence is low (e.g. less than 1%) the probability of finding an infected tree is also lower than 1%, which is useless! The building up of inoculum in the field may be overlooked by the farmers and agricultural services until natural transmission by aphids starts, even in the absence of *T.citricidus*.

The increase in vector-mediated transmission implies a change in the short-term control strategy. Surveys should be much more efficient to be able to follow the situation. Molecular typing of strains is now of utmost importance to concentrate the eradication efforts in the elimination of strains that are able to deteriorate trees grafted onto tolerant rootstocks (e.g stem pitting strains). Besides this selective eradication, the objective of general eradication should be to maintain the inoculum level at a low, economically acceptable, level.

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Toxoptera citricidus (Kirkaldy, 1907) (Homoptera, Aphidoidea), the tropical citrus aphid in continental Portugal

Ilharco F. A.¹, Sousa-Silva C. R.²

¹ Departamento de Protecção de Plantas, Entomologia Estação Agronómica Nacional, Portugal

² Universidade Federal de São Carlos, Departamento de Ecologia e Biologia Evolutiva São Carlos, Brasil

Abstract. Surveys aiming to detect the presence of *T. citricidus* in Portugal mainland have been carried out since 1963. The aphid was detected for the first time in Madeira Island in 1994 and in the mainland in 2003, in the north western region. In the years that followed the aphid could be always detected, and in some places even in the winter months. The infested area is slowly enlarging. The preferred host is *C. lemon*. Predators and parasitoids are present in the period of population growth. A macroscopic key is presented for the identification of the economically important *Citrus* aphids in Portugal.

Keywords. Aphids – Citrus – Portugal – *Toxoptera citricidus* – Tristeza

***Toxoptera citricidus* (Kirkaldy, 1907) (Homoptera, Aphidoidea), Le puceron tropical des agrumes dans le Portugal continental**

Résumé. A partir de 1963, des enquêtes ont été réalisées afin de déceler le *T. citricidus* dans le Portugal continental. Ce puceron a été signalé pour la première fois sur l'île de Madère en 1994 et, en 2003, sur le territoire continental dans le nord-ouest. Ensuite, le puceron a continué d'être signalé et dans certains endroits, il a été mis en évidence aussi pendant l'hiver. La zone d'infestation s'étend progressivement et le *C. lemon* reste l'hôte préféré. Les prédateurs et parasitoïdes sont présents pendant toute la période de croissance de la population du puceron. Une clé macroscopique est proposée pour faciliter l'identification des pucerons des agrumes économiquement importants au Portugal.

Mots-clés. Pucerons – Agrumes – Portugal – *Toxoptera citricidus* – Tristeza.

I – Historical review

1963 - 1993. The first survey on *T. citricidus* in Portugal was carried out in 1963, in the agricultural areas of Coimbra, Santarém, Setúbal, and Algarve (Neves, 1965) and, afterwards by Ilharco (1978), also in the Algarve province. On those occasions, the aphid was not found.

1994 - 2002. *T. citricidus* was reported in Madeira Island (Aguir et al., 1994). The efforts to eradicate it were unsuccessful due to its fast dissemination in the Citrus growing areas of the island and it was considered to constitute a potential threat to Citrus orchards in Continental Portugal. New search for the aphid in the mainland was carried out. Cruz de B oelpaepe and Ferreira (1998) conducted surveys in the Citrus growing areas from the Agricultural Regions of Entre Douro e Minho, Beira Litoral and Ribatejo and Oeste, in the Northern, central and central-southern area of Portugal, respectively. From 1997 to 1999 surveys of *T. citricidus* were done also in Algarve (Ramos et al., 2000), without captures. The aphid was detected on neither occasions.

2003. *T. citricidus* was reported in North Western Portugal in the Entre Douro e Minho region and, in Spain, in Astúrias and Vigo (Ilharco et al., 2005).

2004 - 2006. Various surveys were done to evaluate the dispersion of *T. citricidus* in continental Portugal in citrus and in other plant species and to check the action of their natural enemies.

Toxoptera citricidus was collected directly from the Citrus plants or by yellow water traps; small citrus orchards were inspected, along with single backyard plants or ornamental citrus in towns; other neighbouring plant species were also inspected. The aphid identification was made in the field, aided by magnifying lenses, or in the laboratory, if it was necessary. Samples caught by traps were identified under stereoscopic microscope and nominated vagrants.

In 2004, *Toxoptera citricidus* was caught in Valença, Monção, Vila Verde, Amares, Barcelos, Braga, Vila Nova de Famalicão, Guimarães, Fafe, Santo Tirso, Felgueiras, Celorico de Basto, Amarante, Penafiel, Marco de Canaveses and Santa Marta de Penaguião. In 2005, in Vila do Conde, Paços de Ferreira, Lousada, Vila Nova de Gaia, Espinho, Ovar and Ílhavo and in 2006, in Arcos de Valdevez, Viana do Castelo, Ponte de Lima, Ponte da Barca, Terras de Bouro, Esposende, Vieira do Minho, Póvoa de Lanhoso, Gondomar, Aveiro and Estarreja (Fig. 1).

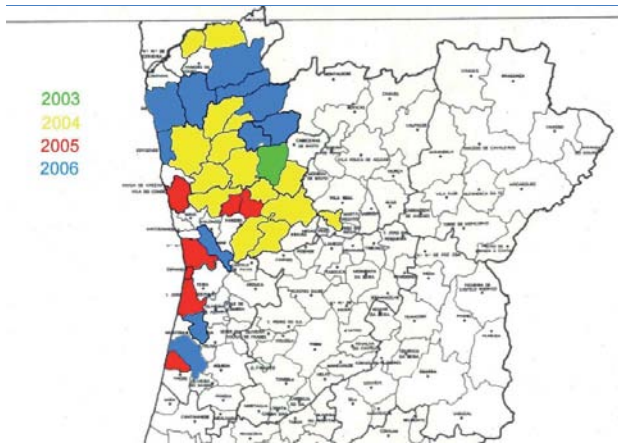


Figure 1. Dispersion of *Toxoptera citricidus* (Kirkaldy), in Continental Portugal. The coloured areas on the map are related to the areas where the aphid was firstly designated.

In this period, the limits of *T. citricidus* dispersion in Continental Portugal are Valença and Monção in the north, Ílhavo in south, and Santa Marta de Penaguião in the east, and its presence was checked all over the year (Tab. 1). This shows the capacity to resist the thermal variations from winter to summer in the studied area. Blackman and Eastop (2000) have mentioned that although *T. citricidus* develops better in hot weather, it can, apparently, tolerate low temperatures better than *T. aurantii* (Boyer de Fonscolombe, 1841), which is a well-adapted species in Portugal.

Table 1. *Toxoptera citricidus* survey in the continental Portugal in the period 2004-2006.

Year/ Month	J	F	M	A	M	J	J	A	S	O	N	D
2004	-	-	-	-	x	x	x	x	x	x	x	x
2005	x	-	x	x	-	-	-	-	x	x	x	x
2006	x	x	0	x	x	x	x					

x: presence of *T. citricidus*
 -: month without survey
 0: absence of *T. citricidus*
 J-D: January – December
 May and June/2004: Samples caught by traps (vagrant aphids)

In 2005, from February to April only one apterous *T. citricidus* was caught on *Citrus aurantium* L., and one alate aphid on *Citrus sinensis* (L.) although it was observed that lemon trees had suitable foliage for the aphids all the year round in opposition to orange trees.

In 2006, during March and early April, no *T. citricidus* specimen was found. However, in the surveyed locations the plants had received phytosanitary treatments against aphids. In later April, 2006, in Guimarães region a large population was observed on a single lemon tree that had not received any phytosanitary treatment. This suggests that the isolated hosts can become a focus of aphid dispersion when weather and citrus foliage conditions ameliorate. This could explain the great amount of *T. citricidus* caught later in May, 2006, in the same areas where in the previous month the aphids were almost absent. Probably during February-April, the adverse conditions, plus the sanitary treatments, can be responsible for the decrease in the aphid populations in the studied areas. Particularly in the orange tree, the lack of suitable foliage is an additional factor affecting adversely the development of *T. citricidus* population. During the period of population growth some natural enemies, predators and parasitoids were observed (Fig. 2, 3). Michaud (1998) reports an extensive literature about this.

T. citricidus has been collected on Citrus only, particularly in *C. limon* (over 60% of the samples). When large populations develop the aphids may be observed also on the top twigs and flowers (Fig. 4).

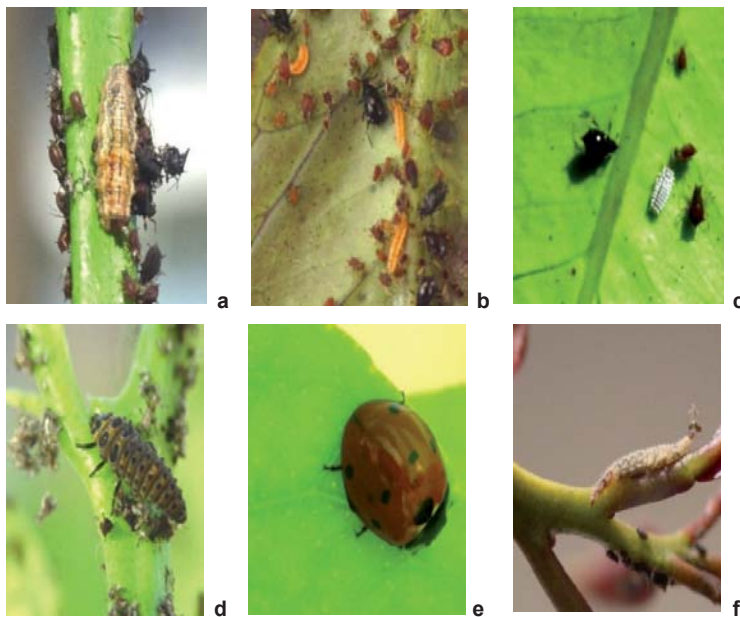


Figure 2. *T. citricidus* predators: a) Syrphids; b) Cecidomyiids; c, d, e,) Coccinellids; f) Chrysopids (Photographs by C. R. Sousa-Silva).



Figure 3. Mummified *T. citricidus*. (Photographs by C. R. Sousa-Silva).

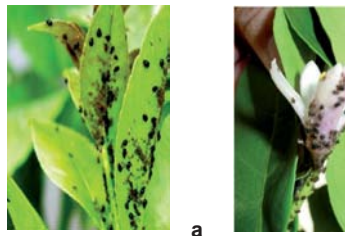


Figure 4. *T. citricidus* (Kirkaldy) on *Citrus limon*: a) along the veins; b) on the flowers. (Photographs by C. R. Sousa-Silva).

Based on Ilharco and Fonseca (1985) a macroscopic key is presented for the identification of the economically important *Citrus* aphids in Portugal:

Apterous forms:

- | | | |
|---|---|--|
| 1 | Body green, yellow- green or brown | 2 |
| - | Body dark reddish-brown or shining black | 3 |
| 2 | Body green to yellow-green. Cauda as dark as the siphunculi | <i>Aphis spiraecola</i> Patch (Fig. 5a) |
| - | Body green, yellow-green or brown. Cauda lighter than siphunculi | <i>Aphis gossypii</i> Glover (Fig. 6a, b) |
| - | Body light-yellow with some dorsal dark markings. Generally small insects | <i>Myzus ornatus</i> Laing (Fig. 7a) |
| 3 | Body dark-brown. Apex of antennal segments III, IV and V, the apical half of base of VI and sometimes also the apex of <i>processus terminalis</i> dark | <i>Toxoptera aurantii</i> (Boyer de Fonscolombe) (Fig. 8a) |
| - | Body reddish-brown or shining black. Antennal segment III and the basal part of IV pale, V and VI dark | <i>Toxoptera citricidus</i> (Kirkaldy) (Fig. 9a) |

Alate forms:

- | | | |
|---|--|--|
| 1 | Abdomen green, yellow-green or brown, with or without compact dorsal plate brown to black | 2 |
| - | Abdomen dark-browened, reddish-brown to dark or shining black | 4 |
| 2 | Abdomen light-green to pale-yellow with compact dorsal plate. Head and thorax brown | <i>Myzus ornatus</i> Laing (Fig. 7b) |
| - | Abdomen without dorsal plate | 3 |
| 3 | Abdomen green to yellow-green. Head and thorax black, cauda as dark as the siphunculi | <i>Aphis spiraecola</i> Patch (Fig. 5b) |
| - | Abdomen yellow-lighted to dark or brown green with a black stripe between the siphunculi. Cauda pale or lighted dark, lighter than the siphunculi | <i>Aphis gossypii</i> Glover (Fig.6c) |
| 4 | Abdomen dark-brown, fore wings with median vein almost always once branched, pterostigma black. Antennal segment III pale with black apex | <i>Toxoptera aurantii</i> (Boyer de Fonscolombe) (Fig. 8b) |
| - | Abdomen dark reddish-brown or shining black with smooth, polished, shining cuticular surface, fore wings with median vein usually twice branched, pterostigma pale, antennal segment III black, in contrast with basal half of segments IV and V, which are pale | <i>Toxoptera citricidus</i> (Kirkaldy) (Fig. 9b) |



Figure 5. *Aphis spiraecola*: a) apterous; b) alate.
(Ilharco and Fonseca, 1985).

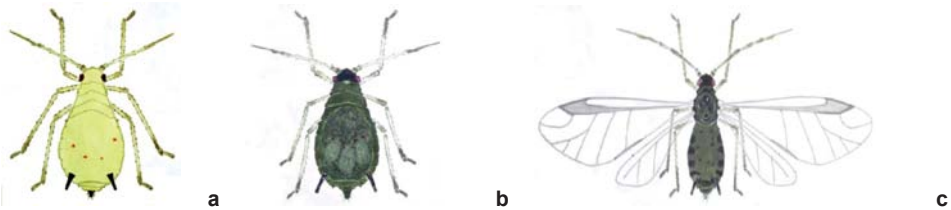


Figure 6. *A. gossypii*: a) apterous, summer form; b) apterous, winter form; c) alate.
(after Ilharco and Fonseca, 1985).



Figure 7. *Myzus ornatus*: a) apterous; b) alate.
(Ilharco and Fonseca, 1985).

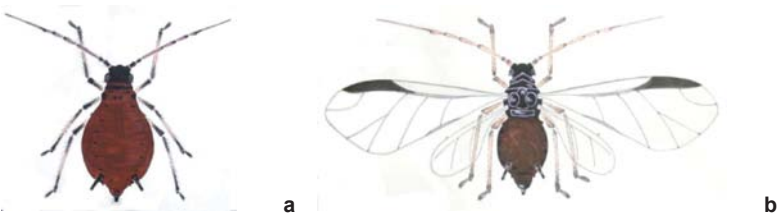


Figure 8. *T. aurantii*: a) apterous; b) alate (Ilharco and Fonseca, 1985).



Figure 9. *T. citricidus*: a) apterous; b) alate.
(Ilharco and Fonseca, 1985).

II – Conclusions

- i. *T. citricidus* is already well established in the north of Continental Portugal, and has been collected all the year round.
- ii. *Citrus limon* is there the preferential host of *T. citricidus*.
- iii. *T. citricidus* was collected only on Citrus hosts.
- iv. Single lemon trees that do not receive any sanitary treatment have an important role in maintaining *T. citricidus* populations during the winter period.

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Historical review of *Citrus tristeza virus* in Italy

Djelouah K., Valentini F., D'Onghia A.M.

CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. A brief introduction on the Italian citrus industry is provided. Tristeza findings are reported from 1955 to date and emphasis is laid on the outbreaks which occurred in Sicily and in Apulia in 2002. Special attention is paid to the Apulian legislation which reinforces the current national regulation for the mandatory control of CTV in Italy.

Keywords. Aphids – Citrus – Citrus tristeza virus – Italy – Monitoring.

Rétrospective historique du CTV en Italie

Résumé. Une brève rétrospective de l'agrumiculture italienne est présentée dans ce travail. Les données relatives à la Tristeza sont passées en revue de 1955 à nos jours, en rappelant, en particulier, les foyers qui se sont développés en Sicile et dans les Pouilles en 2002. Une attention particulière est donnée sur la législation régionale des Pouilles qui renforce la réglementation nationale pour la lutte obligatoire contre le CTV en Italie.

Mots-clés. Pucerons – Agrumes – Virus de la tristeza des agrumes – Italie – Suivi.

I – The citrus industry in Italy

In Italy the citrus industry covers a surface area of 182 000 ha averaging a production of about 3 million tons. The most common species is the sweet orange (61%) followed by clementine (18%) and lemon (20%) whereas minor citrus trees (e.g. grapefruit, bergamot and citron) account for 1%. Citrus trees are mainly grown in southern Italy (Sicily, Calabria, Apulia, Basilicata, Sardinia and Campania) where the CTV-sensitive sour orange is the most common rootstock (about 98%) (ISTAT, 2006).

Over the last years, due to the crisis of the Italian citriculture, the cultivation of citrus trees for ornamental purposes has been playing a primary role with a mean annual production of about 2 500 000 plants (mainly lemon, kumquat, calamondin, myrtle-leaf orange) for the northern European market. National nurserymen guarantee a qualitatively-high production and contribute to consolidating the Italian leadership in the northern European market (ISTAT, 2006).

The crisis of the citrus sector, resulting from the higher economic competitiveness of the Spanish and non-EU produce, has spurred the commercial production of valuable typical citrus trees which characterize peculiar areas to meet the needs of a niche market. At present, in our country, ancient citrus collections are still grown in the gardens of historical palaces and in protected areas where ancient-old citrus varieties may be found. In these sites, citrus trees are also protected by the Department for Cultural Heritage since they are considered as integral part of the Italian landscape.

II – Historical review of Tristeza

1955. The disease was first reported at Acireale (CT) when it was identified on Meyer lemon and Owari satsuma trees introduced from foreign countries and on two Meyer lemon trees kept in the Botanical Garden in Palermo. As known, these species are healthy carriers of tristeza, a disease which originated in China (Russo, 1956).

1967 - 1974. Both in Catania and in Reggio Calabria, some isolated foci of the virus were reported on the above-mentioned species (Servazzi *et al.*, 1967; Catara, 1968; Davino *et al.*, 1998).

1982 - 1986. In Calabria, the first major focus was identified of infected Marsh seedless grapefruit, Golden Buckeye sweet orange, Wase satsuma and Ceylon lemon trees, varieties introduced from countries where the infection is endemic (e.g. Spain) (Davino *et al.*, 1983).

1986 - 1995. With the start of tristeza monitoring activities, carried out by scientific institutions, some 200 cases of infection were discovered (plants and scions) (Davino *et al.*, 1998).

1995 - 1996. In the ornamental nurseries of Pescia (Pistoia) a focus was discovered of about 4000 *Citrus othaitense* plants and of 10 000 calamondin plants. These plants originated from infected sources with scions imported from an English botanical garden (Davino *et al.*, 1998).

1996. Due to the incidence of CTV infected plants, the Decree on Mandatory Control (D.M. n. 285 of 22 November 1996) transposing the EC directive N°.77/93/EEC, was issued. This Decree allows regulating the mandatory control to CTV through systematic monitoring in commercial citrus groves, nurseries, supply sources, mother plants, varietal collections, public and private gardens.

1997 - 2002. Monitoring activities were carried out by the Mediterranean Agronomic Institute of Bari under the umbrella of the regional phytosanitary services and enabled to identify some ornamental citrus plants (all of Sicilian origin) in a nursery located in Apulia. These plants were promptly eradicated.

In Sicily, Apulia and Sardinia, open-field foci were identified in areas kilometres apart, with thousand plants of different species and age. In Sicily, two foci were detected one of which in the district of Siracusa, with "Fortune" and "Nova" hybrids, satsuma and grapefruit plants showing symptoms of dieback, stunted growth and death. In the other focus, in the district of Catania, local Tarocco orange plants, showed symptoms of chlorosis, small-sized fruits and poor pigmentation. Diseased plants did not exhibit symptoms of inverse pitting at bud line on sour orange. Since the virus attacks Tarocco as well, which is a local Sicilian selection, the natural transmission of the virus was also envisaged.

Two foci were identified in Apulia in the district of Taranto, one with a few infected Navelina orange plants, showing reduced size; in the second focus, Navelina orchard had an infection rate exceeding 30% while Common clementine planting recorded a 20% infection. In this case infected plants did not display inverse pitting symptoms at bud line on sour orange. Diseased plants in the first focus and the whole orchard in the second focus were immediately uprooted (Davino *et al.*, 2003; Babarossa *et al.*, 2005).

In the same period, also in Sardinia, a few CTV-infected plants were identified and immediately destroyed (Davino *et al.*, 2003; Babarossa *et al.*, 2005).

2003 - 2005. After the first outbreaks in Sicily and Apulia, and thanks to monitoring activities, other foci were identified.

In Sicily, new outbreaks were reported from the districts of Palermo and Messina. The Regional Phytosanitary Service proposed a strategy other than the one envisaged by the decree of mandatory control given the severity of the disease (Davino *et al.*, 2002).

The Regional Government of Apulia adopted very stringent control measures with two provisions issued by the Regional Council (N° 780 of 5/6/2003 and N° 554 of 20/4/2004) envisaging different procedures according to the destination of orchards and areas (free or contaminated). New working tools were introduced such as orthophotographs with cadastral map for the fast identification of sites to be monitored and of their owners and the application of more thorough hierarchical sampling methods (Gottwald and Hughes, 2000), (25% of plants both in the field

and in the nursery) and faster diagnosis (DTBIA instead of DAS-ELISA). An information campaign was also launched by promoting periodical meetings and handing out leaflets reporting salient clues on the disease and its vectors, on the control methods and on the institutions to get in touch with in case of need.

With Directive 2005/18/EC of 2 March 2005, the European Commission repelled the status of protected area. Citrus produced in Italy can not be sold with the leaves and will be considered on a par with the Spanish citrus fruit.

2006. With the first monitoring in Calabria, CTV foci were identified on Fortune and Satsuma mandarin in Reggio Calabria district. On mandarin, the disease was manifest and numerous plants had already been killed (Caruso *et al.*, 2006; Schimio *et al.*, 2007).

The National Phytosanitary Service set up an inter-regional working group including experts from scientific institutions and regional phytosanitary inspectors with a view to studying all the needed amendments to the decree on the mandatory control.

After the first detection of *Toxoptera citricidus* (main CTV vector) in Northern Portugal and in Spain, a working group was set up by the National Phytosanitary Service, also to issue a national decree for the mandatory control of *T. citricidus* to amend the M.D. on CTV.

In Apulia, Sicily and Calabria, systemic monitoring of aphids was promoted to exclude the presence of *T. citricidus* from citrus-growing areas. The results excluded the presence of *T. citricidus* but highlighted other vectors such as *A. gossypii*, *A. spiraeicola*, *T. aurantii*.

Interestingly, the continuous monitoring activities, the biological and molecular characterization of the CTV isolates in different areas of the country, indicates apparently the presence and diffusion of the mild CTV strain (Barbarossa *et al.*, 2004; Davino *et al.*, 2005).

III – Conclusions

The serious aphid-vector-borne disease outbreaks in the major Italian citrus-growing regions (Sicily, Apulia and Calabria), and the presence of sour orange which is highly susceptible to tristeza, corroborate the hypothesis that drastic measures must be taken to face tristeza which is likely to destroy the Italian citrus industry (fresh fruit, processed products and ornamental citrus plants) and the landscape.

Several factors can impact the success of this battle among which are: (i) an updated regulation on the mandatory control of CTV with procedures which respond to the effective contamination levels; (ii) a new regulation on *T. citricidus* to gain the recognition of area protected from this aphid; (iii) strengthening quarantine measures; (iv) eradication of CTV foci in the Regions where the virus is not endemic yet (Apulia, Calabria); (v) the characterization of CTV isolates to assess their severity and eventually their eradication; (vi) reinforcing faster monitoring methods, strain characterization for a rapid field identification of virulent strains and for the selection of those which match cross protection; (vii) training human resources and empowering the Regional Phytosanitary Services; (viii) setting up a team of national coordination for the application in the Italian citrus-growing regions, of common measures relating to information, interaction, fund raising for those growers obliged to uproot their orchards and other initiatives.

These steps may be undertaken with the joint commitment of the political authorities, inspection boards, research institutions, nurseries' and producers' associations. The citrus industry has a strong impact on the Italian economy; the fate of the citrus industry and the future of our country depend on the timely solution of this crisis.

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Citrus aphids in Italy: historical review

Addante R.¹, Djelouah K.², D'Onghia A.M.²

¹ Dipartimento di Biologia e Chimica Agro-forestale ed Ambientale,
University of Bari, (BA), Italy

² CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Aphids occurring on citrus in Italy have been studied since the second half of the XIX century. Most of the previous papers on the aphids of the Italian citrus groves concerned faunistic and morphological traits whereas the main aspects investigated in the last decades focused on their potential as virus vectors and on tools and strategies used for their control. Eleven are the aphid species reported on citrus in Italy, although most of them can be found only occasionally.

The following review takes also into account the works by authors who have described some aspects relating to the morphology, biology, control, etc. of one or of several citrus aphid species in Italy.

Keywords. Aphids – Citrus – *Citrus tristeza virus* – Italy – Virus vectors.

Les pucerons des agrumes en Italie: bref historique

Résumé. Les pucerons qui infestent les agrumes en Italie ont été étudiés à partir de la deuxième moitié du XIXe siècle. Au début, la plupart des travaux consacrés aux pucerons dans les vergers agrumicoles italiens étaient des études faunistiques ou des études sur les caractéristiques morphologiques alors que, dans les dernières décennies, l'attention s'est concentrée davantage sur leur potentiel en tant que vecteurs de virus et sur les outils et stratégies employés dans la lutte. Il existe onze espèces de pucerons connues sur les agrumes en Italie bien que, pour la plupart, elles ne soient signalées qu'occasionnellement. Ce travail prend aussi en considération les travaux des auteurs qui ont décrit certains aspects de la morphologie, de la biologie, de la lutte d'une ou plusieurs espèces de pucerons des agrumes en Italie.

Mots-clés. Pucerons, agrumes – Virus de la tristeza des agrumes – Italie – Vecteurs de virus.

I – Fauna

Among the scientists who, since the last decades of the XIX century, have reported the presence of aphid species (Hemiptera Aphididae) on some *Citrus*, apart from many other plant hosts, mention shall be made of Passerini (1863), Ferrari (1872), Del Guercio (1900) and Ribaga (1901). In 1917, Del Guercio published the description of some citrus aphid species thought to be new for science. Following review by various aphidologists highlighted that these new species had already been described before. Other contributions on aphid species that can be found also on the citrus were published by Silvestri (1939) and R oberti (1945). In the second half of the XX century, more specific contributions were published on the citrus aphids. The first paper on citrus aphids in Sicily was published by Barbagallo in 1965; the Author reports essential data on *Toxoptera aurantii* (Boyer de Fonscolombe), *A. spiraecola* Patch and *A. gossypii* Glover, and underlines the first finding of *Aphis spiraecola* on the island and its higher harmfulness versus other specific aphid species. In 1966, the same Author (Barbagallo, 1966b) published the first comprehensive review on the citrus aphids concerning only Sicily. The Author reports five more species of Aphididae in the Sicilian citrus-growing areas: *A. craccivora* Koch, *A. fabae* Scopoli, *Macrosiphum euphorbiae* (Thomas), *Myzus (Nectarosiphon) persicae* (Sulzer) and *Rhopalosiphum maidis* (Fitch). The most frequent species in the Sicilian citrus groves are, in decreasing order, *T. aurantii*, *A. spiraecola* and *A. gossypii*. The other species mentioned above are less relevant or occasional (as is the case for *R. maidis* usually associated with graminiae) and their detection may be ascribed to the presence

of other wild or cultivated plants in the citrus grove hosting the same aphids (Barbagallo, 1966b; Patti, 1985). With the exception of *A. fabae*, the above mentioned species were also reported from Calabria (Micieli De Biase, 1975) on various *Citrus* trees. The list of aphid species occasionally found on citrus trees was enriched by Patti (1983 and 1985) who reported *Aulacorthum solani* (Kaltenbach) and *Hyadaphis coriandri* (Das), which usually attack different Umbelliferae species, and by Barbagallo (1986) who found in Sardinia *Myzus ornatus* Laing another highly polyphagous species. To date, the *Citrus* aphid species found in Italy are eleven.

The fauna composition of citrus aphids is quite the same across the Italian citrus-growing regions and does not differ from that reported in the other Mediterranean countries (Barbagallo and Inserra, 1974).

II – Morphology

Several Italian authors have contributed to probing into the morphology of citrus aphid species. As to *A. spiraecola* (1966a), Barbagallo describes in detail also the main morphological traits which help discriminating between the green aphid and the similar *Aphis pomi* DeGeer. Other data on *A. spiraecola* morphology were later published by Micieli De Biase (1970). Again, Barbagallo (1966a) provided an accurate morphological description, complemented with detailed drawings, of eight apterous and alate virginoparous citrus aphid species. Of paramount importance is the morphological, anatomical and histological description of *A. gossypii* (sub *Aphis (Doralis) frangulae* Koch) published by Roberti in 1946. Further contributions to the morphological description and brief information on the geographical distribution, the biological cycle and the ecology of the Italian aphid species, including all those that can be found on the citrus, were given by Roberti (1991) in his work on aphids in Italy.

Dichotomic keys to identifying the main aphid species in the Italian citrus groves, valid for the apterous virginoparous forms, were reported by Barbagallo (1966b), Micieli De Biase (1975) and Patti (1983), who reminded that the colonies on the same shoot may sometimes include several aphid species.

III – Symptomatology and damages

Aphids may become harmful both when their populations attain high density values, thereby causing veritable pullulation, and with a few individuals per plant as pathogen vectors.

In the first case, the damage results from sap sucking and injection of toxic saliva in the tissue, with alterations of the plant cell metabolism which impact the crop yield. The symptomatology displays a lower development of the shoots, leaf deformations of variable entity according to the species, drop of flowers and fruitlets, production of honeydew with the development of sooty mould, which contributes to worsening the damage by reducing photosynthesis and decreasing the gaseous exchange (Zappalà, 2001). The most harmful species reported in Italy is *A. spiraecola* which causes leaf rolling and deformations or stops the growth of shoots. Less harmful, in decreasing order, are *A. gossypii* and *T. aurantii* (Barbagallo, 1966a; Micieli de Biase, 1970; Patti, 1983).

In the second case, the harmfulness is higher due to the ability of some aphid species to transmit virus diseases which are sometimes lethal. Although the importance of aphids as virus vectors (mention should be made of *Citrus tristeza virus*) has been known in Italy for more than fifty years (Russo, 1956; Barbagallo, 1965, 1966a, 1966b) a few studies have been carried out in our country on the ability of the various aphid species to transmit viruses, on the mode of transmission, on the diffusion of virus diseases according to the bio-ethological characteristics of the aphid species, etc. In some trials conducted in Italy by Cartia *et al.* (1980), *A. gossypii*, *A. spiraecola*, *A. craccivora* and *T. aurantii* were not able to transmit CTV. However, it is notorious that in the

citrus-growing areas of other countries, *A. gossypii*, *A. spiraecola* and other aphid species have proved to transmit CTV although less efficiently than *T. citricidus* (Kirkaldy), the most efficient tristeza vector (Lim and Hagedorn, 1977). Few years later, Davino and Patti (1986) and Davino *et al.* (1990) showed that also the Italian populations of *A. gossypii* were able to transmit CTV under restricted environment. More recently, Davino *et al.* (2004) stated that *A. gossypii* is the main responsible for CTV dissemination in the Sicilian citrus groves, with a 5 % increase of infected plants in two years.

IV – Bio-ethology

With the exception of *T. aurantii*, oligophagous on Citrus and on a few other genera of trees or herbaceous plants, citrus aphid species are highly polyphagous (Barbagallo, 1966b; Patti, 1983 and 1985) and can infest, in some instances, several hundred host species (*A. gossypii* can infest about 700 hosts worldwide). The surveys conducted in the Italian citrus-growing regions on the aphid species and their Citrus hosts could confirm that *T. aurantii* does not prefer any specific citrus species whereas *A. gossypii* and *A. spiraecola* clearly prefer clementine, mandarin and orange trees and to a lesser extent lemon and citron (Barbagallo, 1966b; Patti, 1985). Aphid infestations on lemon and citron are mostly ascribable to *T. aurantii* since these citrus trees are rarely infested by other Aphididae species (Patti, 1985). More detailed information on the bio-ethology of the citrus aphids was reported by Barbagallo (1966a) for *A. spiraecola* and by Barbagallo (1966b) and Patti (1983, 1985 and 1996) for the other species found in Italy. In the Italian citrus-growing regions, various aphid species behave as anholocyclic, sometimes obligate (*T. aurantii*) whereas others, such as *A. spiraecola* and *M. persicae*, can complete the holocycle. In Italy, holocyclic species use citrus trees as secondary hosts since the amphigonous generation never grows on them (Patti, 1983).

Aphid infestations usually turn into pullulation in spring and are less intense in autumn (Patti, 1983).

V – Control

The wide resources of natural antagonists of the aphid species living on citrus were reported by several authors, mainly by Patti (1983) and Longo and Benfatto (1987) for predators and by Stary (1967), Tremblay *et al.* (1978 and 1980) and Marullo (1985) for parasitoids. In a study on the complex of parasitoids of *T. aurantii*, Tremblay (1984) reported seven species of Imenoptera Braconidae highlighting the competition relationship between the prevailing species *Lysiphlebus fabarum* (Marshall) and *Lysiphlebus testaceipes* (Cresson). Surveys conducted in the main Italian citrus-growing regions over twenty years have highlighted that *A. spiraecola* colonies are less visited by the parasitoids with respect to those of other citrus aphid species (Tremblay *et al.*, 1978 and 1980). Liotta (1988), apart from evaluating the parasitization activity of *Aphelinus chaonia* Walker at the detriment of *T. aurantii*, gave evidence of the importance of the host-feeding activity of the entomophagan.

For the protection of the natural enemies Barbagallo and Inserra (1974) suggest integrated control strategies along with a reduced application of selective aphicides.

As for integrated control measures, Stary (1964 and 1967) suggested that *Pittosporum tobira* (Thunb.) W.T. Aiton hedges should be replaced with oleander, willows or other species which can favour the development of numerous parasitoids without hosting aphid species (mainly *T. aurantii*) likely to infest the Italian citrus groves. By adopting integrated control measures and maximising the action of entomophagans, Ortu and Prota (1983) could limit the populations of aphids and of other citrus pests with a few insecticidal treatments.

For the control of *A. spiraecola*, 5% and 10% thresholds of infested shoots have been proposed on clementine and sweet orange respectively whereas the threshold can equal 25% for the control of *A. gossypii* and *T. aurantii* infestations (Cavalloro and Prota, 1983; Patti, 1983; Zappalà, 2001). In Sardinia, Delrio *et al.* (1981) adopted thresholds equalling 10-15% of shoots infested by *A. spiraecola*.

Pertaining to aphicides, over the years nicotine sulphate and quassia-wood have been adopted (Silvestri, 1939), along with white oils, whose low impact on the entomophagans in Calabria was highlighted by Micieli De Biase (1975), phosphorus esters and carbammates (Barbagallo, 1966a; Patti, 1983), imidachloprid (Micieli De Biase and Russo, 1996; Nucifora, 1998), a cetamidrid (Domenichini and Roffeni Tiraferri, 1998).

For the control of aphid species on citrus plants grown in the nursery, the following active ingredients are now used: imidachloprid, methomyl, pymetrozine+thiamethoxan, thiamethoxan, tau-fluvalinate (Di Franco and Benfatto, 2007).

As to the biological control of citrus aphids, Tumminelli *et al.* (2004) cast light on the efficacy and selectivity towards natural enemies of a mixture of paraffin oils, pyrethrins and rotenone.

Finally, mention shall be made of the side effects of insecticides on the useful arthropodofauna. Bernardo and Viggiani (2000) could show that pymetrozine, an insecticide active against various phytomyzae, having a new mode of action and thought to be selective towards the entomophagans, is highly toxic on the parasitoids and on the adults of *Rodolia cardinalis* (Mulsant) and spares just the pre-imago stages of the Coccinellid.

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Citrus tristeza virus (CTV) in Greece: historical review

Dimou D.¹, Coutretsis P.²

¹ Directorate of Agricultural Development, Prefecture of Argolis, 21100 Nafplion, Greece

² Control Station for Vegetative Propagative Material, 19300 Aspropyrgos, Greece

Abstract. Tristeza is a very recent problem in Greece raised after the free circulation of goods, such as agricultural products and propagating material, among the EC member countries.

The Greek legislation, issued in 1959, forbids the importation of citrus propagating material into Greece. Only controlled quantities of citrus propagating material, produced in research institutions and universities, were allowed to be introduced under special permission and after laboratory testing (Kyriakopoulou, 1999). The year 1994 may be considered as a milestone for the dissemination of quarantine diseases when the check-in of the imported plant material at the borders was abolished.

Thus, the current situation is very hard because the free circulation of plant material, in Europe, can function as a “Trojan horse” for the dissemination of destructive diseases, like Tristeza, among the EC member states.

Keywords. Citrus Tristeza – ELISA – Greece.

Le virus de la tristeza des agrumes (CTV) en Grèce: aperçu historique

Résumé. La Tristeza est un problème qui s’est posé en Grèce très récemment, à la suite de la libre circulation des biens, tels les produits agricoles et le matériel de multiplication, entre les états membres de l’Union Européenne.

La législation nationale, promulguée en 1959, interdit l’importation du matériel de multiplication en Grèce. Il est possible d’introduire exclusivement des quantités contrôlées de matériel d’agrumes, produit dans des instituts de recherche et des universités, soumis à une autorisation spéciale et testé au laboratoire (Kyriakopoulou, 1999).

L’an 1994 représente une pierre milliaire pour la dissémination des maladies de quarantaine étant donné que tout contrôle aux frontières sur le matériel végétal importé a été aboli.

Par conséquent, aujourd’hui on se trouve confronté à d’énormes difficultés vu que le matériel végétal peut devenir le “Cheval de Troie” de la diffusion de maladies destructrices, comme la Tristeza, sur le territoire de l’Union européenne.

Mots-clés. Tristeza des agrumes – ELISA – Grèce.

I – Introduction

Citrus is one of the most important fruit crops in Greece, grown in 29 of the 54 Prefectures of the country. It is the main cultivation in southern Greece, especially in regions characterized by mild climatic conditions and abundance of water. Citrus industry covers a total area of 52.212 ha, 1/3 of which is located in Argolis prefecture. Sweet orange is the dominant species covering 36.670 ha followed by lemon with 9.700 ha, mandarin with 5.792 ha and grapefruit with 1.080 ha. (Anonymous, 2004).

Commercial orange cultivars, grown mainly in regions protected from the frost, include Washington Navel followed by Navelina, Common orange, Salustiana, New hall and Valencia. As to mandarins, the main commercial cultivar is SRA-63 followed by Common mandarin and the

lately introduced hybrids Nova, Page and Ortanique. In lemons, which are grown in warm regions, commercial cultivars include the local varieties Maglini, Karistini, and Adamopoulou. Grapefruits cover a small area and the main cultivar is Marsh Seedless (Kyriakopoulou, 2002).

II – Review of Tristeza detection in Greece

Citrus Tristeza, the most destructive virus disease attacking the citrus trees, was identified for the first time in Greece, in Argolis county (North East Peloponnese), in June 2000 (Dimou *et al.*, 2002a, b). The disease was detected in one Lane Late orange tree, grafted on CTV-tolerant Carrizo citrange rootstock. That tree was a part of a consignment of Lane Late orange trees labelled as “Conformitas agraria communitatis” (CAC) quality which were imported illegally from Spain in 1994. Fifty trees of that consignment were planted in Argolis county but only 20 trees finally survived because they had been imported bare rooted, a technique used by Spanish nurseries. The survived trees, planted in various plots in the region, were spotted and after testing, 9 of them were found to be infected by CTV (Dimou *et al.*, 2001).

Further surveys revealed 15 more CTV-infected trees, of various varieties, all located close to an infected tree of the initial consignment, strongly indicating aphid transmission. In addition, some of the initial trees were used by the farmers as mother trees to get budwood in order to propagate a given cultivar. Three of those mother trees were showing slight stem-pitting symptoms.

In the same period, 18 more trees from the initial illegal consignment of Lane Late were spotted in Chania, Crete. In addition, 2 of them, which were later found to be CTV infected, had been used as a source of budwood for the establishment of other orchards in the area. Finally, the number of CTV infected trees in Chania prefecture went up to 3,500 (Dimou *et al.*, 2002a, b). In all the above cases, the CTV infected trees were symptomless.

In the year 2000, a total number of 7345 trees were tested for CTV. From those, 1727 trees concerned mother plantations, 543 trees were selected from nurseries and 5075 trees concerned commercial orchards from 14 regions of the country.

2001. The importation of nursery material from EC countries continued. In Spring 2001, a new consignment of 1100 bare-rooted nursery plants from Spain was introduced into the Argolis region. That material was also certified (blue label) and concerned Clemenpons mandarin, a clone of Clementine mandarin, grafted on Carrizo citrange rootstock. Seven of these trees were found to be CTV infected although symptomless.

At that time, characteristic symptoms of the disease were observed for the first time in a 25-year-old Washington navel orange tree, grafted on sour orange, in a commercial orchard in the Argolis region. The symptoms observed concerned small yellow leaves, dried branches, small fruits and a general decline of the tree. In the same orchard, some more trees were found to be CTV infected showing limited blossom in the spring.

As a result of the above findings, the first Ministerial Decree (51/24-1-2001) was issued concerning measures which should be urgently taken for the eradication of Citrus Tristeza disease. Also, with another Ministerial Decree (36229/20-4-2001) the amount of 20.54 euros per uprooted tree was set as compensation for the growers who are forced to uproot and burn their CTV infected trees.

Furthermore, the first pan-Hellenic meeting of the scientists who work in Plant Protection Services took place, in which they were informed about the appearance and distribution of Citrus Tristeza disease in the country, the measures which should be taken for its eradication and the way to enforce the new Ministerial Decrees .

In the year 2001, a total number of 12530 trees were tested for CTV. From those, 324 trees concerned mother plantations, 74 trees were selected from nurseries and 12931 trees concerned

commercial orchards from 13 regions of the country. Those tests showed that 54 trees from commercial orchards were CTV infected, 25 from Argolis region and 29 from Crete.

Finally, in 2001, 60 CTV-infected and their neighbouring trees, from 11 orchards in Argolis region, were uprooted and destroyed with fire.

2002. The testing of citrus trees was carried on in Spring and Autumn. Two methods were used, DAS-ELISA and immunoprinting. PCR-RFLP analysis on a 520 bp fragment of the virus 3' end genome between p20 and p23 genes from all different virus sources in Greece gave the same profile as that produced by the known T385 virus isolate of Spanish origin. The phylogenetic nucleotide sequence analysis of the PCR product of the initial isolate from Argolis revealed 100% identity with T385, which is in agreement with the historical background of the disease introduction in Greece (Dimou *et al.*, 2004).

A total number of 15063 trees were tested for CTV. From those, 120 trees concerned mother plantations and the rest concerned commercial orchards from 14 regions of the country. Those tests showed that 109 trees from commercial orchards were CTV infected, 16 from Argolis region and 93 from Crete.

In May 2002, a meeting was organised by the Argolis Agriculturist Association in collaboration with the Ministry of Agricultural Development and Food. The subject of the meeting was the Tristeza situation in Greece and in the Mediterranean. Specialists like Prof. Bar-Joseph (Israel), Dr Mariano Cambra (Spain) and Anastasia Kyriakou (Cyprus) participated as invited speakers.

2003. Laboratory testing of all the trees of the orchard in Argolis (Katsikania area), in which Tristeza disease was detected for the first time (in 2000) in Greece, showed a spread of the disease inside the orchard from the initially infected tree. In order to eliminate the dissemination of the disease from that focus, 150 Valencia orange trees on Troyer of the particular orchard were cut and burnt. This was the first application of the eradication measures at the scale of an orchard and not of an infected tree.

Extended surveys in citrus growing areas and collection of samples for laboratory testing continued in Spring and Autumn. A total number of 14172 citrus trees were checked. From those, 755 trees concerned mother plantations and 13417 trees concerned commercial orchards from 13 regions of the country. Those tests showed that 18 trees from commercial orchards were CTV infected, 10 from Argolis region and 8 from Crete.

On the Argolis Agriculturist Association initiative, an information campaign started for the Citrus growers by issuing and delivering technical leaflets and informing and advising the growers through local Radio and TV stations.

2004. A total number of 5727 trees were tested for CTV. From those, 679 trees concerned mother plantations, 238 trees were selected from nurseries and 4810 concerned commercial orchards from 11 regions of the country. Those tests showed that 7 trees from commercial orchards were CTV infected, 2 from Argolis region and 5 from Crete.

Characteristic symptoms of Tristeza disease were noticed in an orchard of Washington Navel orange on sour orange rootstock, in Argolis (Argolico area).

2005. Up to this moment, Tristeza infected trees were restricted only to the prefectures Argolis and Chania-Crete. However, in a survey carried out in Arta prefecture (North West Greece) by prof. M. Vovlas, 11 out of 123 trees tested for Tristeza were found to be infected. Those trees were Washington Navel and Navelina varieties on sour orange rootstock. Analysis of the data revealed that the CTV isolates from Arta prefecture have high similarity to mild isolates T30 from Florida and T385 from Spain (Barbarosa *et al.*, 2007a,b).

A total number of 6876 trees were tested for CTV. From those, 618 trees concerned mother plantations and 6258 concerned commercial orchards from 9 regions of the country. Those tests showed that 11 trees from commercial orchards were CTV infected, 9 from Argolis region and 2 from Crete.

In a second orchard, located in Argolis and planted with Washington Navel orange trees on sour orange rootstock, alarming spread of the Tristeza was observed indicating aphid transmission. Eradication measures were applied to that orchard and 150 trees were cut and burnt.

2006. A total number of 6665 trees were tested for CTV. From those, 710 trees concerned mother plantations and 5955 concerned commercial orchards from 13 regions of the country. Those tests showed that 1 tree from commercial orchards in Crete was CTV infected but none in Argolis region.

In a third orchard, located in Argolis (Anifi area) planted with Clemenpons variety on Carrizo citrange (certified material legally imported from Spain) rootstock, alarming spread of the Tristeza was observed indicating aphid transmission. Eradication measures were applied to that orchard and 333 trees were cut and burnt.

A new Ministerial Decree was issued (1416-665/26-9-2006) improving the former one.

2007. During the ordinary annual testing of citrus orchards from all over the country to monitor the spread of Tristeza, an orchard planted with 939 trees was found, in which 230 trees proved to be CTV infected. The trees, Clemenpons mandarins on Carrizo citrange rootstock, concerned certified planting material legally imported from Spain, 4 years ago, by a grower in Skala Laconias (South Peloponnese). Due to the facts that: a) those trees were grafted onto CTV resistant rootstock which minimises the symptom expression, and b) the aphids (*Aphis gossypii*) are very active because of the mild climate of the region, there is an increased possibility for the dissemination of the disease to the neighbouring trees and the nearby orchards. After the new data on the introduction of Tristeza in Laconia, that new region, where most of the biggest and best nurseries of the country are based, enters the adventure of Tristeza. The new introduction of the disease into a healthy region through infected "Certified" propagating material, and the fact that the same clone of Clementine mandarins had been imported in 2001 from Spain in Argolis and part of the material was also found CTV infected, shows that the Spanish system for production of citrus certified material is not so good as they desire to present it.

A total number of 5502 trees were tested for CTV. From those, 872 trees concerned mother plantations and the remaining 4630 concerned commercial orchards from 5 regions of the country. Those tests showed that 235 trees from commercial orchards were CTV infected, 5 from Crete, none from Argolis and 230 from the recently infected region of Laconia (South Peloponnese).

III – Conclusion

At the moment, Tristeza disease in Greece is under control, due to its timely detection through the extensive surveys and laboratory testing as well as due to the prompt application of eradication measures. In Argolis Prefecture, the region where Tristeza disease was first detected, the annual surveys carried out since the year 2000, showed that the disease remained restricted to the initial orchards. Only in one case, a dissemination of the disease in the adjacent orchard had been noticed.

In three consignments sent to Greece, the Spanish system for the production of certified citrus propagating material proved insufficient to guarantee the health of the exported material. This is a real threat for the dissemination of the most destructive disease of citrus trees, Tristeza, to other Mediterranean countries. In addition, the affected countries have to spend huge funds in eradication programmes to control it.

Under the new data, the priority actions are to be the following:

- i. the surveys and laboratory testing of citrus orchards all over the country will be strained;
- ii. more effort in informing the citrus growers for the seriousness of the treat that they will face in the case of uncontrolled dissemination of citrus Tristeza disease in the country;
- iii. careful substitution of the traditional sour orange rootstocks with other resistant to Citrus tristeza virus, taking into account the calcareous soil in Greece;
- iv. collaboration with other Mediterranean countries threatened by *Toxoptera citricidus* (Kirkadly) invasion into the Mediterranean basin (Ilharco *et al.*, 2005);
- v. extensive controls of the propagating material produced by nurseries. Particularly, for nurseries operating in regions where Tristeza has been detected, the propagating material should be produced inside screenhouses under special conditions;
- vi. establishment of a national institution or organization in the country for the production of the demanded certified propagating material.

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Citrus tristeza virus (CTV) survey in the Maltese Islands 1999-2005

Attard D.¹, Gatt M.¹, Agius M.¹, Muscat A.², Leone Ganado C.³

¹ Diagnostic Lab, Plant Biotechnology Centre, Lija, Malta

² Plant Quarantine Unit, Plant Biotechnology Centre, Lija, Malta

³ In Vitro Laboratory, Plant Biotechnology Centre, Lija, Malta

Abstract. The following report illustrates the situation of the Maltese Citrus industry with respect to *Citrus tristeza virus* (CTV) in the last years. Annual surveys for CTV in the Maltese Islands have been carried out since 1999. Results have shown a general absence of CTV from Maltese citrus trees. However, annual testing has shown the presence of infected citrus trees imported from other countries where the disease is present. Thus, there is a high risk of introducing the disease through imported infected citrus trees.

Keywords. Citrus – Insect vectors – Malta – Tristeza – Survey.

Enquête sur la tristeza des agrumes (CTV) dans les îles maltaises 1999-2005

Résumé. Ce rapport illustre la situation de la Tristeza des agrumes (CTV) à Malte au cours de ces dernières années. Depuis 1999, des enquêtes annuelles sur le CTV ont été effectuées sur les différentes îles maltaises. Les résultats ont montré une absence totale du CTV sur les différentes espèces d'agrumes ; cependant, les tests ont révélé la présence du virus sur des plants d'agrumes importés d'autres pays où la maladie est présente. Ainsi, il existe un risque élevé d'introduction de la maladie à travers l'importation de plants d'agrumes infectés.

Mots-clés. Agrumes – Insectes vecteurs – Malte – Tristeza des agrumes – Enquête.

I – Introduction

In Malta citrus trees are grown for commercial fruit production and as ornamental trees in gardens. Sour Orange (*C. aurantifolia*) is the main citrus rootstock used and so most of Maltese citrus is susceptible to infection by CTV. The aphid vectors *Toxoptera citricidus* have never been reported in the Maltese islands, however *T. aurantii*, *Aphis spiraecola* and *A. gossypii* are all present (Plant Health Department records). The Plant Health Department recognised the need to run surveys for this disease and in 1999 started the CTV survey in collaboration with CIHEAM-IAMB and assisted by the 4th Italian Maltese protocol. The European Union (EU) recognised Malta as a Protected Zone with respect to Citrus tristeza virus in 2004, when Malta joined the EU.

II – 1999 - 2005: The CTV survey

Field inspections are done aiming to assess the sanitary conditions of the trees through visual observation with special reference to the *Citrus tristeza virus* and collecting samples for laboratory testing.

CTV monitoring is carried out by ELISA-testing on green bark, collected during November/December; and on flower buds and green bark collected during April and May when daily temperatures are around 18 - 20°C.

1. Sampling methodology

The survey targets private orchards, commercial plantings and nurseries (Fig. 1) . In private orchards compound samples made up of 4-6 green bark twigs or flower buds were collected, stored at 4°C. In commercial plantings compound samples from about 20 % of all plants from each commercial citrus orchard visited were collected. Each sample was made up of 5 green-bark twigs or flower buds from 5 different trees. The small commercial orchards were sampled totally. Regarding nurseries, to date 12 citrus nurseries/importers have been visited from where compound samples were collected from each citrus variety present at each nursery. Compound samples which were taken, consist of 5 twigs or flower buds, from 5 different plants within a homogenous group.

2. Laboratory tests

The serological and molecular tests were carried out at the Virology and Bacteriology Laboratory, Plant Biotechnology Centre. Several commercial polyclonal antisera have been used for the purpose of this survey and the classical ELISA Double Antibody Sandwich technique was applied (Clark and Adams, 1977). The assays were performed as indicated by the protocols given with the kit available; assays included coating of ELISA plates with antigen specific antibodies at the given concentration and incubating in a moist chamber at the temperature indicated by the protocol. The plates were then washed with washing buffer 3 times, for 3 minutes each time and sample extracts were then loaded and left incubating overnight at 4°C. Fresh healthy, positive and buffer controls were also included. The following day the plates were washed again in washing buffer and the conjugated antibodies were loaded. The plates were incubated in a moist chamber at 37°C and again washed for 4 times in washing buffer, finally the substrate (p-nitrophenylphosphate diluted in substrate buffer at 1mg/ml) was added. The readings of the plates were taken with a photometric measurement at 405 nm at ½ hour, 1 hour, 1 ½ hours and 2 hours after substrate deposition. Samples that gave unclear results were re-tested singly by ELISA and RT PCR.

RT-PCR was run by using primers pairs PIN-1 and PIN-2 following the procedure cited in SMT project SMT4-CT98-2252 (EPPO, 2004).

III – Results

CTV was never diagnosed on Maltese citrus trees between 1999 and 2004; however during this period 4 infected CTV plants were intercepted, on imported lot of citrus trees. In February 2003, 3 interceptions were made - 2 on plants imported from Sicily, and 1 on plants imported from Calabria. The last interception was made in January 2004 on plants imported from Sicily. All the plants within the homogenous consignment found to contain infected plants have been destroyed (Tab. 1).

In April 2005 the first CTV infected trees were found in a private garden at Wardija. Both trees were uprooted and kept under greenhouse conditions for further testing. Another small group of imported kumquats was also diagnosed infected by the virus and were destroyed by the Plant Quarantine Section (Tab. 1).

Table 1. Results of the CTV survey

Period	Local Citrus			Imported Citrus		Results
	Sampled areas	No. of Samples	Varieties sampled	Total N°. of samples	Varieties sampled	
Dec.1999- Jan.2000	8	83 compound samples, 31 single samples (195 trees).	Common, Vaniglia Navel, Blood, Sweet Oranges; Mandarin; Lumicella; Sweet Lime; Perpetual Lemon.	48 compound samples (157 trees).	Sweet, Valencia, Tarrocco Oranges, Calamondin; Clementine; Mandarin; Kumquat; Lime; Lemon.	CTV negative.
Dec. 2000 -Jan 2001	6	122 compound samples, 50 single samples (456 trees).	Common; New Hall, Navel, Washington Navel, Blood, Sour Oranges; C lementine; Mandarin; G rapefruit; Common Lemon.	154 compound samples, 38 single samples (440 trees).	Sweet, Navel, Vaniglia, Valencia, Tarocco oranges; Mandarin; Tangelo; Kumquat; Grapefruit; Pompelmo; Citron; Lemon; Cedro Diamante; Limoncello; Limone stilocotta.	CTV negative.
Dec. 2001- Jan 2002	12	84 compound samples (423 trees).	Sweet, Common, Sour Oranges; Mandarin ; Grapefruit; C ommon Lemon.	155 compound samples (834 trees).	Common, Blood, Vaniglia, Navel, Tarocco oranges; Mandarin; Kinetto; Clementine; Kumquat; Pompelmo; Lima di Spagna; Bergamotte; Common Lemon; Limoncello.	CTV negative.
Dec. 2002- Jan 2003 May 2003	13	187 compound samples (937 trees).	Sweet, Sour, Blood, Navel Oranges ; Clementine; Grapefruit ; Lumicella/ Rumicella ; Common Lemon.	173 compound samples (864 trees), 134 single samples.	Orange, Washington Navel, Blood, Vaniglia, Sweet, Navel, Valencia, Tarrocco Oranges; Mandarin Fortune; Tangelo; Clementine; Common Grapefruit; Desiderio Grapefruit; Kumquat; Mandarin; Satsuma; Moro; Pompelmo; Cedro; Tacle; Bellezza; Perpetual, Common Lemons.	Three interceptions of CTV infected plants: 1 kumquat imported from Calabria; a group of 5 citrus trees imported from Sicily; infected plants of Desiderio, Tacle, Pompelmo, Cedro, Moro, Tarrocco, Bellezza, Marisol Clementine, Femminello Comune Lemon and Navellina Orange varieties. imported from Sicily.
Dec. 2003- Jan 2004 May 2004	6	125 compound samples (622 trees); 91 single samples.	Common, Valencia, Vaniglia, Sour Oranges ; Clementine; Mandarin; Common Lemon.	388 compound samples (1984 trees); 390 single samples.	Common, Tarocco Gallo, Vaniglia, Tarocco, Washington Navel, Valencia Campbell, Variegated, Moro Nucellare oranges; Cami Clementine; Mandarin; Kumquat; Star Ruby Grapefruit; Diamante Lime; Femminello Zagara Bianco, Perpetual, Common Lemons; Limoncello; Rosso Lunario; Cedro.	A consignment imported from Sicily was found to contain CTV infected Mandarin, Sweet Orange, Tarrocco orange, and Common Lemon.
Dec. 2004- Jan. 2005	16	406 compound samples; 16 single samples (2027 trees).	Sweet, Vaniglia ; Sour Oranges ; Mandarine; Clementine; Grapefruit; Common, Perpetual Lemons.			CTV negative.
Apr.-June 2005	16	111 compound samples (517 trees).	Sweet, Vaniglia, Sour Oranges ; Mandarin ; Clementine ; Grapefruit ; Common, Perpetual Lemons.	127 compound samples (664 trees).	Sweet, Brazilian Washington, Valencia, Tarrocco, Tarrocco Gallo; Kumquats; Mandarin; Clementina; Nova Tangelo; Diamante; Star Ruby Pompelmo; Femminello Comune; Femminello Zagara Bianca; Limone lunario/C. Volkameriana, Marsh Seedless/ Arancio amaro, Limoncello. Navellina/C. Troyer, Moro Nucellare/C.Troyer.	2 CTV infected trees in a private garden A group of 21 kumquats traded from Italy resulted positive to the CTV
Oct.-Dec. 2005	23	216 compound samples (638 trees).	Sweet, Vaniglia, Sour Oranges ; Mandarin ; Clementine ; Grapefruit ; Common, Perpetual Lemons.			CTV negative.
Total	100	1522 samples (5906 trees)		1607 samples (4943 trees)		

IV – Conclusions and limitations of the survey

CTV has been found on 2 isolated citrus trees in Maltese orchards, both of which were eradicated. Currently the area is under contingency measures and intensive testing is being applied to confirm if other infected trees are present. However there is a high risk of importing the disease into the Maltese islands through movement of citrus trees from other countries (especially southern Italy). The trees come from nurseries in compliance with a certification or CAC scheme but still the disease has persisted.

A total of 71,000 citrus trees have been moved/imported into Malta mainly from Sicily and Calabria (Italy) between 1999 and 2004 (Plant Quarantine records, 1999-2004). It is not possible to test annually all the imported citrus trees.

Presently the budget to run the annual survey is limited to 2 ELISA kits of 480 tests each, due to a low budget and lack of human resources. This means that a maximum of 4000 citrus trees per year may be tested covering nurseries, the fruit production areas and private/public gardens. The risk of missing infected consignments and introducing the disease remain high.

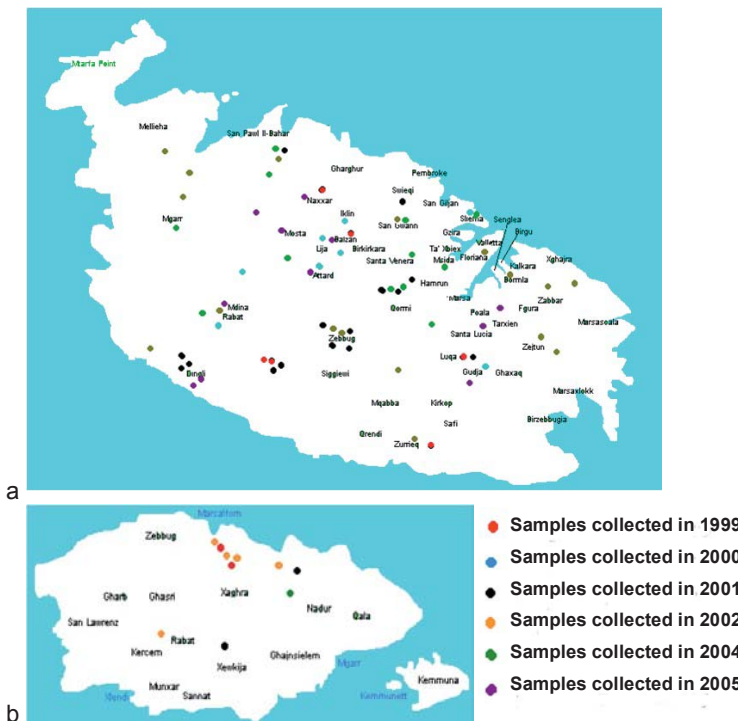


Figure 1. Distribution of the Citrus sampled areas in Malta (a) and Gozo (b) for the period Dec 1999 – Jan 2005 .

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Citrus tristeza virus (CTV) in Cyprus, 1992-2004

Kyriakou A.¹, Kapari.Th. I.¹, Gavriel I.², Papayiannis L.¹, Ioannou N.¹

¹ Agricultural Research Institute, Lefcosia, Cyprus

² Department of Agriculture, Ministry of Agriculture, Natural Resources and Environment, Lefcosia, Cyprus

Abstract. A historical review of CTV in Cyprus is presented with special reference to the epidemics that occurred in the period from 1992 to 2004. Monitoring was based on ELISA assays and the whole groves were eradicated when CTV incidence was equal to or lower than 15 %. The general CTV infection rate equaled 4.43%; CTV biological and molecular characterization highlighted the south-African origin of the isolates which were introduced in the district of Ammochostos. A law was issued to prohibit the movement of citrus planting from this district to other areas. Ortanique and the red-flesh grapefruits were the most infected. The presence of CTV was the main driving force for the elaboration and implementation of a citrus certification programme in the 1990's.

Keywords. Biological indexing – Certification – Citrus – Citrus tristeza virus – Cyprus – ELISA – RT-PCR.

Le Virus de la tristeza des agrumes (CTV) à Chypre, 1992-2004

Résumé. Dans le présent travail est présenté un 'historique du CTV à Chypre, en mettant l'accent, tout particulièrement, sur les épidémies qui se sont déclarées entre 1992 et 2004. Le suivi a été réalisé sur la base du test ELISA et des vergers entiers ont été arrachés lorsque l'incidence du CTV était égale ou inférieure à 15 %. Globalement, le taux d'infection du CTV s'élevait à 4,43 %, la caractérisation biologique et moléculaire du CTV a permis de démontrer que les souches introduites dans la région d'Ammochostos étaient d'origine sud-africaine. Une loi a été promulguée en vue d'interdire le transfert du matériel d'agrumes dans d'autres régions. La variété Ortanique et les variétés de pomélo à pulpe rouge se sont avérées être les plus infectées. La détection du CTV a indiscutablement contribué à l'élaboration et à la mise en œuvre du programme de certification des agrumes au cours de années 1990.

Mots-clés. Indexage biologique – Certification – Agrumes – Virus de la tristeza des agrumes – Chypre – ELISA – RT-PCR.

I – Introduction

Citrus has been growing in Cyprus since the 1st century B.C. In the 1970's citrus cultivation reached an area of 13000 hectares and was considered the "yellow gold" of the island. However, over the last years the citrus industry has been declining, mainly as a result of water deficiency, increase in labour costs and decrease in export markets. In the year 2004, citrus covered an area of 5543 hectares which represents 3.9% of the total cultivated area and 15% of the irrigated surface. Total production reached 142 576 tons of which about half was exported as fresh fruit (Markou and Papadavid, 2007). The main citrus species are sweet oranges (valencias and navels), mandarins and hybrids, grapefruits (Marsh seedless and Star Ruby mainly) and lemons (mostly the local Lapithou); the main rootstock used is sour orange (*Citrus aurantium* L.). They are grown on the coastal area and in the central Lefcosia-Morphou plain.

II – Historical review of CTV in Cyprus

Before 1992. On the island, CTV was first detected by Papisolomontos and Economides (1968) when 27 trees of five citrus species were found infected and destroyed. During a virus survey, conducted since 1986, CTV was initially detected in four out of 156 groves surveyed by the use

of Mexican lime (*C. aurantifolia* Swingle) indicators (Kyriakou and Polycarpou, 1989; Kyriakou *et al.*, 1992).

1992-2004. Given the danger of an epidemic spread of CTV and the prevalent use of CTV-sensitive sour orange rootstock, a project for the control of this disease was initiated in 1992 (Kyriakou *et al.*, 1996; Kapari *et al.*, 2000). The basic objectives of the project were: a) the mapping of CTV infection through a systematic survey of citrus; b) the removal of infected trees or groves where this was feasible against compensation to the growers; and c) the establishment of a viable citrus certification programme. In the island, CTV is presently transmitted in nature by *Aphis gossypii* in a non epidemic form (Kapari *et al.*, 2000). However, in case of *Toxoptera citricidus*, the efficient vector of the virus which is already present in Portugal and Spain, spread throughout the Mediterranean region, then the CTV eradication efforts on the island will probably fail and alternative solutions should be available to be applied. Discrimination of prevailing CTV strains is a key element to predicting disease impact and devising appropriate control strategies suitable to specific regions (Niblett *et al.*, 2000).

III – Survey and eradication

The survey was conducted by indexing with ELISA tests on 10 to 20% of citrus trees of each grove in the five citrus-producing districts of Cyprus (Fig. 1), making efforts to include all varieties contained in a single grove.



Figure 1. Survey for citrus tristeza virus in the main citrus-producing areas of Cyprus.

When CTV was detected in a grove, then all trees were tested in order to determine the actual disease incidence in the infected grove. When CTV incidence was equal or lower than 15%, it was recommended that only infected trees be removed, whereas when infection was higher than 15%, then it was recommended that the whole grove be destroyed. Compensation given was based on a prescribed formula given by Chr. Papayiannis, taking into account the variety, the age and the general condition of the tree, with an average amount of € 45.00 per tree.

Antisera were obtained from the laboratory of Prof. M. Bar-Joseph, Volcani Center, Bet Dagan and the method used was that developed by Hadjinicolis *et al.*, (1995). Several CTV isolates were grafted on plant indicators, including Mexican lime, sweet orange (*C. sinensis* (L.) Osbeck), sour orange and grapefruit (*C. paradisi* Macf.) in a temperature-controlled greenhouse (16 –33° C). Results of the survey showed a CTV incidence of 4.43% (Tab. 1).

Table 1. Survey for citrus tristeza virus in five districts of Cyprus, 1992-2004.

	Lefcosia	Ammokhostos	Larnaca	Lemessos	Paphos	Total
No. groves indexed	403	29	50	186	98	766
No. groves infected	70	21	17	45	17	170
% groves infected	17.37%	72.41%	34.00%	24.19%	17.35%	22.19%
No. trees indexed	29 019	2 140	3 758	25 866	8 044	68 827
No. trees infected	1 109	374	272	821	475	3 051
% trees infected	3.82%	17.48%	7.24%	3.17%	5.90%	4.43%
No. surveyed trees	127 758	16 358	35 454	211 337	69 028	459 935

Indexing was done by enzyme-linked immunosorbent assay (ELISA).

Among the 68 827 trees indexed, surveyed from 766 groves with 459 935 trees, 3 051 trees were found to be CTV-infected. A hundred and seventy of the 766 groves surveyed had CTV-infected trees. Disease incidence and prevalence ranged in the different districts from 3.17% to 17.5% and from 17.3% to 72.4%, respectively. The highest proportion of infected trees and groves was noted in the district of Ammochostos (Table 1), where it was decided that eradication was no longer feasible. The practice of removal of infected trees was applied in the other areas, with compensation to the growers. Approximately 4 500 trees have been uprooted to the present, including seven entire groves. With regard to the heavily infested district of Ammochostos, a regulation was issued (131/93) which forbids the movement of citrus planting material from this district to other areas of the island. In addition, efforts have also been made to clear the area of Ammochostos from trees infected with severe CTV isolates.

With regard to the incidence of CTV in the different citrus species and varieties, infection was found in nearly all citrus accessions, but Ortanique and the red-flesh grapefruits, the most popular varieties of the last 25 years, appeared the most heavily infected.

IV – Symptoms

Field symptoms of CTV-infected trees varied from inconspicuous to severe. The most intense symptoms were noted on Marsh seedless and Star Ruby grapefruit and included stunting, chlorosis, fragility and dieback of twigs, pitting of branches and general decline. Tristeza caused severe decline and death of 40 to 50 year-old grapefruit and Valencia orange trees in certain groves in the districts of Ammochostos and Lemessos. With regard to the greenhouse indexing tests, usually the intensity of field symptoms related well to the severity of CTV symptoms on Mexican lime in the greenhouse. No seedling yellows symptoms on sour orange or grapefruit were observed.

V – Citrus Certification

A mandatory certification programme has been established and functioning since the mid 1990's; it was described by Gavriel (2002). The responsibility for the implementation of the relevant legislation rests with a nine-member Board under the Minister of Agriculture. The foundation or pre-basic block is kept and maintained under insect-proof screen by the Agricultural Research Institute, whereas the multiplication and mother blocks, protected also under insect-proof screen, are maintained by the Department of Agriculture. In addition, the private nurseries are obliged to

keep their mother plants and the production of seedlings and budded treelets also under screen. The foundation block provides virus-tested material to the mother blocks of the Department of Agriculture which, in turn, provide the private nurseries or directly the growers with budwood. Citrus budwood, which is introduced from overseas sources, is kept in a post-entry quarantine station and undergoes thorough indexing for the known virus and virus-like diseases before entering the foundation block. Local varieties are being cleaned from the known virus problems by micrografting. At present, the foundation block, which is at the Agricultural Research Station in Akhelia, Paphos, includes about 50 imported and local citrus species, varieties and/or clones.

VI – Molecular characterization of CTV isolates from Cyprus

A study was conducted for the molecular characterization and strain differentiation among several CTV isolates, which were selected from the main citrus-growing areas of the island on the basis of different symptomatology on host trees and on the Mexican lime indicators. The 673 bp CP gene was amplified from infected material by one step Immunocapture-Reverse-Transcription (RT) Polymerase Chain Reaction (PCR) according to procedures and primers reported by Nolasco *et al.* (2002). Agarose gel electrophoresis showed that a single band of the expected size was obtained. All amplified products were subjected to Single strand conformation polymorphism (SSCP) (Rubio *et al.*, 1996) in order to help choosing characteristic isolates for cloning and sequencing. Results showed that the haplotypes obtained from the Cypriot isolates were distributed in five of the previously reported seven CP groups (Fig. 2).

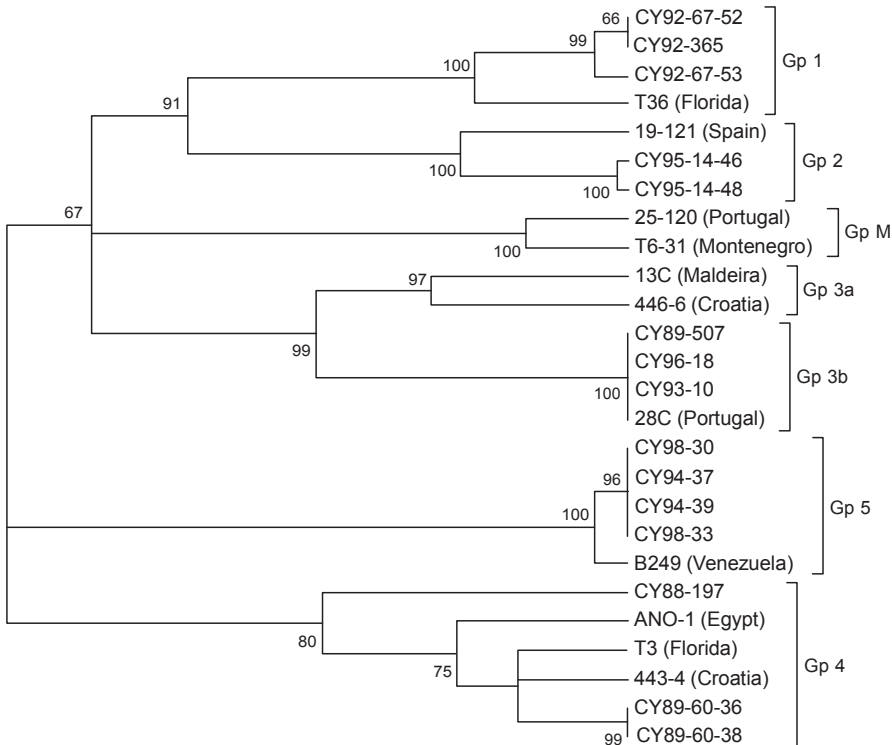


Figure 2. Dendrogram showing the genetic relationships among the coat protein genes of Cypriot CTV isolates and CTV world-wide reference isolates. (Papayiannis *et al.*, 2007).

None of the above groups is specific for Cypriot isolates, ruling out geographic speciation and therefore indicating introduction of CTV from multiple sources which is consistent with historical data (Papayiannis *et al.*, 2007). An origin of the disease is South Africa, as material was officially introduced from this country by the local Ministry of Agriculture in the 1930's and established in a nursery in the Ammokhostos district, explaining the heavy infection of the area. However, the history of some infected groves showed that there were in addition other infection sources, as material introduced illegally from Israel.

VII – Conclusions

The proportion of infection in most areas of the island showed that the control of CTV is still possible by removal of infected trees and use of virus-free planting material for the establishment of new groves. The destruction of the CTV foci, especially the severe virus isolates, or their confinement as much as possible, would diminish the danger of dissemination of CTV, in case *Toxoptera citricidus*, the most efficient vector of the virus, and which has already entered Portugal and Spain, invades the island.

The presence of CTV on the island and the strife to control the disease were undoubtedly the main driving force for the formulation and application of a certification programme which will hopefully protect the citriculture of the island and in general the Mediterranean area from invasion of more severe strains of CTV, or strains which are more effectively spread in nature by *Aphis gossypii*, the vector of CTV present on the island and the region. The certification programme will also prevent the introduction of exotic devastating diseases, including greening, which is caused by *Liberobacter* spp., Citrus canker (*Xanthomonas campestris*), and citrus variegated chlorosis (*Xylella fastidiosa*).

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Historical review of *Citrus tristeza virus* (CTV) and its vectors in Turkey

Baloglu S.¹, Birisik N.²

¹ Plant Protection Department of Agricultural Faculty, University of Cukurova, Adana, Turkey

² Plant Protection Research Institute, Department of Phytopathology, Adana, Turkey

Abstract. *Citrus tristeza virus* (CTV) has been officially reported from Turkey since 1963. After the first findings, technical and scientific studies were carried out in different citrus-growing areas, and this, to determine infected areas, discriminate between CTV strains and understand the presence and distribution of the CTV vectors, in the country. Today, it is very clear that CTV and its vectors (except *Toxoptera citricidus*) are widely distributed in Turkey; however, no report reveals the presence of severe CTV strains in the country. According to the latest monitoring results, the situation of CTV during the last two decades is under control but it is still considered as the most harmful threat to the Turkish citrus industry.

Keywords. Citrus – Tristeza – Turkey – Virus vectors – Virus.

Aperçu historique du virus de la tristeza des agrumes (CTV) et de ses vecteurs en Turquie.

Résumé. En Turquie, le virus de la tristeza des agrumes (CTV) a été officiellement signalé en 1963. Après les premiers signalements, de nombreuses études scientifiques et techniques ont été réalisées dans les différentes zones agrumicoles afin de déterminer les zones infectées, discriminer les souches de CTV et évaluer la présence et la distribution des vecteurs de CTV dans le pays. Aujourd'hui, il est très clair que le CTV ainsi que tous ses vecteurs (sauf le *Toxoptera citricidus*) sont largement distribués en Turquie, mais il n'y a aucun rapport qui indique la présence d'une souche sévère de ce virus dans le pays. D'après les enquêtes récentes réalisées au cours des deux dernières décennies, la situation du CTV reste sous contrôle, mais il s'agit là toujours de la menace la plus grave pour l'agrumiculture turque.

Mots-clés. Agrumes – Tristeza –Turquie – Vecteurs de virus – Virus.

I – Citrus industry in Turkey

Citrus production in Turkey has increased over the last two decades due to high demands of local consumption and exportation. The total citrus production of Turkey is about 2.492.650 tons/year.

Citrus production is mainly concentrated in the Mediterranean, Aegean and Black Sea regions. Generally, sour orange rootstock is used in all areas because of its vigour and compatibility with common cultivars except for the Aegean region in which trifoliate orange predominates.

The citrus industry faces many problems due to plant pests and pathogens. At present, about 15 virus and virus-like diseases have been reported in the Turkish citrus production area e.g. Psorosis complex, Infectious variegation (crinkly leaf), Chlorotic dwarf, Tristeza, Satsuma dwarf, Cristacortis, C oncave gum, Impietratura, Gummy bark, Woody gall, C achexia-xyloporosis, Exocortis.

II – Review of CTV History in Turkey

CTV most probably has been introduced into Turkey via imported material from far-east Asia such as China to the Black Sea Region in the northern part of the country. For the southern part of the country, e.g. the Mediterranean Region, another source of introduction could be Israel.

1958. Reichert reported that Turkish local citrus varieties were CTV free.

1959. Dickson and Flock published that CTV was present in the Mediterranean countries and most probably it has been present in the region since 1890.

1961. Cengiz observed some suspicious trees in Adana and Mersin whose symptoms could be attributed to CTV but he had no possibility to confirm his suspicion (Dolar, 1976).

1963. Norman, as an FAO expert, made a visual survey throughout the Turkish citrus-growing areas. He observed some CTV-like symptoms in Adana, and reported that Turkey was a CTV-free country.

1965. Moreira did some biological indexing on Mexican lime and reported that all results were negative and that the Turkish citrus industry was free from CTV.

1967. Ozalp and Azeri were the first to report the presence of CTV on Satsuma Mandarins from the Aegean Region in Izmir province.

1974. A voluntary certification system was implemented under the control of the Ministry of Agriculture and Rural Affairs.

1976. Cengiz *et al.* reported that the first CTV - infected citrus trees were a Yafa sweet orange in Adana, and five mandarin trees in Mersin. The CTV detection in Adana was confirmed by biological indexing.

Dolar carried out a survey in the eastern Mediterranean region and reported 2.6 % of CTV infection. Most of the infected trees were 15 to 20 year - old Yafa and Washington Navel sweet orange trees, Duncan grapefruit and kumquats. Even grafted on susceptible rootstock all infected trees exhibited mild symptoms. No inverse pitting could be observed in the bud union of infected trees.

1978. Azeri and Hepper reported 16 % of CTV infection on Satsuma Mandarins in the Aegean Region.

1984. Azeri completed a survey in the Aegean Region (western part of Turkey). He reported the presence of five different CTV biotypes in the region by means of biological indexing. In comparison with the Mediterranean region, he attributed the smaller incidence of the virus to the use of trifoliolate rootstock and climatic conditions not favorable for CTV dissemination.

1988. Baloglu tested by ELISA 112 suspicious trees in the Mediterranean region, from which 71 were positive. All infected trees originated mild vein clearing symptoms when indexed on Mexican lime. A polyclonal antiserum was produced following partial purification of the virus. Electron microscopy studies confirmed the presence of CTV.

1990. Gullu carried out a general survey on navel sweet oranges and mandarins in the Turkish Mediterranean Region. Tristeza symptoms were observed both on navel and satsuma trees. The disease incidence ranged from 0.06 % to 2.1 % in Navel oranges; it was 0.08 % in Satsumas. The diseased trees occurred individually or in small groups and were older than 15 to 20 years.

In the same year Yilmaz *et al.* reported that the aphid species present in infected orchards was *Aphis gossypii*. Aphids were collected and used in successful transmission assays to Mexican lime. Although no symptoms of CTV could be seen on test plants, the virus was detected by ELISA. The virus could also be detected by ELISA in *A. gossypii* but not on *A. ruborum* or *A. solanella*.

1993. Yumruktepe carried out an aphid survey in the eastern Mediterranean Region. *Aphis citricola*, *A. Gossypii*, *A. craccivora*, *Toxoptera aurantii* and *Myzus persicae* were detected in the region. *A. citricola* and *A. gossypii* were found to be the major aphid species among the others.

1996. Akbulut *et al.* cloned and sequenced the capsid protein genes of five Turkish isolates of CTV. Cluster analysis indicated that the sequences were closely related to the known severe stem pitting isolate (B53) from Japan. These isolates also reacted with the monoclonal antibody MCA13, which reacts predominantly with severe CTV isolates. The biological characteristics of these isolates have not been assessed, and stem pitting was not obvious in orange trees in the field.

1997. Satar did experiments on the transmission of the CTV isolate from Mersin by *A. gossypii*. Transmission averaged 7.9 %, but greatly varied between 0.0 % and 21.5 %.

1998. Ulutas tried to obtain CTV tolerant rootstock plants via somatic hybridization. Yılmaz *et al.* (1998) used immunoprinting to detect CTV in samples from different Turkish Provinces.

1999. Ince detected CTV infection in field samples from diverse hosts from the Mediterranean Region using dsRNA.

2000. Kamberoglu produced a monoclonal antiserum against Turkish CTV isolates. The antisera produced were successful for the detection of local CTV isolates.

2002. Bozan conducted a new survey (using ELISA and DTBIA methods) to determine the incidence of CTV in the citrus-growing area in the East Mediterranean Region. The CTV incidence was found to total 0.04 % in the surveyed area.

III – Conclusion

Citrus production is a vital branch of agriculture in the country. Improvement of production quality and control of the pests and diseases with environment-friendly techniques are the major strategies for a sustainable and profitable citriculture. CTV is endemic in the country and remains a major threat to the citrus industry due to the use of susceptible rootstocks. Apparently most of the characterized isolates appear to be mild. Although *T. citricidus* is not present, the other vectors such as *Aphis gossypii* can transmit the local isolates.

A voluntary certification system under the control of the Ministry of Agriculture and Rural Affairs is operating. For an effective control of CTV, research on tolerant citrus rootstocks and varieties, and the strengthening of quarantine measures as well as participation in international control and research activities are of utmost importance. Application of a harmonized certification system will be the major part of a control strategy.

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Presence of *Citrus tristeza virus* in Croatia

Černi S.¹, Krajačić M.¹, Hartl D.², Gatin Ž.², Škorić D.¹

¹ Department of Biology, Faculty of Science, University of Zagreb, Croatia

² Institute for Adriatic Crops and Karst Reclamation, Split, Croatia

Abstract. Forty-five samples, mostly Satsumas, from 11 orchards, 2 nurseries and a collection planting were tested by ELISA or IC/RT-PCR for the presence of Citrus tristeza virus (CTV). This preliminary survey confirmed the presence of CTV in 17 out of 45 field samples that were mostly asymptomatic, as expected in plants grafted onto trifoliolate rootstocks. For assessing the sanitary status properly, a more systematic approach including indexing should be applied.

Keywords. Citrus tristeza virus – Croatia – ELISA – IC/RT-PCR – *Poncirus trifoliata* – Satsuma.

Présence du virus de la Tristeza des agrumes en Croatie

Résumé. Quarante-cinq échantillons, en grande partie de Satsuma, prélevés dans 11 vergers, 2 pépinières et une collection variétale ont été analysés par ELISA ou IC/RT-PCR pour déterminer l'éventuelle présence du virus de la tristeza des agrumes (CTV). Cette enquête préliminaire a confirmé la présence du CTV dans 17 des 45 échantillons prélevés en plein champ, et qui, pour la plupart, ne montraient pas de symptômes, comme c'est normalement le cas des plants greffés sur des porte-greffes d'oranger trifolié. Afin d'évaluer l'état sanitaire de ce matériel d'une manière appropriée, il serait nécessaire d'adopter une démarche plus systématique incluant aussi les techniques d'indexage.

Mots-clés. Virus de la tristeza des agrumes – Croatie – ELISA - IC/RT-PCR - *Poncirus trifoliata* - Satsuma.

I – Introduction

The citrus industry in Croatia, with an acreage of about 1500 ha, is as interesting as probably the northernmost commercial citrus growing areas in the world. The climatic conditions between 42° and 43° 30' of the northern latitudes on the Dalmatian coast and the islands impose constraints on the citrus variety choice. The main commercially grown variety is the most cold-tolerant Satsuma (*Citrus unshiu* Marc.). Its production is estimated up to 30000 tons a year. Local citrus producers are mostly oriented towards the very early and early ripening Satsuma varieties like Zorica Rana (locally obtained bud mutant from an old Japanese early variety Kawano Wase), Wakiyama, Chahara, Okitsu and others (Gatin, 1997). Lemon and sweet orange are grown only in the spots with warmer microclimate, or in the family gardens. The beginnings of modern citrus industry in Croatia have already been described as well as the foundations for the assessment of the citrus sanitary status (Škorić *et al.*, 2002a).

II – Historical review of CTV in Croatia

Before 1990. According to Šarić and Dulić (1990), CTV in Croatia was originally reported in the paper of Davino and Catara (1986). They detected it from the material that had been introduced in Croatia in the 1980s from Japan. The majority of Satsuma trees are still propagated from this material. They are mostly grafted on *Poncirus trifoliata* rootstock and, with a few exceptions, they do not show tristeza symptoms in the field. It is believed that tristeza had been introduced in Croatia even earlier with Satsumas that had arrived from Japan as early as 1933-1934. The lack of apparent symptoms in Satsumas grafted onto *P. trifoliata* was probably the reason why

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the disease went unnoticed by the nurserymen and spread throughout the whole growing area. The old citrus foundation block and new introductions from the 1970s until the late 1980s were monitored regularly by ELISA (Šarić and Dulić, 1990) but at the beginning of the 1990s that practice stopped and the foundation block was destroyed during the war in former Yugoslavia.

1995 - up to present. In the post-war period, with the replanting of citrus, the demand for the nursery trees raised. Several projects on citrus certification have been proposed with less success than necessary to meet the needs of the growers (Škorić *et al.*, 2002a). Nonetheless, the attempt to survey for CTV has been successful.

Since tristeza is one of the most devastating diseases of citrus apparently reemerging in the Mediterranean (Dimou *et al.*, 2002; Davino *et al.*, 2003), our intention was to concentrate primarily on the detection of this viral agent by using ELISA and occasionally IC/RT-PCR, directly from the field trees in the absence of the facilities for biological testing. Meanwhile, the attempts to build a new mother block and to enable biological testing, as well as sanitation by thermotherapy and shoot-tip grafting are under way. Aside from that, new research projects have been proposed aiming at the detailed molecular and biological characterization of the Croatian CTV isolates.

Forty-five samples from 11 orchards, 2 nurseries and a collection planting (Tab. 1) were tested by ELISA or IC/RT-PCR. Collected orchard samples from Dalmatia encompassed both island and coastal locations.

Table 1. Citrus plantings surveyed for the presence of CTV in Croatia.

Plantings N°	Location	Mandarins	Lemons	Sweet Oranges	Others	Rootstocks for tested trees
Orchards						
4	Vis island	<i>C. unshiu</i> Kuno, <i>C. deliciosa</i> Tenore Willowleaf unknown variety	Lisbon or Lisbon type, Eureka, an unknown variety	Sanguinello, Washington navel		sour orange, except Kuno (grafted on <i>P. trifoliata</i>)
2	Split-Kaštela	<i>C. unshiu</i> Chahara, Ichimaru, Zorica rana			kumquat	<i>P. trifoliata</i>
1	Brač island		Lisbon type			<i>P. trifoliata</i> or citrumelo Swingle 4475
3	Opuzen	<i>C. deliciosa</i> Carvalhal SRA, <i>C. unshiu</i> Saigon SRA-29	Lisbon type	Washington navel		<i>P. trifoliata</i>
1	Metković	<i>C. unshiu</i> Ueno				<i>P. trifoliata</i>
Nurseries						
1	Kaštel Štafilić		Meyer improved, Lisbon, Eureka	Bonanza navel		Bonanza on <i>P. trifoliata</i> , Eureka on Cleopatra, Citrumelo SW4475 for the rest
1	Kaštel Štafilić- Nehaj			Fukumoto		<i>P. trifoliata</i>
Collection						
1	Split-Institute for Adriatic Crops	<i>C. unshiu</i> Cleopatra	<i>C. wilsonii</i> (Ichang lemon)		citrumelo SW 4476, <i>C. aurantium</i> , <i>P. trifoliata</i> , <i>C. limon</i> Etrog	seedlings, except self- rooted Etrog from cuttings

The source trees were mostly asymptomatic with a few exceptions reported in Table II. Leaf midrib tissues from the majority of field samples were tested by DAS-ELISA (Clark and Adams, 1977) using commercial antisera according to the manufacturers' protocols (Loewe Phytodiagnostica Biochemica, Sauerlach, Germany). The same extracts were tested by IC/RT-PCR performed by a standard protocol using primers for the CTV coat protein gene (Nolasco *et al.*, 2002).

The preliminary survey of CTV by DAS-ELISA and/or IC/RT-PCR (Tab. 2) confirms the presence of tristeza agent in 17 out of 45 field samples. Although almost 38% of the tested plants proved positive for CTV, only three of them showed symptoms, but those can primarily be attributed to the infection with citrus exocortis viroid (Škorić *et al.*, 2002b).

Table 2. CTV infected samples detected by ELISA and/or IC/RT-PCR in Croatia.

Location	N° of positive samples	Sample type/ rootstock	ELISA	IC/RT-PCR	Field symptoms
Orchards					
Vis island	2	Washington navel/SO	+	+	-
	2	Kuno/Pt	+	+	-
Split-Kaštela	2	Ichimaru/Pt	+	+	-
	1	Chahara/Pt	+s	+	-
	5	Zorica rana/Pt	+	not tested	-
Brač island	1	Lisbon type/Pt	not tested	+	Severe rootstock bark shelling and yield reduction. Mixed infection with citrus viroids confirmed.
Opuzen	1	Washington navel/Pt	not tested	+	Rootstock bark shelling, stunted tree, yield reduction. Mixed infection with CEVd suspected.
	1	Saigon SRA-29	not tested	+	-
	1	Lisbon type/Pt	not tested	+	Rootstock bark cracking, yield reduction. Mixed infection with CEVd suspected.
Nursery					
Kaštel Štafilić-Nehaj	1	Fukumoto/Pt	+	+	Stunted tree
Total	17				

Pt – *Poncirus trifoliata*, *SO* – *Sour orange*

This biological manifestation of the disease is not surprising considering the tolerance of trifoliolate rootstock to CTV and its sensitivity to citrus viroids. The case of Washington navel sweet orange from the island of Vis stands out because it shows no CTV symptoms regardless of being grafted on sour orange rootstock. It is possible that this tree hosts some mild tristeza strains and even that the presence of mild symptoms was overlooked at the time of sampling. Four out of 6 sweet oranges tested were positive for CTV, while this ratio for Satsumas was 11/14 and for lemons 2/13 (Table 2). A few of these samples were further characterized by indexing on Madam vinous and molecular analysis (Černi *et al.*, 2005). These harbored a mixture of CTV coat protein gene sequence variants encompassing some clustering close to mild types while others clustered close to severe strains. Sweet orange stem pitting symptoms could be observed. These findings were later confirmed by direct observation of stem pitting in the branches of Satsumas in the Neretva Valley during the spring of 2004. It is interesting to note that as these symptoms are not externally conspicuous they may pass unnoticed to farmers.

The high percentage of CTV-infected Satsumas with the apparent lack of symptoms and the highest rate of its propagation for commercial purposes make Satsuma the best candidate for CTV reservoir host in our region. Two Mediterranean mandarins, kumquat and all the 9 samples planned to be used as indicators or rootstocks from the collection planting from Split were CTV negative.

III – Conclusions

It is encouraging that with the above percentage of CTV infected trees most of the nursery samples tested negative. Nonetheless, in order to assess the tristeza sanitary status of the nursery plantings properly, a more systematic approach including indexing should be applied. The results of this survey are only an indication of the CTV incidence in the Croatia-grown citrus. No local studies are available on the natural spread of the virus by *Aphis gossypii*, known to be a very efficient vector of CTV in the Mediterranean, or by less efficient aphid vectors like *Myzus persicae*. Regardless of the results of future studies and considering the potential damages that can be caused by CTV in Citrus, it is necessary that strict CTV control measures in the citrus propagation and introduction are taken. These measures should eventually lead to the development of a certification program for our locally important Satsuma cultivars, a step we are legally bound to take by the year 2007 according to our present laws.

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Citrus tristeza virus (CTV) in the State of Montenegro

Papic T.¹, Nolasco G.²

¹ Ministry of International Economic Relations, Mihajla Pupina 2, 11000 Belgrade, Montenegro State

² Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Abstract. Citrus production in the Montenegro State is mainly concentrated in the coastal area with predominance of satsumas and lemons varieties. Citrus tristeza virus was detected in several trees but symptoms are mostly absent due to the traditional grafting of citrus plants onto a CTV tolerant rootstocks, *Poncirus trifoliata*. Over the years, CTV has been continuously spreading by the propagation of infected material.

Keywords. Citrus – Citrus tristeza virus – Montenegro – *Poncirus trifoliata* – Satsuma.

Le virus de la tristeza des agrumes (CTV) au Monténégro

Résumé. Au Monténégro, la production d'agrumes est essentiellement concentrée dans la zone côtière, où prédominent les variétés de satsuma et citronnier. Le virus de la tristeza des agrumes a été décelé chez plusieurs arbres, mais les symptômes sont assez rares étant donné que, traditionnellement, le greffage a été effectué sur un porte-greffe tolérant au CTV, le *Poncirus trifoliata*. Dans le temps, le CTV a été disséminé sans arrêt par la diffusion du matériel de multiplication infecté.

Mots-clés. Agrumes – Virus de la tristeza des agrumes – Monténégro – *Poncirus trifoliata* – Satsuma.

I – Introduction

Citrus production in the State Union of Serbia and Montenegro has a strategic importance for the agricultural sector. About 400,000 trees are now grown in the major citrus producing region which is the coastal area of Montenegro. Satsumas and lemons grafted on *Poncirus trifoliata* are the most cultivated varieties. About one third of these are new plantations.

II – Historical note on the detection of CTV

Sudden and serious decrease in citrus yield was detected during the 2003 harvest ranging between 10 and 60 % in some areas. Affected trees were showing bark-gummosis, leaf yellowing and strong deformation of the fruits. In December, eight samples, taken from the coastal region close to the towns of Bar and Ulcinj, were analyzed by ELISA and Immunocapture RT-PCR targeting the whole coat protein (CP) gene, according to standard protocols. Seven out of the eight samples analyzed were found to be CTV infected by both techniques, including two samples that were symptomless.

The PCR products of two samples were cloned in *E. coli* cells and the CP inserts analyzed by SSCP and sequencing. In both cases, the SSCP analysis of several clones indicated a variety of different patterns, suggesting the occurrence of infections with a mixture of genomic variants. Sequence analysis of different variants showed a true-to-type CP gene with 669 nucleotides. One sequence obtained was deposited at the GenBank under the accession number AY764154. This genomic variant is closely related (1.5 % distance) to the mild strain T30 from Florida (GenBank accession AF260651). However, other sequences obtained differed as much as 7 % from this one

and were closer to others from Croatian isolates. Although a very small number of samples were analyzed in this study, CTV appears to be common in the Satsuma groves. This could be due to the traditional use of the trifoliolate rootstock which prevents the appearance of tristeza decline, thus enabling the unnoticed propagation of infected material. The fact that symptomless trees are also infected, and the kind of symptoms referred, which are not typical for CTV, suggests that the virus is not responsible for the symptoms observed in the field. Most of these data were previously published in Papic *et al.* (2005).

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Historical review of Citrus Tristeza in Morocco

Zemzami M.¹, Benali D.², Moukhtari A.², Soulaymani A.²

¹ DDA-UCP, Domaine Maamora, Salé, Morocco

² Université Ibn Tofaïl, Dpt de Biologie, Kénitra, Morocco

Abstract. This review outlines important facts and dates which marked the history of the long and laborious struggle to fight against citrus tristeza virus in Morocco. CTV is widespread worldwide. It has been detected in Morocco several times in various varieties of germplasm introduced to Morocco during the second quarter of the 20th century. Meyer lemon is the only infected variety planted to a large extent. This variety was tracked and eradicated everywhere in the country. Some recent importations of new citrus varieties were also CTV-infected. However, each time CTV was found, immediate eradication actions were taken. Eradication has, up to now, been quite effective. Legislation has been enforced to backup CTV control strategies by mandatory eradication, limitation of citrus importations, compulsory quarantine, and finally instauration of a certification program. Significant efforts were devoted by public and private institutions to develop appropriate means and facilities for efficient CTV control. MAbs were produced in Morocco and as well, new diagnostic techniques were acquired. These means were used for extensive tristeza surveys and eradication in the country. Awareness towards the danger of CTV and its efficient vector is growing, but more is still needed to secure the future of citrus industry in Morocco.

Keywords. CTV – Eradication – Morocco – Tristeza.

Revue historique de la tristeza des agrumes au Maroc

Résumé. Cette revue retrace les faits et dates importants qui ont marqué l'histoire de la lutte laborieuse contre le virus de la tristeza des agrumes (CTV) au Maroc. Le CTV est répandu à travers le monde. Il a été décelé au Maroc à plusieurs reprises dans diverses variétés de collection introduites au Maroc durant le second quart du 20ème siècle. Le citronnier Meyer est la seule variété atteinte qui a été cultivée à large échelle. Il a été traqué et éradiqué partout dans le pays. Certaines introductions récentes de nouvelles variétés ont aussi été infectées par le CTV. Cependant, chaque fois que le CTV est mis en évidence, des actions immédiates pour son éradication ont été prises. L'éradication a jusqu'à présent été très efficace. La législation a été renforcée pour renforcer la stratégie de lutte contre le CTV, en instaurant l'obligation d'éradiquer le CTV, la limitation des introductions des citrus de l'étranger, la quarantaine obligatoire, et enfin, l'instauration du règlement de la certification. Des efforts louables ont été dédiés par les institutions publiques et privées au développement de moyens et d'infrastructures pour une lutte efficace contre le CTV. Ainsi, des anticorps monoclonaux ont été développés au Maroc, de même des nouvelles techniques de diagnostic ont été acquises. Ces moyens ont servi pour des prospections étendues pour l'éradication de la tristeza dans le pays. La prise de conscience vis-à-vis du danger du CTV et de son vecteur le plus efficace « *Toxoptera citricidus* » est grandissante. Cependant, beaucoup reste à faire pour sécuriser l'avenir de l'agrumiculture au Maroc.

Mots-clés. CTV – Éradication – Maroc – Tristeza.

I – Introduction

Citrus trees are a major fruit crop in Morocco. They cover about 80 thousand ha with an average yearly production of 1.5 million metric tons of which about 50% is exported as fresh fruits. Commercial varieties and cultivars include early and late clementines, Navels, Valencias, blood oranges and very little of lemon and grapefruit. The main rootstock by far is sour orange. Nevertheless, other rootstocks are now commonly used in new plantings, in particular Citranges (Carrizo, Troyer, C-35), Volkameriana and Macrophylla.

II – Tristeza historical events in Morocco

CTV was probably introduced in Morocco early in the 1930's with citrus culture development in the country. Furthermore, commercial germplasm repositories were established before 1951, with material introduced from abroad, when the aetiology of tristeza disease was still unknown. Hence, there is no realistic reason to preclude the introduction of CTV-infected material with the massive number of citrus varieties existing in germplasm blocks, most of which are of foreign origin (Nadori and Zemzami, 1987). Irrefutable proof for that is the commercial planting of Meyer lemon, known to harbour CTV (Wallace and Drake, 1955) to a large extent. This variety was later confirmed in Morocco to harbour CTV (Chapot and Cassin, 1961).

1930 - 1950. Establishment of Souihla Germplasm block with the supervision of Chapot, which contains 368 varieties and selections imported mainly from California, Florida, South Africa, Australia, Argentina, Brazil, Spain and other neighbouring countries (Nadori and Zemzami, 1987).

1951. Issuing of a decree to regulate importation of plant material of *Rutaceae* to Morocco (Anonymous, 1951).

1955. Rebour reported the results obtained by Wallace and Drake which proved infection of the original Meyer lemon budwood source by CTV in the USA. He alerted all Mediterranean countries which imported Meyer lemon. This alert was reiterated for Morocco by Mendel in 1956.

1959 - 1961. Cassin conducted CTV indexing on Mexican lime (Wallace, 1959) of Meyer lemon and some other varieties of foreign origin. Meyer lemon and 8 other accessions were found infected with CTV. They were eradicated from germplasm blocks (Cassin, 1963).

1964. Chapot and Delucchi (1964) reported infection of some exotic varieties with CTV, including all Meyer lemons in germplasm repositories as well as in commercial plantings, various Satsumas and Kumquats in Morocco, Sicily, Israel and Egypt.

1967. Bové confirmed the introduction of tristeza into Morocco in budwood of exotic varieties including Ovari Satsuma and King Mandarin imported from Florida between 1945 and 1948. Preservation of that material was done by grafting sour orange rootstocks established in the field. It happened to graft more than one variety on a single rootstock and to re-graft sour orange plants with other varieties when the first grafting failed. He adverted against the risks of having CTV-infected Meyer lemon trees planted to a large extent in the Marrakech valley.

1968. The Citrus Committee of the Franc Zone (CAZF) stressed out the urgent need to eradicate all known sources of CTV in the country. A governmental decree was issued for mandatory eradication of Meyer lemons in the region of Marrakech. Professionals and farmers cooperated fully with the authorities to launch an eradication campaign of Meyer lemon that included 18 farms. Subsequent surveys and indexing of neighbouring orchards indicated a absence of any signs of natural propagation of tristeza. This action may be questionable as whether it was fully successful. However, the awareness raised among Moroccan vis-à-vis the danger of tristeza that has stood strong since then.

1969. The False Alert of Tristeza: in an indexing block of budwood from nucellar varieties introduced in 1964/65 from the USA, a Hamlin orange grafted onto Mexican lime with typical CTV symptoms was discovered by Bourge and Nhami at El Menzeh Experimental Citrus Research Station.

Since budwood from Hamlin orange was largely distributed, its potential infection with CTV was an issue of concern. The first expertise survey made an alarming assessment, indicating that tristeza was largely present in various orchards in Beni Mellal area. This same year, a decree was issued to regulate circulation of budwood and nursery plants of citrus and citrus relatives between different regions in the country (Anonymous, 1969).

1970. The CTV alert triggered a general mobilisation among officials and the professionals (INRA, SODEA OCE, ASPAM, SASMA). The CTV alert enigma was further investigated by other worldwide experts. The outcome of the surveys and study tours conducted by these experts indicted unanimously that the Hamlin/Mexican lime tree had unequivocal CTV symptoms. However, none of the Hamlin trees produced from budwood of US origin was infected with CTV. Hamlin material collected from the field in Morocco was indexed in France and two Hamlin trees in California. All results were negative. It was shown that the initial expertise confused severe Stubborn symptoms with those of tristeza. Therefore, the alert of tristeza being widespread in Beni Mellal region was a false one. Experts revealed that they found during their surveys some former Meyer lemon trees topworked with other varieties, which is a real danger for the dissemination of CTV. They requested immediate eradication. Again, no signs of natural spread of CTV were noticed (Calavan, 1969). The false Tristeza alert raised awareness among officials and professionals for the need to set a strong CTV control strategy by devoting adequate means (fund, trained human resources, appropriate equipment and infrastructure) for this mission.

1978. Nhami, from Société de Développement Agricole (SODEA), conducted a survey for the sanitation of SODEA farms, with special consideration of Meyer lemon. Seven Meyer lemon trees were found in 3 farms. Their eradication was swift (Nhami, 1981).

1980. Fourteen Meyer lemon trees were again found in 3 citrus orchards in Marrakech area. They were eradicated as well (Nhami, 1981).

1983. Setting up of a certification program for citrus nursery plants (Anonymous, 1984). A scheme was inspired by the Californian and the Spanish certification programs, requiring systematic indexing of CTV for initial and pre-basic material and screening by ELISA and molecular techniques of increase blocks. No CTV positives were ever revealed during the subsequent decade (Nhami *et al.*, 1993).

1984. The first quick declining tree was found in the citrus germplasm block at INRA-Station of El Menzeh. A tree of Pan American mandarin declined suddenly exhibiting typical quick decline symptoms (Fig. 1). A systematic testing of the entire germplasm block was ordered.

The remaining trees of Pan American mandarin were found infected with CTV. However, the survey could not be completed due to the lack of means (Nadori, personal communication).



Figure 1. Quick decline symptoms observed in the CTV infected trees.

1987. A thorough survey was undertaken to test the citrus germplasm block of INRA-Souihla, near Marrakech (Nadori and Zemzami, 1987). A total of 1749 trees were tested; 67 trees of 19 varieties were positive. After the preservation of plant material for research purposes and sanitation of infected varieties, eradication of all CTV-infected trees was completed meticulously (Fig. 2).

A similar survey was carried out in El Menzeh germplasm block in the Gharb valley near Kénitra. A total of 2130 trees were tested; 21 trees of 6 varieties were positive. They were also immediately eradicated (Nadori and Zemzami, 1992).

Similar surveys were accomplished to control the other INRA-germplasm of citrus: Aïn Chaïb and Melk-Zhar in the Souss valley near Agadir and Aïn-Taoujdate in the Saïss valley near Fès. Mainly varieties duplicated from Souihla germplasm were in these blocks; 9 positives were found all among the duplicated trees of P an American at Aïn-Chaïb and Aïn-Toujdate (Zemzami and Nadori, 1989).



Figure 2. Eradication steps of CTV infected trees.

1988. The first massive importation of certified citrus nursery plants from Spain to Morocco was made. It concerned 60 thousand plants of Fortuna, Nova and Marisol varieties. The plants had the label of the Spanish certification authority; nevertheless, a systematic testing by ELISA was imposed by the Plant Protection Services of Morocco. Huge efforts were deployed by the importer to fulfil the required tests. Plants were maintained for quarantine under an insect-proof screen-house in an isolated locality far from citrus orchards (Fig. 3).

All plants were tested systematically by ELISA in autumn 1988. Several hundreds of positive cases were found, representing percentages varying from 5 to 10% depending on batches and varieties (unpublished data). All the infected material was eradicated (Fig. 3).

Released plants were tested again the following spring before undergoing further increase in the nurseries.



Figure 3. Overview of the imported certified plants maintained in screenhouse and the following eradication of some CTV infected plants.

1990. Domaines Agricoles created a facility (Unité de Contrôle des Plants-UCP) devoted to the development of disease diagnostics means and sanitary surveillance of citrus and other crops in their farms. Work on the development of monoclonal antibodies for CTV detection was initiated. Immuno-reagents of high quality were produced (Zemzami *et al.*, 1993; Zemzami, 1995) and it became possible to undertake large surveys for CTV wherever suspected declines were reported. A total of 400 thousand ELISA tests were done from 1994 to 1998.

Impressive efforts were made to raise awareness for an open dialogue about tristeza through seminars and workshops animated by sound international CTV experts. Unfortunately, the subject of tristeza remained a taboo and every suspected CTV case is eradicated in silence (Lbida *et al.*, 2005).

1995. Domaines Agricoles extended the activities of UCP to conduct a certification program for their citrus nurseries. Indexing and sanitation facilities as well as molecular and serological laboratories were set up (Fig. 4). Hundreds of local selections were regenerated by thermotherapy/shoot-tip-grafting.

A sound certification program was operative. Tactics for Tristeza control strategies based on meticulous sanitary control and substitution of sour orange with tolerant rootstocks were fully integrated. Thorough indexing as well as serological and molecular testing were adopted for detection of viruses, and CTV tolerant rootstocks including Carrizo, Troyer and C-35 citranges, Citrumelo sacaton, and Volkameriana lemon were forced in the plantings with outstanding results.

Joined efforts were deployed by UCP and Plant Protection Services to provide Quarantine service for imported new varieties to other Citrus producers. Several Clementine varieties were released to the farmers through this collaboration.



Figure 4. Indexing facilities at the UCP Domaines agricoles.

1997 to date. UCP engaged in regional networks cooperating in coordinated actions to control CTV in the Mediterranean region. UCP participated actively in a CFC project for the control of CTV by new efficient RT-PCR techniques and the Mediterranean Network for Certification of Citrus. Characterization of Moroccan isolates of CTV was carried out. It showed presence of highly severe strains capable of causing disastrous ravages if they become efficiently disseminated (Zemzami *et al.*, 2002).

1998. Interception of CTV in citrus material introduced legally from Spain to Morocco by Plant Protection Quarantine Service (Lbida *et al.*, 2005).

1999. The Plant Protection Service was informed by SODEA technicians about abnormal dwarfing of 12 trees of Nova mandarin and Lane Late navel in a topworked orchard in the Souss valley. Presence of CTV in samples collected from suspected trees was confirmed by ELISA at both SODEA and Plant Protection laboratories. The owner of the orchard cooperated fully with the authorities and eradicated the whole plot composed of 900 trees. The origin of the material used for topworking could not be traced back to any local source. Tests conducted in the neighbouring

plots the next spring showed no positive cases, indicating again that no natural spread had occurred (Plant Protection Services, CTV-Team report, 1999).

2000. Again, interception of CTV in citrus material introduced legally from Spain to Morocco by the Plant Protection Quarantine Service (Lbida *et al.*, 2004).

2004. Isolates of CTV (P1 and P2) intercepted in citrus material imported from Spain by Quarantine Service in 1998 and 2000 were characterized. They showed genomic variability (Lbida *et al.*, 2004) as well as biological and serological differences (Lbida *et al.*, 2005).

2005. The General Council of the Ministry of Agriculture organized a meeting for national experts for an open debate about CTV control strategy to be adopted in Morocco in the light of the appearance of the Brown Citrus Aphid in Spain and Portugal. The input of Mr Nadori and Mr Nhami was highly enriching, both men have devoted much of their outstanding careers to the development of Moroccan citrus culture and its preservation from a disaster by *tristeza*. It was clear that CTV was found and eradicated to a higher extent than we knew. Fortunately, from both experiences, we drew 2 important conclusions:

- CTV foci found were all limited to some varieties and their distribution patterns do not reflect the presence of any natural propagation. This was reported on many occasions by various experts who surveyed Morocco.
- Accomplished eradications were always very successful. Further testing up to 2 springs after eradication never revealed any positive cases. This is of significant importance because many question if CTV eradication is ever possible!

Two of her meetings were organized at the Plant Protection Headquarters in Rabat and at Domaines Agricoles in Casablanca. Both were open to a large audience including the Growers' Association ASPAM and major stakeholders of the citrus industry. The general consensus was that in the Mediterranean Basin we are all facing a serious threat as reflected by the widespread of CTV foci in all Mediterranean countries, the presence of severe strains and the arrival of *T. citricidus* in the region. Enthusiasm for implementing immediate CTV control actions was everyone's desire. However, until now the follow-up is weak.

III – Conclusion

Citrus *tristeza* virus was introduced into Morocco early in the last century in budwood introduced from abroad. CTV infected Meyer lemon was propagated largely in homogeneous plantings which were maintained until 1967 (Bové, 1995). It didn't fortunately find favourable conditions to become endemic. Infected trees remained isolated foci until their eradication. The numerous eradications that were carried out, helped to contain the disease and to prevent its diffusion. However, it would be naïve to think that Morocco is undoubtedly free of CTV. Characterization studies showed that some of the strains found are highly severe. They could cause extensive damage to our citrus culture if they get disseminated efficiently. Remarkable efforts have been deployed to develop adequate means for quick and efficient detection at affordable costs. A dependable certification program has been established since 1984. Substitution of the CTV-sensitive sour orange rootstock is underway. In spite of these achievements, we still have to remain alert and double our efforts at all levels of action:

- raising awareness of the growers and the public;
- engaging in germplasm exchange to make it safely accessible to the growers;
- intensifying efforts in quarantine, surveys and eradication of the virus;
- enforcing surveillance at the borders;

- stimulating substitution of sour orange and implementation of additional rootstock trials;
- strengthening of common harmonized regional actions with neighbouring countries throughout the Mediterranean basin.

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Historical review of *Citrus tristeza virus* (CTV) in Libya

Abukraa H.^{1,2}, Djelouah K.¹, Kafu A.²

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

² Agricultural Research Center, Tripoli - Libya

Abstract. Citrus orchards in Libya are known to have been affected by *Citrus tristeza virus* (CTV) for nearly 35 years. However, since the first findings, no further investigation has been carried for several years. During the spring 2008, citrus growing areas were surveyed to assess the presence and distribution of CTV. The results highlighted the wide distribution of the virus in the most important Libyan citrus-growing areas. A historical review of CTV in Libya is presented with a special reference to the surveys carried out in 2008.

Keywords. Libya – Citrus – Citrus tristeza virus – Survey.

Bref historique du CTV en Libye

Résumé. En Libye, les vergers d'agrumes sont censés être infectés par le virus de la tristeza des agrumes (CTV) depuis presque 35 ans. Cependant, après les premiers signalements, aucune autre enquête n'a été réalisée pendant de longues années. Au cours du printemps 2008, les principales régions agrumicoles ont fait l'objet d'une prospection pour évaluer la présence et la distribution du CTV, ce qui a permis de confirmer la vaste distribution du virus. Dans le présent travail, on va parcourir l'histoire du CTV en Libye, en mettant l'accent sur les enquêtes menées en 2008.

Mots-clés. Libye – Agrumes – Virus de la tristeza des agrumes – Prospection.

I – Introduction

Citrus is considered the only winter fruit produced in Libya. The total area devoted to citrus growing is about 7.000 ha (Anonymous, 2001) with an average size of 1-2 ha of citrus plantation.

Citrus growing is concentrated mainly around the coastal areas of the Mediterranean sea where the climate is mild and rainfall abundant. There are also some plantations in the South of the country near Sebha region (Abu-Daba and Abu-Ziada, 1978).

All citrus produced is consumed locally as fresh fruit. On the other hand, no citrus is imported; the main rootstock is the sour orange, however, other rootstocks were introduced in the country during the seventies as experimental trials and some of them were recommended as alternatives to the sour orange for their disease resistance and soil tolerance.

Since most citrus cultivars, currently cultivated in Libya, were imported from Italy, Spain, Egypt, Palestine and from neighbouring countries, it must be expected that many other pathogens were inadvertently also introduced.

Based on visual observations, several diseases induced by virus and virus-like agents were observed such as various forms of psorosis, cachexia, gummy bark of sweet orange, woody gall, impietratura and exocortis (Chapot, 1975; Fudl-allah, 1978; Nour-Eldin, 1975; Nour-Eldin and Fudl-Allah, 1976; Salibe, 1985; Khalil *et al.*, 1994; Bové, 1995).

II – CTV situation

CTV was first detected by Nour-Eldin and Fudl-Allah (1976). Two cases of CTV were found at the research station of El-Ftah University in Tripoli. These cases were confirmed through biological indexing onto Mexican lime indicator, the trees were eradicated and after those findings no further investigations have been carried out.

In 2007, in the framework of a mobility MSc Thesis under the supervision of the CIHEAM-IAMB, a CTV monitoring was carried out in the main citrus cultivated areas in Libya.

Survey was carried out in nurseries, varietal collections, mother trees blocks and commercial orchards in the main citrus growing areas.

During the survey, a total of 515 samples were collected in four regions from various farms and different areas; meanwhile, the main citrus species belonging to four nurseries producing 30,000 to 90,000 seedlings were selected to be surveyed, the total samples belonging to the mother block of two nurseries were collected, while, in the two other nurseries, in the absence of mother block, the samples were collected randomly.

Moreover, two varietal collections were surveyed, one belonging to Zahra region, the second to Sabha region, both maintained by the Agriculture Research Center (ARC). CTV monitoring was carried out on the total of the existing trees (569).

Surveys carried out in nurseries registered 5.6 % a s overall CTV incidence, whereas, all the infected trees were present in just one mother block located in Tripoli with a 25% incidence.

The CTV infected plants were detected in four varieties: sukkari orange, Navel orange, blood orange and Jaffa; no CTV infected plants were detected in nurseries located in Tajora region.

In commercial orchards, an incidence of 3.5% was registered. The infected trees were found in just three areas represented by Zawia region (having the highest infection rate (8%), followed by Zahra region (2.5%), and Grabolli region (1%). The infected trees in this field were showing a general decline which could be associated to CTV. Blood orange was a highly infected variety (3%), followed by navel orange (0.58%).

Regarding the varietal collection, the CTV was detected only on 2 trees out of the 569 sampled trees, representing an infection rate of 0.35%

The overall CTV infection rate in all surveyed sites (nurseries, mother blocks, commercial orchard and varietal collections) reached 3% and the CTV-infected trees were all belonging to sweet oranges. Dammi orange (Sanguina) showed the highest number of infection (1.25%), followed by navel orange (0.75%), Sukari (0.45%) and Jaffa (0.2%).

A preliminary molecular characterization of the CTV isolates was carried out; several clones were selected and their partial coat protein was sequenced.

In order to have a genetic comparison with some retrieved sequences used as references in the GenBank, the sequences obtained were compared to other well known CTV isolates in the world. The partial coat protein gene nucleotide sequence, obtained from 4 clones, revealed a highly nucleotide homology with well studied isolate VT (Mawassi *et al.*, 1993) (accession E 937519), the Jordanian isolate (accession AY550 252) and the Syrian isolate STV 6 (Abu Kubaa *et al.*, 2008) (accession EU626 555). Interestingly, all these isolates belong to the Middle East countries and were in some cases responsible for epidemics in those areas.

III – Conclusion

The assessment of the sanitary situation of the main citrus growing areas in Libya, with particular reference to CTV, reported for the first time the presence of CTV, and its wide distribution.

The presence of CTV in several areas (Zawia, Zahra, Tripoli, Grapoli and Sabha) can contribute to the fast and long spreading of the virus into the country, since nurseries are producing and selling CTV-infected propagating material in different areas of Libya. Indeed, tristeza is the most important virus disease of citrus in the world and in the Mediterranean countries causing reduced fruit quality, rapid decline and death of trees.

Additionally, first investigations on the CTV characterization of local isolate, put in evidence interesting results. The sequence analysis of the partial CPg clustered the virus in a group including several Mediterranean CTV isolates, most of them are infecting the citrus trees in the Middle East, and were responsible for epidemics in those areas; based on these considerations, it's of utmost importance to investigate the vector capability and efficiency to transmit the virus.

Considering the presence and distribution of vectored CTV which can endanger the Libyan citriculture, the situation is alarming and urgent actions need to be taken to avoid a serious crisis and deterioration of the citrus industry in Libya.

Libyan administration should soon adopt urgent actions to continue such surveys, by establishing laws, regulating the control of CTV and their main vectors, since this is the only tool to control and to stop the introduction of new emerging diseases, as greening and witches' broom which are already in the neighboring area.

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Historical review of *Citrus tristeza virus* (CTV) in Algeria

Larbi D.¹, Ghezli C.², Djelouah K.³

¹ University of Skikda, Algeria

² Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAFV) Boufarik, Algeria

³ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Since the early cases of tristeza disease reported in the Algerian citrus orchards, probably introduced with infected budwood from abroad, the disease has never shown particular problems for the local citriculture. In the past, the disease was monitored only based on symptoms observations. Recently, the use of biological indexing and serological assays have contributed to detecting other CTV infected trees. Management policy and strategies for disease control and quarantine were undertaken in order to avoid the introduction or spread of the disease in citrus orchards.

Keywords. Algeria – Citrus – Satsuma – Tristeza.

Aperçu historique du virus de la tristeza des agrumes en Algérie

Résumé. Depuis la détection des premiers cas d'infection par la tristeza en Algérie, probablement causés par l'introduction de matériel de multiplication infecté de l'étranger, la maladie n'a pas posé de problèmes majeurs pour l'agrumiculture locale. Par le passé, la tristeza faisait l'objet d'un suivi exclusivement basé sur l'observation des symptômes. Récemment, l'utilisation de l'indexage biologique et des techniques sérologiques a contribué à déceler d'autres arbres infectés par le CTV. Des politiques et des stratégies de gestion et lutte et des mesures de quarantaine ont été adoptées afin d'éviter l'introduction et la diffusion de cette maladie dans les vergers agrumicoles.

Mots-clés. Algérie – Agrumes – Satsuma – Tristeza.

I – Introduction

For centuries, citriculture has been considered as the most important fruit crop sector in Algeria and part of its traditional agriculture. Before the French colonisation (1830), more than 22 000 citrus trees, mainly orange trees, were already grown in the Mitidja area.

Until the end of the 2nd world war, the Algerian citriculture was considered as one of the most important in the Mediterranean basin and showed fluctuations in production, with a positive peak in 1950 (Rebours, 1950).

At that time, mother blocks were already established by the growers and the relative trees were subjected to varietal and sanitary assessment ensuring the true-to-type of the selected clones and the absence of virus and virus-like pathogens (Rebours, 1950).

Unfortunately, due to an inadequate reorganization in the 1970s, the cultivated area and the citrus production have considerably decreased over the last twenty-five years from 450,000 tonnes (1974) to less than 250,000 tonnes (2000).

Algeria has always been aware of the problems of virus diseases that damage the citrus orchards, adopting a continuous monitoring of the propagative material of the different nurseries and the use of resistant and tolerant rootstocks (Taleb, 1974).

Currently, citrus orchards cover an area of 45,040 ha, or 0.6% of the agricultural land. This crop is located along the coastal zones mostly concentrated in the Mitidja area (44%). The production is intended for local consumption; the citrus orchards are mostly of the major orange and mandarin varieties, while most plantations are grafted onto sour orange.

The biggest mother block is situated in the Beni Tamou farm Institut Technique d'Arboriculture Fruitière (ITAF) which was created during the 80s from plant material of the ITAF Boufarik which has a collection of 256 varieties and clones. Regional mother blocks are also available introduced by the French from the INRA Corsican station (ITAF, 2003).

Particular emphasis was given to the sanitary status of the propagated citrus varieties in Algeria; the decline of this crop in Algeria was the result of several constraints including the aging of the orchard, the low turnover rate of plantations, inadequate care of the crop, water stress observed over the past decade and poor health status (Bové, 1995). About the health status, many fungal, bacterial, virus or mycoplasma diseases, which have led to a decline in quality and quantity, were reported.

For over 40 years, the existence of viral diseases was and still is among the factors which have promoted the decline of this crop in Algeria (Bové, 1995).

The first virological control of the mother block was made at the time of its establishment by the *Institut Technique d'Arboriculture Fruitière* (ITAF) in conjunction with the French research center (CIRAD).

II – CTV situation

Since 1948, quick decline probably associated to the CTV has been reported in some commercial groves; particular attention was given to these declining trees.

It seems that all the early cases of tristeza found in the citrus-growing areas of the Mediterranean Basin can be traced back to the introduction of infected budwood from abroad. All countries included Algeria, which have introduced the Meyer lemon variety, have also introduced tristeza (Bové, 1966).

In fact, between 1955 and 1957, a number of Meyer lemon trees in Algeria were carrying *Citrus tristeza virus* (Frezal, 1957). No natural propagation of the disease was observed at that time, except for bud propagation. The eradication of Meyer lemon and the characteristics of the virus strain showed a low risk of spread of this disease.

Moreover, other varieties imported from Australia, South Africa, Japan and the United States of America have also been reported to have introduced tristeza into the Mediterranean countries (Bové, 1967).

Later, Farraj and Omar (1969) suspected the presence of tristeza disease on 30 trees, which was confirmed later by the Mission COFROR in 1971 (Anonymous, 1971). During a technical visit to Algeria, French researchers found indications that tristeza virus is present in some citrus trees in the Mitidja area. The trees affected were two "Marsh" seedless grapefruits and two Salustiana oranges budded on sour orange, about 22 years old. The "Marsh" seedless grapefruit displayed a foliage of normal green colour, but with an overgrowth of the scion trunk and typical honeycombing below bud union. The "Salustiana" orange trees were stunted with twig dieback, trunk overgrowth above bud union and honey combing in the sour orange trunk (Bové, 1995).

During a visit to a large state-owned citrus orchard in Massouma, 40 sweet orange trees probably of "Verna Pereta" were found showing dieback, leaf vein yellowing and wilting, while in some trees, honey combing was observed in the trunk. In the same orchard, about 500 m away, another

group of about ten declining trees of Clementine mandarin and Pereta orange, budded on sour orange rootstocks, were observed. All the trees were over 30 years old, some were dying and few were already dead. The cause of the abnormality was not determined, but it was suggested that ELISA should be used to index the trees.

In 1967, Bové, on a mission in Algeria, found tristeza in some trees of the varietal collection (Owari Satsuma, Tangerine) and some clementine trees belonging to the same collection of the experimental station of Boufarik.

The opinion was formulated on the fact that the disease is not spreading, while the Meyer lemon and Satsumas are not conducive to the dissemination of the infection source and are insufficient to allow diffusion with the vector *Aphis gossipii* (Taleb, 1974). During the 1970s on the request of the Algerian government, some visits and studies on the health status of citrus were also conducted by French experts who were familiar with the citrus situation in Algeria. Solutions and some recommendations were provided to better manage the situation; since then the plants have only worsened.

In 1982, the French expert Bové detected two trees showing CTV symptoms in the citrus varietal collection of Boufarik. These trees have been confirmed by biological indexing and destroyed; since then and till 2000, no symptoms of CTV have been observed in the citrus mother blocks and in the citrus orchards (ITAF, 2003).

In 2001, the ITAF reported again the presence of CTV in 11 mandarin and sweet orange trees belonging to the mother trees maintained on Beni Tamou farm. The positive trees were pulled out, strict and preventive measures were taken by national technical institutions and precautionary measures strengthened in the field (ITAF, 2003; EPPO, 2003).

III – Control

The complete elimination of Meyer lemon, the absence of the main vector *Toxoptera citricidus* and of natural transmission by other aphid species, have probably removed the risk of spreading the disease in Algeria. However, CTV was and is still a major problem and a danger to this crop, mainly because surveys for this disease have never been performed in commercial orchards.

In the late 80s, the Centre National de Contrôle et Certification (CNCC) and ITAF started to control the mother plants belonging to the multiplication plot; however, during the 90s and given the political situation prevailing in the country, the activities had been limited at all levels.

Since the introduction of *T. citricidus* in Portugal and the appearance of new outbreak of tristeza in some other Mediterranean countries, Algeria has adopted a new technical guidance of its citrus fruit such as the selection of healthy local varieties, the importation of disease-free varieties and the adoption of new tristeza-tolerant rootstocks: Cleopatra mandarin, *Poncirus trifoliata*.

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State of the art of *Citrus tristeza virus* (CTV) in Egypt

Fahmy H.¹, Salama E.S.², D'Onghia A.M.³

¹ Certification Center Bahtem, Cairo, Egypt

² Horticulture Research Institute, ARC, Giza Egypt

³ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Citrus industry is the major fruit crop in Egypt; *Citrus tristeza virus* (CTV) was reported in the country in 1957, where 4 trees were found to be infected. In 2000, a large-scale CTV survey was carried out, reporting several other CTV outbreaks, in different governorates. Preliminary data based on CTV molecular characterization suggest that some CTV isolates are very similar to the severe strain from Florida, that causes quick decline and stem pitting. In contrast, the seedling yellows component seems not be present. The possible entrance of *Toxoptera citricidus* and the use of a CTV susceptible rootstock (sour orange) pose today a major threat to the Egyptian citrus industry.

Keywords. Citrus – Citrus tristeza virus – Egypt – Sour orange.

Le CTV en Egypte: état de l'art

Résumé. Les agrumes constituent la principale culture fruitière en Egypte ; le virus de la tristeza des agrumes (CTV) a été signalé pour la première fois en 1957 dans ce pays, où quatre arbres étaient infectés. En 2000, une enquête sur le CTV a été réalisée sur une grande échelle, permettant de déceler plusieurs autres foyers d'infection dans de nombreux gouvernorats. Les données préliminaires, basées sur la caractérisation moléculaire du CTV, indiquent une similitude entre certains isolats de CTV et la souche sévère connue en Floride, induisant le quick decline et le stem pitting. En revanche, la souche qui cause le seedling yellows semble ne pas être présente. La possible introduction de *Toxoptera citricidus* et l'utilisation d'un porte-greffe sensible au CTV (le bigaradier) semblent représenter une menace considérable pour l'agrumiculture égyptienne.

Mots-clés. Agrumes – Virus de la tristeza des agrumes – Egypte – Bigaradier.

I – Introduction

Citrus industry is the major fruit crop in Egypt. It covers about 380,000 acres along the Delta of the Nile region and the new cultivated areas (new modern farms in desert areas), with a total production of 3,200,000 tons and export about 796,000 tons in 2007.

Several graft-transmissible pathogens were reported in Egypt, including citrus psorosis, stubborn, citrus variegation, concave gum, citrus tristeza, citrus exocortis and citrus cachexia. Among all of them, still citrus tristeza is considered the main threat to the Egyptian citriculture,

Sweet orange (*Citrus sinensis* L.) grafted on sour orange (*C. aurantium* L.) rootstock, represents the first citrus growing combination. Although the absence of natural spreading and the main vector *Toxoptera citricidus*, the CTV infected trees were uprooted.

In 2000, the Egyptian Ministry of Agriculture and Land Reclamation (MALR) established a program for citrus development and certification in cooperation with the the German Cooperation (GTZ) and the technical support from CIHEAM- Mediterranean Agronomic Institute of Bari (MAIB). Infrastructure have been established in Bahtem area that includes conservation and premultiplication facilities besides diagnostic units (serology , molecular techniques). A Ministerial

decree that organizes the production of certified material and requirements through the nurseries, has been issued. The CTV monitoring was also included.

II – Historical review of citrus tristeza in Egypt

In **1957**, CTV presence in Egypt was reported for the first time by Nour-Eldin and Bishay (1958). Four trees, belonging to a varietal collection of the MALR at Kanater, were found infected after indexing. Two of them were Bergamot orange grafted on sour lime rootstock, the third was a Tenerife orange grafted on sour lime rootstock, while the fourth was a Valencia orange.

All were indexed onto Mexican lime and Baladi lime, which developed vein clearing symptoms, typical of CTV (Nour-Eldin, 1957; Nour-Eldin *et al.*, 1958).

Later, another four trees were discovered in two varietal collection farms at Giza and also on the same farm of the first report; the CTV infected trees were eradicated.

In **1961**, Knorr detected 41 trees showing CTV symptoms at Giza and Kanater and these trees were uprooted (Knorr, 1961).

In **1984**, four CTV infected trees were discovered at the Horticulture Research Institute - Agricultural Research Center (HRI-ARC, Giza), three of them being sweet orange (Thompson navel, Tenerife and Clean William), the fourth a Cleopatra mandarin. All these trees were grafted on sour orange and showed typical symptoms of honeycombing below the bud union (Eid *et al.*, 1984a).

The same authors reported other 33 infected trees in Sharkia Governorate where they described symptoms of two other types of stem pitting on Navel sweet orange trees grafted on sour orange rootstocks. In the first type, pegs and pits were observed only on rootstock, whereas in the second type the pitting extended to the Navel scion (Eid *et al.*, 1984b).

In **1990**, CTV infected trees were found in Anshas and Beni Suef regions, on Navel orange and Valencia (Abou-Zeid *et al.*, 1990). All infected trees showed typical symptoms. However, CTV-infected trees were not found in any commercial orchards or among any of the varieties of local origin (Eid *et al.*, 1984a; Bové, 1995).

In **2000**, by using ELISA tests for the first time in a large scale, 43 CTV infected trees were found in the varietal collections of the Horticultural Research Institute, Giza and Kourashia varietal collection farm (Ministry of Agriculture) on Navel orange and Valencia (Fahmy, 2000; D'Onghia, 2001). The infected trees were symptomless but the results were confirmed by indexing to Mexican lime. Vein clearing and leaf cupping symptoms appeared about 25 days after inoculation. None of the assayed CTV sources induced symptoms (stem pitting, stunting or yellowing) on Duncan grapefruit, Madam vinous orange and Sour orange seedlings. These sources apparently show differences from other CTV sources characterized in the previous work by Abdel Salam (1998), which induced stem pitting in sour orange. Preliminary data suggest that seedling-yellows component is not present in the Egyptian sources.

In **2002**, in the framework of the project "Improvement of the citrus production in Egypt" sponsored by GTZ, 13000 plants were tested for CTV from which 2% were found infected (Cambra, 2002).

In **2006**, CTV characterization was completed (Amin *et al.*, 2006). Three Egyptian isolates of CTV were collected at 2 locations from rough lemon trees (*C. jambhiri*) grafted on sour orange, showing symptoms of decline. The capsid protein gene was amplified by RT-PCR, and analysed by SSCP (single stranded conformation polymorphism) and coat protein gene sequencing.

Comparison with reference sequences (of isolates coming from other parts of the world) showed that the Egyptian isolates were very similar to a severe strain from Florida which causes quick

decline and stem pitting. These results suggested that isolates causing severe quick decline are present in Egypt.

In the same year, a complete nucleotide sequence of the Qaha isolate with 19296nt was submitted to the genebank (Abdelmaksoud, and Gamal El-din, unpublished) under the accession number AY340974.

III – Conclusion

Citrus tristeza is still a major threat to the citrus industry in Egypt although an epidemic has not been reported and only very small foci have been discovered. The presence of *A. gossypii* and sweet orange grafted on sour orange could change the situation in the future as happened in other countries around. It is therefore clear that strengthening the quarantine measures, surveying coupled to an eradication program besides exchanging information with other countries which face a similar situation is the only way to prevent the threat of citrus tristeza disease.

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Occurrence and distribution of *Citrus tristeza virus* (CTV) and its vectors in Syria

Abou Kubaa R.¹, Addante R.², Jamal M.³, D'Onghia A.M.¹

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

² Dipartimento di Biologia e Chimica Agro-forestale e Ambientale, University of Bari, Italy

³ General Commission for Scientific Agricultural Research, Damascus, Syria.

(present address: International Center for Agricultural Research in the Dry Areas, Aleppo, Syria)

Abstract. A survey of *Citrus tristeza virus* was carried out in the main Syrian citrus-growing areas of Lattakia and Tartous by *Direct Tissue Print Immunobinding Assay* (DTBIA) in 2006. Several citrus nurseries, budwood source fields and groves of the main citrus varieties were visually inspected and samples collected for laboratory analysis. 3.5% of the tested plants, coming from two nurseries, two budwood source fields and six groves, were found CTV-infected. Partial characterization of CTV local isolates was carried out using biological indexing and serological assays. Aphids monitoring in the same areas, showed a population diversity, but no evidence of the presence of *Toxoptera citricidus*. CTV was widely reported in Syria, but apparently, the virus has not been spread by the vectors.

Keywords. Aphids – Citrus – DTBIA – Syria – Tristeza.

Presence et distribution du virus de la tristeza des agrumes et de ses vecteurs en Syrie

Résumé. En 2006, une enquête a été menée pour évaluer la présence du virus de la tristeza des agrumes dans les principales régions agrumicoles de la Syrie, à savoir les régions de Lattakia et de Tartous, en utilisant la technique du *Direct Tissue Print Immunobinding Assay* (DTBIA). Un certain nombre de pépinières d'agrumes, de parcs à bois, et de vergers, où sont cultivées les principales variétés d'agrumes, ont été soumis à des observations visuelles et des échantillons ont été prélevés pour des analyses au laboratoire. 3,5 % des plants testés, provenant de deux pépinières, deux parcs à bois et six vergers, étaient infectés par le CTV. Une caractérisation partielle des isolats locaux du CTV a été réalisée à travers l'indexage biologique et des essais sérologiques. Dans les mêmes zones, les pucerons ont fait l'objet d'un suivi qui a permis de révéler une diversité de la population, sans pour autant confirmer la présence du *Toxoptera citricidus*. Le CTV a été signalé en Syrie sur une grande échelle, mais apparemment, le virus n'a pas été disséminé par les vecteurs.

Mots-clés. Pucerons – Agrumes – DTBIA – Syrie – Tristeza.

I – Introduction

Citrus, one of the main fruit crops of Syria, is distributed throughout the country on a surface of about 30,000 hectares with a total production of 850,000 tons. The majority of citrus groves and nurseries are located in the coastal region (Lattakia and Tartous). More than 95% of citrus species are grafted on sour orange (*Citrus aurantium* L.), the most represented being sweet oranges (Navels, Valencia and Jaffa), followed by mandarin, lemon and grapefruit.

II – Tristeza historical events in Syria

Citrus tristeza virus (CTV), an aphid-transmitted closterovirus, is the most economically important viral disease of citrus worldwide which has destroyed millions of citrus trees throughout the world, mainly where sour orange was the rootstock. CTV foci have been found in all Mediterranean countries, mostly as isolated foci and without showing clear-cut symptoms (Bové, 1995; Djelouah and D'Onghia, 2001). It is likely that CTV can be present in Syria, too. Nevertheless, the occurrence

of CTV in the neighbouring countries in Turkey (Norman, 1963) and in Lebanon (D'Onghia *et al.*, 1988) represents a serious threat to the Syrian citrus industry.

In fact, few data are available on the presence of the main virus and virus-like diseases which are only based on visual observations (Bové, 1995); moreover, no surveys for the monitoring of CTV and its vectors were conducted before the present one; some limited surveys were done annually by citrus board in the country using some traditional methods. Therefore, we realized that there is a need to enforce this activity together with a rapid eradication of the infected trees and of *T. citricidus* if soon identified. The surveys were conducted by IAMB jointly with the cooperation of Citrus board and the General Commission for Scientific Agricultural Research (GCSAR) in Syria.

In autumn 2005 and in spring 2006, nationwide surveys were conducted to determine the incidence of CTV and relative vectors in different citrus-growing regions including eight nurseries, 2 budwood source fields and 19 groves.

A total of 1,055 plants from the nurseries, 1,134 trees in 13 selected areas, and 464 trees from budwood sources fields were sampled. Sampling in the fields was done according to the hierarchical method of Gottwald and Hughes (2000).

All collected samples were analyzed by DTBIA for the detection of CTV using stems and leaf petioles in the nursery survey (Bar Joseph *et al.*, 1979; Cambra *et al.*, 2000), whole flower explants (Djelouah *et al.*, 2002) in the grove survey. The CTV positive samples were confirmed using biological indexing. Serological characterization following DAS and TAS-ELISA protocols (Bar Joseph *et al.*, 1979) was applied to 10 CTV sources for their geographical distribution and as a represented variety; two sweet orange CTV sources of Lebanese origin were also included in this study.

Monitoring for aphids was carried out in 18 citrus groves and in 2 nurseries in order to identify aphid species living on citrus. About 15 trees/grove were selected randomly on the diagonals of the fields and two infested shoots/tree (when present) were collected every three weeks, choosing them at different height and orientation. Then nine aphids per each grove were singly mounted on slides and were identified using keys developed by Blackman and Eastop (2000), Heie (1980, 1986), and Stoetzel (1994).

As shown in Table 1, about 3.5 % of the trees tested (of 2653) were found to be CTV-positive (3,5 %), 76 from commercial groves and 13 from nurseries: Overall, in the field, sweet orange proved to be the most infested showing an infection rate of 9,1 %, in Valencia, 6.8 % in Jaffa and 3,6 in Navel; in the nursery the highest infection rate was found in Navel orange (5,4 %). 12 Navel oranges were collected in a single nursery (Alhannadi) in Lattakia, 1 clementine was found in a private nursery in Tartous, 60 samples were detected in 6 groves located in Tartous (mainly in the Southern part of the Governorate), whereas the remaining 16 were from the 2 fields used as budwood sources in Lattakia, which were labelled, and eradicated in the year 2007 to avoid using them as an infected CTV sources - in these two budwood sources - for propagation materials to be sold.

Most of the infected trees were symptomless, whereas the others showed a general dieback and stunting, but no evidence of clear-cut tristeza symptoms was observed. CTV indexed sources showed vein clearing in Mexican lime two months after inoculation. Few of them induced leaf cupping or stem pitting or general stunting. Only one Valencia source showed all the above mentioned symptoms in moderate and severe form. The result of serological characterization evidenced a great variability between the tested CTV isolates, while it showed a close correlation with the Lebanese ones.

The green citrus aphid, *A. spiraecola*, was found to be the dominant species in the citrus orchards in the study area, representing 50.0 % of the total number of identified aphids, followed by

A. gossypii, with 27.3 %. *T. aurantii*, the black citrus aphid, ranked third with 20.3 %, whereas *Aphis fabae* (Scopoli) represented only 2.3 % of identified aphids.

III – Conclusions

This study reported the presence and wide distribution of CTV in Syria, but fortunately an outbreak of the disease has not occurred yet. The only symptoms observed are associated to a general dieback and stunting of some infected trees, but no evidence of clear-cut tristeza symptoms (i.e. inverse stem pitting on Sour orange and necrosis at the bud union).

The presence of CTV in the budwood sources of Navel orange as well as in the nursery plants of the same variety highlights that apparently the infection has been spread by infected material. This variety has been also found infected in commercial groves located in Tartous, but not in Lattakia where infected propagating material was detected.

In the case of the Navels it is known that all budwood sources of the tested field were imported from the French programme several years ago, whereas a Lebanese origin was assessed for the infected Clementine plant tested in the nursery. It is generally known that most of the citrus species grown in Syria were introduced from other 'certification programmes' in the seventies and eighties, when virus detection was not so accurate.

Knowing that the infected field of Navel budwood sources is located in an area where no CTV was detected, that this field is 25 years old, and that most of the citrus propagating materials produced in Syria are sold in Lebanon, where the infection was reported since 1998, it is likely that CTV infection has been present in Syria for several years.

From the results obtained in the identification of citrus aphid populations, *T. citricidus* is not present and *A. spiraecola* is the most spread aphid species, followed by *A. gossypii*. The latter is known to be responsible for tristeza outbreaks in Spain and apparently in Italy, too. Fortunately, *A. spiraecola* is a low efficient virus vector, therefore it could be assumed that the low virus incidence reported (3.5%), which is affecting most of citrus species and varieties of different age, located in different areas, could be due to a slow virus spread by this or other aphid species.

Considering the socio-economic and environmental importance of a tristeza outbreak in the Syrian citriculture and the difficulty to manage the severe strains of the virus, once they get established, the Syrian Government should soon adopt urgent measures to continue such an activity by law establishing an annual programme for the mandatory control of CTV and its main vectors. The production of CTV-tested plants should be part of this programme and it will represent the first step toward the establishment of a certification system of citrus propagating materials aimed at controlling all citrus graft-transmissible pathogens.

Table 1. CTV situation in nurseries and commercial groves.

Species	Inspected trees		
	N. tested	N. infected	% infected
GROVES			
Sweet orange (<i>Citrus sinensis</i>)			
<i>Navel</i>	663	24	3.6
<i>Valencia</i>	165	15	9.1
<i>Jaffa</i>	204	14	6.8
Total	1032	53	5.1
Mandarin-like			
<i>Common clementine (<i>Citrus reticulata</i>)</i>	233	2	0.8
<i>Ortanique (<i>Citrus reticulata</i> X <i>C. paradisi</i>)</i>	3	2	66.6
<i>Satsuma (<i>Citrus Unshiu</i>)</i>	108		
Total	344	4	1.2
Meyer lemon (<i>C. meyerii</i>)	24	4	16.6
Grapefruit (<i>C. paradisi</i>)	166	14	8.4
Pomelo (<i>C. grandis</i>)	24		
Kumquat (<i>Fortunella margarita</i>)	7		
Sour orange (<i>C. aurantium</i>)	1	1	100.0
Total	222	19	8.6
Total groves	1598	76	4.7
NURSERIES			
Sweet orange			
<i>Navel</i>	220	12	5.4
<i>Valencia</i>	181		
<i>Jaffa</i>	105		
Total	506	12	2.3
Meyer lemon	101		
Interdonato lemon (<i>C. limon</i>)	55		
Total	156		
Common clementine (<i>C. reticulata</i>)	152	1	0.6
Grapefruit	84		
Pomelo	30		
Sour orange	38		
Others	89		
Total	393	1	0.2
Total nurseries	1055	13	1.2
TOTAL GROVES AND NURSERIES	2653	89	3.5

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Historical review on *Citrus tristeza virus* (CTV) in Palestine

Djelouah K., D'Onghia A.M.

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Citrus is an important crop in Palestine. Citrus groves are mainly concentrated in the West Bank and Gaza strip. Given the similar conditions of citrus industry across the Middle East area (Jordan and Israel), CTV is thought to have been introduced through infected plants imported from the neighbouring countries or through viruliferous aphids. Recent surveys have demonstrated that today CTV poses a major threat to the Palestinian citrus industry.

Keywords. Citrus – CTV – Palestine – West Bank.

Bref historique du virus de la tristeza des agrumes en Palestine

Résumé. Les agrumes représentent une culture importante en Palestine. Les verges d'agrumes sont pour la plupart concentrés en Cisjordanie et dans la Bande de Gaza. Vue que l'agrumiculture présente des caractéristiques similaires dans toute la région du Moyen-Orient (Jordanie, Israël), fort probablement, le CTV a été introduit en Palestine à travers l'importation de plants infectés des pays voisins ou à travers des pucerons virulifères. Des enquêtes récentes ont démontré qu'actuellement, le CTV constitue une menace considérable pour l'agrumiculture palestinienne.

Mots-clés. Agrumes – CTV – Palestine – Cisjordanie.

I – Introduction

Despite the small geographical area covered, Palestine is characterised by a great diversity in topography and altitude, as it occurs in the West bank, where the altitude ranges between 1020 meters above sea level in the mountains and 375 meters below sea level, in the Jordan valley. Such a variation makes it possible to cultivate fruit crops.

Fruit trees represent the main production in the West bank (106 thousand hectares). Citrus covers more than 7000 ha in the West bank and Gaza strip. It is widespread in the coastal area in Gaza, Qalqilia, Talkarem and Jericho thanks to the water availability and the suitable climate.

In 2005, the production was evaluated to 758 thousand Metric tons (PCBS 2004/2005) and the most famous varieties grown in the country are 'Shamouti', 'Fransawi', 'Dam Alzeghloul' sweet oranges, Palestinian sweet lime and the local cultivar Youssef afandi.

This crop is facing serious problems such as the lack of water resources, marketing constraints, the tree age as well as the bad sanitary status of the plants; the latter is not completely unknown given the similarity with the citrus industry conditions in the neighbouring countries (Jordan, Israel), which have thoroughly been investigated.

II – Citrus tristeza virus

The first record of CTV in Palestine was officially reported by Jarrar *et al.* (2000); the infected trees were found in the Western West Bank at the Israeli border but no infection was assessed in the Eastern zone.

A total of 154 samples belonging to different varieties and species were collected from the Eastern and Western areas of the Palestinian West Bank. These samples were grafted onto sour orange, all the sources were maintained at the CIHEAM-MAIB lab facilities, and analysed by Direct tissue blot immunoassay (DTBIA) (Garnsey *et al.*, 1993).

These samples were also grafted onto Mexican lime and sweet orange and maintained in a conditioned greenhouse as reported by Roistacher (1991). Later, observations were made also by electron microscopy on negatively stained grids of virus particles from concentrated partially purified extracts obtained through cortical scrapings of some samples as described by Milne, (1993).

Twenty-two samples out of 154 trees proved to be infected with CTV, and showed a clear-cut reaction on the nitrocellulose membrane. All CTV-infected samples were collected from the Western area of the West bank; sweet orange showed the highest level of infection followed by mandarins and lemons (Fig. 1).

The incidence of CTV infection in sweet orange indicated that 'Valencia' was the most infected variety in the area surveyed, followed by 'Shamouti' and 'W. navel', whereas the local orange 'Fransawi' was virtually free from CTV.

Three weeks after grafting, the new flushes of Mexican lime seedlings, inoculated with CTV-infected plants, displayed strong vein clearing and cupping of the leaves; 3 months later, stem pitting was observed in some Mexican limes, and, interestingly, also on sweet orange. In addition, CTV-like particles were observed under the electron microscope.

CTV was identified in the samples from Western West bank bordering Israel and none of the samples surveyed coming from Eastern West bank was found to be CTV positive, thus confirming the presence and distribution of this virus in the western area (Jarrar *et al.*, 2000). It is likely that CTV entered this area through infected plants imported from neighbouring countries or through viruliferous aphids or in both manners. Relatively to the Eastern zone, no data have been recently reported.

It is difficult to clearly determine the presence of CTV decline in the field and as most of the Palestinian citrus industry is on sour orange rootstocks, CTV represents a serious threat.

At present, no legislation has been issued for the mandatory control of citrus tristeza virus and for the certification of citrus propagating material. Accordingly, an immediate mandatory eradication program is needed in view of the proposed establishment of a citrus certification program for Palestine.

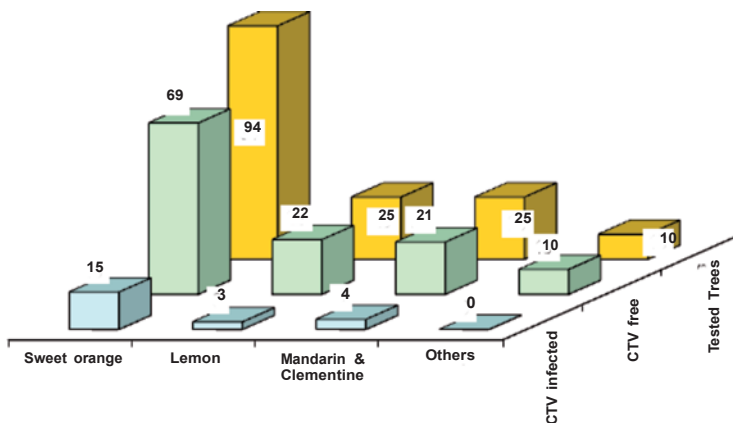


Figure 1. Distribution of CTV infected trees.

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Current status of *Citrus tristeza virus* in Lebanon

Choueiri E.

Department of Plant Protection, Lebanese Agricultural Research Institute, Tal Amara, Lebanon

Abstract. This paper summarizes the field status of citrus tristeza virus (CTV) in Lebanon. Based on the first virus survey conducted in citrus commercial groves and nurseries in autumn of 1996, CTV was reported officially in Lebanon with an overall infection rate of 1.43%. Another survey was undertaken between 1998 and 2000 in Mount Lebanon in addition to the adoption of four experimental plots in the South to assess the CTV incidence. CTV was only detected in the South with an increased infection rate from 1.2 to 3.8% in plot II, and from 2 to 5% in plot III whereas the CTV infection rate in plot I was stable (1.1%) with no concomitant infection in plot IV. The vectors *Aphis gossypii* and *Aphis citricola* were identified whereas *Toxoptera citricidus*, the most efficient CTV vector, was not encountered. With the aim to establishing a citrus mother block for the production of plant certified material, another field survey was carried out between 2003 and 2004 in the South and North of Lebanon close to LARI stations and CTV was found only in the South. Interestingly, no evidence of the disease was observed in the CTV-infected trees as ascertained by repeated observations in the field. Further work and preventive measures should be applied to secure the future of the citrus industry in Lebanon.

Keywords. Citrus – CTV – Lebanon – Vectors.

Situation actuelle du virus de la tristeza au Liban

Résumé. Cette étude résume l'état du virus de la Tristeza des agrumes (CTV) au Liban. Sur la base d'une première prospection menée en automne 1996 dans les vergers commerciaux et pépinières des agrumes, le CTV a été signalé officiellement au Liban avec un pourcentage d'infection égal à 1.43 %. Une autre enquête a été réalisée entre 1998 et 2000 au Mont Liban, ainsi que dans quatre parcelles expérimentales au Sud afin d'estimer l'incidence du CTV. Le virus a été détecté uniquement au Sud où le pourcentage d'infection a évolué durant les trois années d'expérimentation en passant de 1,2 à 3,8 % dans la parcelle II, et de 2 à 5 % dans la parcelle III, tandis que le pourcentage d'infection dans la parcelle I est resté stable (1,1 %). Aucune infection n'a été signalée dans la parcelle IV. Les vecteurs *Aphis gossypii* et *Aphis citricola* ont été identifiés, tandis que *Toxoptera citricidus* n'a pas été retrouvé. Dans le but d'établir une parcelle de pieds mères destinée à la production de matériel certifié, une autre prospection a été effectuée aux alentours des stations de l'IRAL au Sud et au Nord du pays. Le virus a été trouvé seulement au Sud. Par ailleurs, les symptômes typiques n'ont pas été observés chez les arbres infectés. Des mesures préventives sont à appliquer dans l'avenir afin de préserver l'agrumiculture au Liban.

Mots-clés. Agrumes – CTV – Liban – Vecteurs.

I – Introduction

With an area of about 16,940 ha, representing 6.3% of the total agricultural area, citrus ranks second among fruit trees grown in Lebanon (Anonymous, 2005). The average annual production is estimated at 395,000 tons (Anonymous, 2005). The main citrus growing areas in the country are located primarily in the South and North along the coastal area. Currently, Washington Navel, Valencia and Shamouti sweet oranges, lemons, grapefruits, and mandarins are the main species grown in Lebanon. Little is known about the phytosanitary status of these species with respect to virus and virus-like diseases, the only available information stems from a limited survey conducted in 1996 (Saade, 1997; D'Onghia *et al.*, 1998). The lack of a certification program in the past, the exchange of citrus plant material of unknown sanitary status, and the lack of phytosanitary measures increased the incidence of viral problems, in particular the risk of citrus tristeza diffusion due to the alarming presence of CTV in Israel and recently in Syria (Abou Kubaa, 2006). This

situation urged local investigators to assess the occurrence of citrus tristeza virus in Lebanon. The current report summarizes the tristeza virus historical events in Lebanon.

II – Tristeza virus historical events in Lebanon

Citrus tristeza virus (CTV) is one of the major graft-transmissible pathogens limiting profitable citrus production worldwide (Garnsey and Lee, 1988; Bar-Joseph and Lee, 1989; Bar-Joseph *et al.*, 1989). It causes a decline of citrus on sour orange (*Citrus aurantium*) rootstock (CTV-decline) and stem pitting of many cultivars regardless of the rootstock. In Lebanon, more than 90% of citrus species are grafted onto sour orange; for this reason, a first investigation was carried out in 1996 to evaluate the incidence and distribution of CTV throughout the citrus-growing areas of the country (Saade, 1997).

1996. In autumn 1996 field surveys were carried out in commercial citrus groves and nurseries of the main Lebanese citrus-growing areas that included the districts of Akkar and Tripoli in the North and Wasta, Saida, Ghazieyyeh, Najjarieyyeh, Aakaibeh, Gibehit, Addousiyeh, and Maamoura in the South. A total of 3427 samples from the orchards and other 1110 samples from the nurseries were collected and tested. The number of samples from each region was proportional to the area cultivated with citrus and to the economic importance of the species or varieties. DAS-ELISA was used for the detection of CTV (Clark and Adams, 1997). With respect to the groves, 62 samples representing 1.8% were infected with CTV whereas the virus was detected in most of the species monitored. The highest incidence of CTV was 2.4% in mandarin and mandarin-like (*C. reticulata*) species with a high prevalence in Ortanique variety (19%) followed by sweet orange (*C. sinensis*) species (2.3%) with the highest infection in Washington navel variety (5.3%). However, lemon (*C. limon*) had a low infection incidence of about 0.6% and that of Kumquat (*Fortunella margarita*) was 0.3% (D'Onghia *et al.*, 1998). For the nurseries, the incidence of infection was lower than that found in the groves (0.3%) and the species with the highest infection was mandarin-like (0.46%) followed by lemon (0.4%) trees (D'Onghia *et al.*, 1998). CTV was not detected in grapefruit, pummelo and lime species. Although grafted onto sour orange, none of the CTV infected trees showed decline symptoms. Moreover, different CTV sources were graft-inoculated for biological characterization to seedlings of Mexican lime.

1998-2000. In 1998, field surveys were undertaken in Mount Lebanon Iklim AlKharoub, Chouf and a total of 300 samples was randomly collected from 6 commercial fields of sweet oranges and mandarins representing 10% of the field trees (Jawhar and Choueiri, 2000). Four to five budsticks were sampled from the four sides of each tree and stored at 4°C until analysis. ELISA test following Clark and Adams (1997) method was used for the detection of CTV. During that year, weekly visits were made to record the symptoms (stem pitting, decline, yellowing, wilting, etc.). Also, a "iodine test" field diagnosis was applied to detect starch depletion in the rootstock below the bud union. No symptoms were observed and all ELISA tests came out to be negative.

In the same year, four experimental citrus groves were selected to assess citrus tristeza virus incidence. Plot I was a grove of Clementine in LARI station, Tyre; plot II was a grove of sweet orange in the same location; plot III was a commercial grove of grapefruit in Tyre region; and plot IV was a commercial grove of sweet orange in Alman region. Each plot consisted of 100 trees in a grove containing 300 trees. Field inspections and collection of samples from all trees were carried out in these plots during three growing seasons (May-September 1998, 1999 and 2000). DAS-ELISA (Clark and Adams, 1997) was used for the detection of CTV. Monitoring of aphids was also taken in consideration. The infection rate of CTV increased from 1.2 to 3.8% in plot II, and from 2 to 5% in plot III during the three years of inspection; however, CTV infection rate was stable in plot I (1.1%) and no infection was encountered in plot IV. Moreover, *Aphis gossypii* and *A. citricola* were identified in all plots (Jawhar and Choueiri, 2000).

2003-2004. In the framework of cooperation between Lebanon and Italy regarding the certification project entitled “Project for the Production and Delivery of Certified Plant Material in Lebanon” and financed by the Italian Government through the Ministry of Foreign Affairs to upgrade the fruit sector, the Department of Plant Protection at Tal Amara station conducted two surveys: one in the south of Lebanon around Tyre station and the other in the North around Abde station in order to assess the presence of CTV and to establish the location of the citrus mother block. Around 500 samples from all locations were collected and tested by direct tissue blot immunoassay (DTBIA) (Garnsey *et al.*, 1993). CTV was detected in the South with an overall infection rate of 1.2%; however, no infection was found in the North (Choueiri, unpublished data). The infected trees found in the South were symptomless. These results prompted the establishment of a citrus mother block in Abde station.

III – Conclusion

The surveys carried out in Lebanon provided a relatively clear picture of the sanitary status of citrus, particularly citrus tristeza virus. An extremely low level of virus infection was detected in citrus samples from commercial groves and nurseries. The infection rate of CTV was low and there was no evidence of spreading although there was a limited increase in the rate of incidence in experimental trials. The most efficient CTV vector, the brown citrus aphid (*Toxoptera citricidus*), was not encountered in Lebanon; however, only *A. gossypii* and *A. citricola* were identified. It seems that the evidence of natural spread by *A. gossypii*, one of the most efficient CTV vectors in the Mediterranean region, is confined to Lebanon. The graft inoculation results and field observations had shown no apparent signs of decline in infected trees assuming that the CTV isolates present in Lebanon were not considered as severe strains. Furthermore, detailed monitoring of CTV is necessary to gather information on its development in Lebanon especially because the possibility of a sudden outbreak cannot be excluded as reported in other countries (Kyriakou *et al.*, 1996) as well as considering that the majority of citrus trees are grafted onto sour orange which is the most sensitive rootstock to CTV. The implementation of a national certification program for fruit trees and for citrus requires preventive measures aiming at the implementation of CTV eradication, at raising the awareness of citrus growers on CTV, on other virus and viroid disease risks, at introducing new rootstocks that are more tolerant or resistant to CTV, and at extending a continuing CTV monitoring survey.

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Tristeza disease and its vectors in Israel: past and current status

Soroker V.¹, Sadovsky A.², Drishpon Y.³, Wise M.⁴

¹ Department of Entomology, Agricultural Research Organization, The Volcani Center, Israel

² Citriculture, Extension Services. Ministry of Agriculture, Israel

³ Department of Entomology, Agricultural Plant Protection, Extension Services. Ministry of Agriculture

⁴ Plant Protection and Inspection Services, Ministry of Agriculture, Israel

Abstract. The historical review of Citrus tristeza findings in Israel is reported since 1956, showing the first outbreaks in 1970 by *Aphis gossypii*. After the failure of the virus eradication programme, tolerant rootstocks were used for the disease control. Nowadays very few declining trees are reported and, apparently, *Toxoptera citricidus* is not present yet. A national regulation provides preventive measures (primarily the use of CTV-free propagating materials) for the control of citrus tristeza.

Keywords. *Aphis gossypii* – Citrus – CTV – Israel – *Toxoptera citricidus*.

La Tristeza et ses vecteurs en Israël: passé et état actuel

Résumé. Une illustration des données historiques de la tristeza des agrumes en Israël à partir de 1956 est présentée, ceci en décrivant les premiers foyers qui ont été signalés en 1970 et qui étaient associés à la présence de l'*Aphis gossypii*. Après l'échec du programme d'éradication du virus, des porte-greffes tolérants ont été utilisés pour lutter contre cette maladie. A l'heure actuelle, on ne signale que quelques arbres en dépérissement et, apparemment, le *Toxoptera citricidus* n'est pas encore présent. La réglementation nationale prévoit des mesures préventives (en premier lieu, l'utilisation du matériel de multiplication indemne de CTV) pour combattre la tristeza des agrumes.

Mots-clés. *Aphis gossypii* – Agrumes – CTV – Israël – *Toxoptera citricidus*.

I – Introduction

Currently, citrus cultivation in Israel is spreading over about 17,500 ha and some additional 2000 ha are planted annually in the country. A wide variety of sweet oranges, grapefruits and lemons, as well as a variety of more exotic citrus fruit are being cultivated; Israel's major citrus product by volume is the traditional Shamouti orange.

II – Historical review of CTV and its vectors in Israel

1956. Tristeza disease, caused by citrus tristeza virus (CTV), was first detected in Israel in 1956 in Meyer lemon and later in some other varieties. The infected trees were eradicated.

1970. *Aphis spiraeicola* was introduced into Israel in the late 1960s. Natural spread of CTV was noted in 1970. An eradication project was set up.

1963 - 1978. An aphid extensive survey in citrus groves of Israel was conducted with the main task to detect the major CTV vector *Toxoptera citricidus*. This aphid was not found (Swirski and Amitai, 1999). Other aphids found to be potential vectors were *A. spiraeicola*, *A. gossypii* and *T. aurantii*. *A. gossypii* was imputed to transmit some strains of CTV in Israel (Raccah *et al.*, 1976).

1986 - 1987. A CTV survey conducted in these years indicated that the eradication project conducted for a period of 16 years had been only partially effective. CTV infestation in the main orange-growing areas, in the coastal plain of the country was found high and widely spread and could no longer be contained by eradication (Lobenstein, 1993). The management efforts were directed to reduce tristeza-induced damages by uprooting the infested trees and by grafting scions onto rootstocks tolerant to CTV. The approach of CTV tolerant rootstocks did not prove valid, since the resistant rootstocks suffered from low water quality, were susceptible to other diseases and some produced lower yield and fruit quality.

Present status. The occurrence of CTV varies considerably. In the Coastal Plain, most of the infected trees that are 7 years old or older are symptomless and only a few cases of rapid decline were reported. In the south and northern parts of the country outbreaks of CTV are not common. No systematic surveys on CTV spread were conducted in the last decade. Moreover, the Israeli growers are well aware of the problem and suspicious plant material is brought for diagnosis at the laboratories of Plant Protection and Inspection Services (PPIS).

A. spiraecola that was introduced into Israel in the late 1960s is not considered an efficient vector, but its population may reach high rates which in turn accelerate CTV spread. *T. aurantii* failed to transmit CTV in Israel.

In the last years no vector survey has been conducted; however, since it was not recovered in the routine orchard inspection and monitoring by the extension service and PPIS, we assume that *T. citricidus* is not present in Israel.

Management of tristeza today is largely based on preventive measures, quarantine control which includes heat treatment and shoot-tip-grafting, production of tristeza-free propagation material at all production stages and on-site inspection. The official regulations of PPIS for the prevention of CTV spread include:

- i. maintenance of all propagation material under insect-proof screenhouse;
- ii. germplasm blocks tested for CTV one by one twice a year by ELISA following ISO17025 protocol, using CTV reagents from BIOREBA, Switzerland, once every four years the infestation is verified through bioassay-indicators using Key lime.
- iii. foundation blocks tested for CTV once a year at random or by suspect.
- iiii. sampling in the nursery once a year before marketing; the sample size depends on the frequency of CTV past events. The infected plants (currently about 1 in 1000) are immediately eradicated. Only certified CTV-free plants are allowed to be planted.

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Citrus tristeza virus and its vectors in Northern Sudan

Moawia E. M.¹, Nagat Mubarak E.²

¹ Agric. Res. Corporation Shambat Res, Khartoum North, Sudan

² Plant Protection General Directorate of the Ministry of Agriculture, Khartoum North, Sudan

Abstract. Most of citrus varieties in Sudan have been imported from other citrus improvement programmes particularly USA and Spain. This germplasm has also been maintained in varietal collections and found CTV-free. *Toxoptera citricidus* is apparently present only in the Southern and Western part of Sudan, whereas in 2005 CTV was firstly reported in the Northern area of Sudan.

Keywords. Citrus – CTV – *Toxoptera citricidus* – Sudan.

Le virus de la Tristeza des agrumes dans le nord du Soudan

Résumé. La plupart des variétés d'agrumes introduites au Soudan proviennent des programmes d'amélioration du matériel végétal réalisés dans d'autres pays, en particulier des Etats-Unis et d'Espagne. Ces ressources phylogénétiques, exemptes du CTV, ont été conservées dans des collections variétales. Le *Toxoptera citricidus* n'est apparemment présent que dans le sud et dans l'ouest du Soudan, alors que le CTV a été signalé pour la première fois en 2005 dans le nord du pays.

Mots-clés. Agrumes – CTV – *Toxoptera citricidus* – Soudan.

I – Introduction

There is a high potential for citrus expansion in Sudan. Grapefruit, lime and mid and late sweet orange varieties perform well under Sudan conditions. The citrus plantings grown in Sudan are mainly of the following species and varieties: Beladi lime, grapefruit, mainly foster pink but also marsh seedless, Valencia-like Beladi sweet orange and willow-leaf mandarin.

Almost all citrus trees commercially grown in Sudan are old lines. These were introduced from Egypt, Palestine, Trinidad, USA, Kenya and Rhodesia (Bové, 1988). Old lines are known to be infected with virus and virus-like diseases (Roistacher, 1991). In the period 1967-1970 diverse introductions occurred from California, USA, but were not cultivated commercially. Most of the trees are on sour orange rootstock while Beladi lime is propagated from seeds. In 1995, a new virus-free budwood collection was introduced from the National Repository of Citrus and Dates of Riverside, California, USA and IVIA, Spain. This collection is kept at Shambat Research Station (Mohamed, 2001). The collection includes introduced citrus varieties (Tab. 1), Troyer and Carrizo citrange rootstocks, Rough lemon, Volkameriana lemon and *Citrus macrophylla* and the standard indicator plants.

Table 1. List of the introduced citrus varieties.

Species	Cultivar	Source	Year
Sweet Orange	Frost Marsh	Riverside, CA	1995
	Shamber	Riverside, CA	1995
	Rays Ruby	Riverside, CA	1995
	Hudson foster	Riverside, CA	1995
	Rio Red	(IVIA, Spain)	2002
	Olinda	Riverside, CA	1995
	Cutter	Riverside, CA	1995
	Campbell	Riverside, CA	1995
	Gillette	Riverside, CA	1995
	Mandarin	Willowleaf	Riverside, CA
Honey		Riverside, CA	1995
Clementine		Riverside, CA	1995
Wilking		Riverside, CA	1995
Kinnow		Riverside, CA	1995
Lime	Bears	(IVIA, Spain)	2002

II – Historical review of CTV and its vectors

1964. Childs (1964) surveyed the disease status of citrus plantings. He mentioned a few suspected CTV infected trees in Northern Sudan. *T. citricidus* was reported by this author in Western and Southern Sudan but not in the North.

1969 - 1983. The previous observations regarding *T. citricidus* were confirmed by Schmutterer (1969) and Krezdron (1983). Schmutterer refers the existence of legislation aiming to prevent the introduction of pests and diseases of crops from foreign countries. Imported plants and fruits are inspected by the plant quarantine service in offices along the borders and in the capital airport. Other laws aiming to reduce the possible sources of infection were established.

1986. Bové (1988) examined several lime trees and found no symptoms of CTV infection. He came to the conclusion that, at the time of his survey, natural spread of CTV was probably not occurring or at least not to a large scale. The reason for this might have been the absence of the tropical citrus aphid *T. citricidus*, in northern Sudan.

1997. A survey by using Immunoprinting test (Garnsey *et al.*, 1993), was carried out in the Gezira State targeting the citrus germplasm introduced in 1995 (Mohamed, 2001). No evidence of CTV infection was found. Later, 3 trees of Foster Pink grapefruit and Nuri 16 clone, a local sweet orange variety, were indexed by grafting onto acid lime. There was no evidence of the disease, as judged by the absence of stem pitting or leaf vein clearing.

2005. More recently, Abubaker *et al.* (2005) reported the presence of CTV in a survey carried out in the northern State. The virus was detected in 13 samples of sweet orange and in one sample of each mandarin and lime by using a mixture of 3DF1 and 3CA5 antibodies (Plantprint Diagnostics, Spain). While, an RT-PCR approach substantiated the presence of CTV in four trees (3 sweet orange and one lime) which had tested positive by serological tests.

Surveys for *T. citricidus* (Dr. Mousa Abdalla and Dr. Ahmed Hassan, Agricultural Research Corporation, Sudan, Personal Communication) showed no evidence of the aphid in central and northern Sudan. *Aphis gossypii*, the cotton aphid, and *A. craccivora* are present in Northern Sudan.

Sweet orange introductions had been received from Kenya and Rhodesia. Citrus in these countries is known to be infected with citrus tristeza virus (Bové, 1988). Hence, some CTV- infected trees of Kenyan or Rhodesian origin might have been propagated in Sudan.

III – Present concerns and suggested activities for the CTV control

The recent report of Abubaker *et al.* (2005) confirming the presence of CTV in Northern Sudan is very alarming because i) sour orange is the main rootstock used in Sudan, ii) acid lime is grown extensively iii) beside *T. citricidus*, other aphid vector species are widely distributed.

The presence of *T. citricidus* in Southern and Western Sudan, which is known for its high adaptability to various climatic conditions and its rapid spread, is also alarming.

Others reported the wide occurrence of citrus exocortis disease in local germplasm, a situation that would not freely allow the use of alternative CTV-tolerant rootstocks to control the disease as is the case with citranges. Every effort should be made to contain the disease and prevent its spreading.

IV – Proposed measures to address the situation of CTV

- i. Delimiting survey to define the extent of CTV spread and distribution. CTV strains typing as early as possible and destruction of infected trees.
- ii. Regular CTV vectors survey and management.
- iii. Strengthening the on-growing virus-free citrus budwood project
- iv. Indexing citrus germplasm, to ensure CTV freedom and conserve the germplasm under protected conditions.
- v. Evaluating an alternative to the sour orange rootstock
- vi. Enforcing quarantine measures and regulations.
- vii. Prohibition of the importation of budwood from countries where CTV occurs and fruits imported should be free from peduncle and leaves,
- viii. Education and extension program to raise the awareness of the danger of CTV to citrus cultivation.
- ix. Establishment of cooperative relations with regional and international citrus improvement and certification networks.

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Historical review of *Citrus tristeza virus* and its vectors in Iran

Delkhosh H. R., Tousi N.

Plant Protection Organization PPO Tehran, Iran

Abstract. This review outlines the importance of citrus industry in Iran where the different species have been grown for many centuries. Emphasis is laid on the introduction of CTV following the import of large amounts of Satsuma varieties from Japan in the 1970s. A number of surveys were carried out from 1978 to 2007 in the main citrus growing areas across the country and a low level of natural CTV transmission was determined. *Aphis gossypii*, *A. citricola*, *A. craccivora*, *Toxoptera aurantii* and *Myzus persicae* proved to be the most common vectors involved in CTV spread. In contrast, *T. citricidus* has never been reported to date.

Key words. Aphids – Citrus – Citrus tristeza virus – ELISA – Iran.

Rétrospective historique du virus de la tristeza des agrumes et de ses vecteurs en Iran

Résumé. Dans cette rétrospective historique, l'accent est mis sur l'importance de l'agrumiculture en Iran, où de nombreuses espèces sont cultivées depuis des siècles. Dans ce pays, le CTV a été introduit à la suite des importations massives de satsuma du Japon au cours des années 70. Plusieurs enquêtes ont été réalisées entre 1978 et 2007 dans les principales régions agrumicoles iraniennes et elles ont permis de mettre en évidence le faible niveau de transmission naturelle du virus. En plus, l'*Aphis gossypii*, l'*A. citricola*, l'*A. craccivora*, le *Toxoptera aurantii* et le *Myzus persicae* se sont avérés être les vecteurs les plus communs du CTV. En revanche, la présence du *T. citricidus* n'a jamais été signalée jusqu'à présent.

Mots-clés. Pucerons – Agrumes – Virus de la tristeza des agrumes – ELISA – Iran.

I – Introduction

Citrus has a long history in Iran. Citron was the first citrus fruit to be cultivated in the country more than 2500 years ago, in the times of Median Empire. Sour orange, lemon and small fruited lime were spread throughout Persia and Near East around AD 1000. Sweet orange, called “Portugal” in many Arab countries as well as in Iran, reached the Persian Gulf ports with Portuguese trade vessels during the middle centuries (Ebrahimi, 1996).

At present, citrus orchards cover an area of 268,000 ha with a production of 4,300,000 tons in three major citrus-growing regions in Iran. The Caspian Sea belt (Golestan, Mazandaran and Gilan provinces 106 000 ha) extends in the north over 400 km from east to west, and lies between the shore of the Caspian Sea (29 m below sea level) and the first slopes of the Alborz mountain range. Various citrus varieties are cultivated, seedy local sweet oranges (*Citrus sinensis* L.) (55%); seedy local orange trees grafted on sour orange rootstock (*C. aurantium* L.) (20%); Washington navel, Thompson navel and blood oranges, all on sour orange, (5%); Satsuma (*C. unshiu* Marc.), Clementine (*C. reticulata* Blanco) and local and imported tangerine (*C. reticulata* x *C. sinensis*), on sour and trifoliate orange (*Poncirus trifoliata* Raf.) rootstock, (15%); and others (5%).

Citrus orchards of the Southern Inland Belt (116 000 ha) are scattered through the low valleys of the southern Zagros mountain range, essentially in the provinces of Khuzestan, Fars and Kerman. The main characteristics of these areas are low annual rainfall (100-300 mm) and excessive heat in summer. The main citrus species and varieties are seedy local sweet orange trees on sour orange, Bakravi or lemon (*C. limon* Burn. f.) rootstocks, (51.8%); small-fruited acid seedling trees

(28.1%); seedy local tangerine trees on Bakravi or lemon rootstock (9.6%); Palestine sweet lime (*C. limettoides* Tan.) cutting trees (8.4%); and others (2.1%).

Bakravi is said to be a local natural hybrid between mandarin (*Citrus reticulata* Blanco) and small-fruited acid lime (*Citrus aurantifolia* Chrism).

About 46 000 ha of citrus are spread along the coasts of the Persian Gulf and the Gulf of Oman named Southern Coastal Belt. The climate is tropical with no frost, and well suited to acid lime, sweet lime and lemon. In this area acid lime represents 90 percent of production. Salinity of water and high pH are the limiting factors.

Until 1918 there was no real "citrus industry" and all citrus varieties were propagated by seed. The heavy losses due to foot rot prompted growers to use the phytophthora-resistant sour orange rootstock, but sweet orange seedling still constitute 60% of all orange trees in the Caspian Sea area. Acid lime tolerates a high pH (8.5) and is more resistant to drought than sour orange; therefore, it is often used as rootstock in the southern citrus-growing area.

In the country local cultivars are still predominant, in spite of many introductions that have been made since 1933. As part of the economic development of Iran, new citrus species and varieties were introduced for the first time into the Caspian Sea area (Ramsar) in 1933, from Turkey, Italy, Lebanon and Palestine. At that time no attention was paid to the presence or absence of virus diseases. In February 1962, a countrywide frost killed thousands of citrus trees. To rehabilitate the Iranian citrus industry, 700,000 certified buds were imported in 1963-64 from the Willits and Newcomb nursery in California. Additional varieties were introduced into Mazandaran province 1969 from Australia and Japan and in 1971 from California and Morocco. After 1967, a period of heavy frost caused severe damage to citrus in the Caspian Sea region, 40,000 in 1968 and 15,000 satsuma trees in 1970 were imported from Japan. Tristeza virus being endemic in Japan, it could be predicted that each single plant of the 55,000 Japanese satsuma trees was infected with *Citrus tristeza virus* (CTV). These trees were grafted on *P. trifoliata*, a rootstock tolerant to the virus and commonly used in Japan. Hence, the 55,000 Japanese trees have grown well since, but it seems that they were responsible for the introduction of CTV into Iran (Bové, 1995).

The 1985 survey showed that only very limited spread of Tristeza had occurred. The low level of natural transmission is probably due to the fact that *T. citricidus* is absent in the country. *Aphis gossypii*, *A. citricola*, *T. aurantii*, *A. craccivora* and *Myzus persicae* are known as CTV vectors in Iran.

II – Historical review

Ebrahim-Nesbat et al. (1978) reported CTV for the first time from Iran by indexing *Citrus unshiumaro*, imported from Japan, on Keylime; this result was confirmed using ISEM method.

Minassian (1983a) used ELISA for detection of CTV in aphids from the north of Iran. He detected the virus in the sap of *T. aurantii* and *A. citricola* and showed that out of 22 aphid colonies from both healthy and diseased plants, 15% carried CTV, while 45% of colonies from diseased plants carried CTV.

Minassian (1983b) determined the spread of CTV in the north of Iran using ELISA. He tested over 400 citrus trees from different regions in the province of Mazandaran. He showed that in Mahdasht orchards, 90 to 100% of Satsumas on Poncirus stock, introduced from Japan, were infected. He found that 10% of old line of Satsumas on sour orange and less than 10% of Clementines on Poncirus or sour orange were also infected.

Minassian et al. (1983) reconfirmed the occurrence of CTV on Satsuma using indexing on key lime which showed vein clearing symptoms, cupping and chlorosis. They confirmed the presence of CTV using ELISA and ISEM.

Ghorbany (1983), detected CTV in its aphid vectors including *T. aurantii*, *A. spiraeicola* and *A. gossypii* in northern Iran by ISEM technique.

Ghorbany (1986) showed that fruit pericarp has the highest CTV content followed by tree bark and leaves using ISEM method. He didn't see any virus particles in fruit flavedo and seed.

Rahimian (1994) detected strains of CTV in Mazandaran Province in two Mahdasht orchards, considered to be the main infected foci on the basis of biological properties. He identified three strains of CTV: seedling yellows, tristeza and stem pitting. He deduced that a high proportion of Satsuma trees in this area are infected with CTV and the majority of infected trees carry tristeza or stem pitting strains. Infection with the seedling yellows strain was rare.

Aghajanzadeh et al. (1996) studied the transmission of CTV by major citrus aphids (*A. citricola*, *A. gossypii* and *T. aurantii*) using *C. unshiu* and *C. aurantifolia* as donor and recipient host species in Mazandaran. None of aphid species examined could transmit CTV from any donor species.

Zarei et al. (1997) eliminated CTV from two cultivars of Satsuma (cvs. Miyagawa and Sugiyama) through shoot-tip grafting.

Shayeghan et al. (1997) detected CTV in the suburb of Babol (Mazandaran Province) on *C. unshiu* as a new site of infection.

Shafiee and Izadpanah (1998a) purified CTV from the bark of local mandarins in Fars Province and produced an antiserum which reacted with virus isolates both from northern and southern Iran.

Shafiee and Izadpanah (1998b) detected distribution of CTV in southern Iran. Kharf, Kazeroon, Fasarood, Janatshahr, Dalaki, Roodfaryab, Khaeez and Jam regions were infected.

Rahimian et al. (2000a) showed that the decline of sweet orange on sour orange rootstock is an evidence for possible onset of natural transmission of CTV in Mazandaran.

Alavi et al. (2000a) differentiated CTV isolates by electrophoretic analysis of cDNA prepared to CTV-RNA. Most severe strains produced a cDNA band with an estimated size of 4.2 kb but shorter than 1 kb about mild strains.

Alavi et al. (2000b) evaluated various ELISA and DTBIA methods in order to detect CTV. They found that DAS and PTA-ELISA are more sensitive than indirect ELISA methods and the direct and indirect DIBA was as sensitive as DAS-ELISA. They also showed that a 3% gelatin or a 5% triton X-100 solution gave a clear background; the best results were obtained when used together.

Alavi et al. (2000c) purified CTV by sucrose density gradient electrophoresis which could provide preparations of sufficient purity and devoid of contaminating host proteins suitable as immunogen for preparations of CTV specific antisera.

Alavi et al. (2000b) reported *A. gossypii* as a vector of CTV in the north of Iran.

Pakniat et al. (2002a) investigated a decline of Navel orange characterized by yellowing and browning of veins in Darab (Fars Province) since 1998. They detected CTV in affected trees. They could eliminate the disease by shoot tip grafting (STG), while CTV was still present. So, they deduced that although CTV may play a role in the development of Navel orange disorder, other factors, which were excluded by STG, are also involved.

Pakniat et al. (2002b) studied the aphids ability in the transmission of CTV. They found that *M. persicae* can be a dangerous vector in the north while *A. gossypii* and *A. craccivora* are vectors in Fars Province.

Pakniat et al. (2002c) investigated the distribution of CTV in Fars and Bushehr provinces. Fasaroud was the most infected district with 50% infection in Key lime and Talha the lowest one

with 20%. Their data also showed the role of vector in CTV transmission in the area as Key lime and sour orange trees are grown from seeds.

Rahimian et al. (2002) found seedling yellows as the predominant CTV strain transmitted by aphids in Mazandaran Province.

Barzegar et al. (2005a) characterized 22 isolates of CTV collected from the north and the south of Iran based on CP gene sequences. They showed high similarity between Iranian isolates, California SY568 severe stem pitting and Japanese NUagA seedling yellows strain up to 97% based on RFLP profile, nucleotide and deduced amino-acid sequences. Also they couldn't obtain major dissociation between the isolates from northern and southern region of Iran.

Barzegar et al. (2005b) investigated the biological and molecular properties of four CTV strains isolated from sweet orange trees in north of Iran. Sequence analysis showed 98-99% sequence homology of Iranian isolates with the Californian CTV severe stem-pitting isolate SY568 and 97-98% homology with the Japanese seedling yellows isolate NUagA.

Alavi et al. (2005) divided seven isolates of CTV collected from the north of Iran into two groups based on symptoms in different host plants: the first causing stem pitting and severe decline and the second, seedling yellows. Isolates in the first could be transmitted by *A. gossypii*. Based on the phylogenetic analysis of CP gene on the isolates from the eastern part of Mazandaran Province, they suggested at least two independent introductions of CTV into Iran.

Ahmadi et al. (2006) determined distribution and analysis of genetic diversity of CTV isolates in Kerman Province. They found that Jiroft and Hosseinabad were the most infected regions in this province. They divided the isolates in two distinct clusters. They grouped all isolates from Kerman, except Bam isolate, in one cluster and the isolate of Bam with other isolates from Iran in another cluster.

Alebouye et al. (2006) determined the prevalence of CTV infection in the Mahdasht area in Sari. Their results showed that all Satsumas in one plot in Mahdasht, which were among the ones imported from Japan over 35 years ago, were infected and there was an inverse relationship between the proportion of infected trees in the adjacent and more distantly located groves and their distance from the Mahdasht grove.

Pakniat et al. (2006a) detected CTV infection in all southern provinces of Iran (Fars, Kohgiluyeh-Boyerahmad, Hormozgan, Sistan-Baluchestan and Kerman). They couldn't detect any severe strain of this virus in these regions. In contrast, northern isolates recently introduced from Japan included severe CTV strain. They found the highest rate of infection in sweet orange followed in decreasing order by local mandarin, sweet lime and lime.

Pakniat et al. (2006b) demonstrated that an unidentified strain of CTV is associated with vein yellowing and browning in navel sweet orange varieties using biological methods.

Barzegar et al. (2006a) compared sequence analysis of CP27 gene of aphid *A. gossypii* transmissible and aphid non-transmissible CTV isolates. They found 73 nucleotide changes and 5 amino-acid substitutions between them. Three of these amino acids were important for interaction of CP27 and aphid style. In their study comparison of the CP27 deduced amino-acid sequence with their pfam members revealed the conservancy of the Arg and Asp amino-acids needed for formation of salt bridge.

Barzegar et al. (2006b) revealed sequence variability of CP25 gene of 15 CTV isolates by single strand conformation analysis. They observed 8 distinct patterns. SSCP profile of some isolates was in accordance with CP25 gene sequence. But, totally there was no direct relationship between nucleotide differences and SSCP patterns as, sometimes two isolates with a few nucleotide differences produced distinct patterns.

Maghsoudi et al. (2006) evaluated 5 natural citrus rootstocks to CTV in north of Iran. They found that natural Kotra hybrid, Shell mahalleh and Khoram abad lemon hybrid are fairly tolerant, off-type sour orange is tolerant and Ramsar lemon hybrid is susceptible to CTV.

Fifaei et al. (2007) produced Washington navel sweet orange free of tristeza virus using STG technique with four treatments and four replications. All of them were not infected and T-budding method was more successful. They showed that there was no significant difference between used rootstocks.

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Second part

**Advances in CTV detection,
characterization and control**

Improved biological indexing of *Citrus tristeza virus* (CTV)

D'Onghia A.M.¹, El-Sayed T.^{1,2}, Djelouah K.¹, Brandonisio R.¹

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

² Certification Center of Bahteem, Cairo, Egypt

Abstract. Due to the low rooting ability of Mexican lime, the universal CTV indicator, buds were grafted onto Volkameriana lemon. After the inoculation with 3 virus isolates from the MAIB collection and a short IBA treatment for rooting, the cuttings were kept in Jiffy pots under plastic bags at 22-24°C for virus detection. Traditional biological indexing was compared. Starting from 15-20 days after inoculation, clear-cut tristeza symptoms were observed on the new emerging leaves of the indicator, whereas the same results were delayed when inoculated seedlings were used. Results of biological indexing by using cuttings were confirmed by using serological and molecular assays.

Keywords. Biological indexing – Citrus – Citrus tristeza virus – Cuttings – ELISA.

Amélioration de l'indexage biologique de la tristeza des agrumes

Résumé. Vu le faible pouvoir rhizogène de la lime mexicaine, utilisé comme indicateur universel du CTV, les bourgeons ont été greffés sur le citronnier Volkameriana. Après l'inoculation avec 3 isolats du virus originaires de la collection du MAIB, et un bref traitement à base d'IBA pour favoriser l'émission des racines, les greffons ont été placés dans des pots Jiffy, scellés dans des sachets en plastic et maintenus à 22-24°C pour la détection du virus. Cette technique a été comparée à l'indexage biologique traditionnel. Quinze à vingt jours après l'inoculation, des symptômes évidents de tristeza ont été observés sur les nouvelles feuilles développées par l'indicateur, alors que les mêmes résultats ont été obtenus plus tard quand on a utilisé des semis inoculés. Les résultats issus de l'indexage biologique utilisant des greffons ont été confirmés à travers des tests sérologiques et moléculaires.

Mots-clés. Indexage biologique – Agrumes – Virus de la tristeza des agrumes – Greffons – ELISA.

I – Introduction

Citrus tristeza closterovirus (CTV) is the most destructive virus disease of citrus worldwide. It is spread by infected propagating materials, but its outbreaks are primarily due to its transmission by aphids. Many laboratory techniques can be successfully applied for its detection by serological (Bar Joseph *et al.*, 1979; Garnsey *et al.*, 1993) and molecular means (Mawassi *et al.*, 1995; Pappu *et al.*, 1993; Cevik *et al.*, 1996; Nolasco *et al.*, 2002) but none of these assays can totally replace the use of biological indexing on Mexican lime, the universal indicator of CTV. This index is still compulsory for a reliable detection of this pathogen when a primary source is produced in the framework of the certification program. Moreover, characterization of the virus strains is still mainly based on the use of biological indexing, even if laboratory assays may provide some indications.

Constraints of traditional indexing by graft transmission are space and time needed for the production of indicator seedlings and, after graft inoculation, for symptom expression. High skills are the major ingredient for the success of this technique which is based on the production of excellent indicator plants.

A new system of biological indexing based on the use of indicator cuttings instead of seedlings was developed for the detection of the main citrus virus and viroids (Elbacki *et al.*, 2005; El Sayed, 2005).

In this study, the use of Mexican lime cuttings is applied in the detection of three CTV sources in comparison with the conventional method (Roistacher, 1991).

II – Materials and methods

Three virus sources, two from Italy and one from Egypt were provided from MAIB collection which represented typical CTV inoculum of Mediterranean origin.

Ten semi-hard wood and hard wood cuttings were used of Mexican lime containing 4-6 nodes and were inoculated by chip budding using the bark tissue collected from the CTV sources. After labelling, all the cuttings were grafted onto Volkameriana lemon as rootstock, then enclosed inside a plastic bag in order to keep high humidity. The inoculated plants were placed in a Jiffy supporter and were maintained in an air conditioned greenhouse at cool temperatures (22-24°C). After ten days, the plastic bags were opened at the top in order to reduce the inside humidity and grafting success was evaluated. After 20 days, the Jiffy supporter was placed in a small flask inside plastic bags and were held for watering and fertilization (Fig. 1).



Figure 1. Grafting steps of indicator stem cuttings: (a) hard, semi-hard cuttings; (b) Volkameriana lemon cutting chip budded with *M. lime* bud and graft inoculated with CTV; (c) plants in the Jiffy pots inside the plastic bag.



Figure 2. Severe leaf vein clearing induced by CTV in *M. lime* grafted onto Volkameriana lemon cutting.

Comparison with traditional biological indexing, as described by Roistacher (1991), was also performed using one year old Mexican lime seedlings. These indicator plants were chip budded

with two blind buds from each selected CTV source. After sealing the graft with parafilm and labelling, the inoculated plants and the negative controls were grown with the inoculated cuttings in our air conditioned greenhouse.

Serological detection was carried out on the symptomatic and symptomless plants using DAS ELISA and DTBIA (Bar Joseph *et al.*, 1979; Garnsey *et al.*, 1993).

ELISA plates were coated with polyclonal antibodies using the Agritest-Italy commercial kit; at the concentration indicated by the Company; samples were grinded in extraction buffer at 1/10 concentration, using bark or petiole tissue

Diluted linked antibodies (as reported by the company) were added to each well. P-nitrophenyl phosphate in substrate buffer was used and readings of the absorbance values were made by using automatic plate reader at 405 nm with a Titertek Multiskan plus MKII reader; CTV sources were considered positive if the OD405 values were more than 2,5 times above the values of healthy extracts.

Samples were analyzed by DTBIA for the detection of CTV using the commercial kit of Plantprint-Spain (Garnsey *et al.*, 1993). Five tender shoots from each indicator were cut transversely with a sterile razor and the sections were pressed carefully on the nitrocellulose membrane. After blocking with 1% bovine serum albumin (BSA), the membrane was incubated with the Mabs 3DF1+3CA5 mixture conjugated with alkaline phosphatase (PlantPrint). Membranes were developed by using BCIP-NBT (Sigma fast tablets), then read under a light microscope at 10x and 20x magnification. The positive reaction was revealed by the presence of purple-violet blots in the region of phloem tissue cells.

Molecular detection was carried out on the CTV negative plants using RT-PCR test (Nolasco *et al.*, 2002).

Total nucleic acid from about 100mg of young citrus infected barks were extracted by using the RNeasy Mini Kit, Qiagen, according to the manufacturer's instructions. cDNA was synthesized using TNA extracted as template and the PCR mix reaction contained 10mM Tris (pH:8.8), 50 mM KCl, 3mM MgCl₂, 4U RNA Guard ribonuclease inhibitor, 7.5U MuLV reverse transcriptase (Applied Biosystems, Roche), 1U Taq polymerase (Promega), 0.2 mM of each dATP, dTTP, dGTP and dCTP, 200nM CTV1 forward primer, 200nM CTV10 reverse primer (Nolasco *et al.*, 2002) and 3ml of extracted TNA.

cDNA synthesis was performed at 38°C for 45 min followed by a denaturation step and inactivation of reverse transcriptase at 94°C for 2 min. The amplification process consisted of 30 cycles at 92°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec, followed by a 10 min elongation cycle at 72°C. The amplification products were analysed in 1% agarose gel electrophoresis.

III – Results and discussion

Graft success was higher in Volkameriana lemon and shoot flushing usually occurred in 20 – 30 days after inoculation. Symptoms development differed in terms of time of symptom appearance and the number of symptomatic plants for each CTV source. Most of the symptoms with rooted cuttings developed after 20-23 days, while the inoculated one year old seedlings of the tested indicators showed the first symptoms in some plants one month after inoculation.

Twenty days from inoculation clear-cut tristeza symptoms were observed on the new emerging leaves of both indicators. Results of biological indexing were confirmed by ELISA, DTBIA and PCR on the indicator leaves.

IV – Conclusion

Based on these preliminary results, CTV can be successfully detected by the use of inoculated indicator cuttings instead of seedlings. Due to the low rooting ability, Mexican lime must be chip-budded onto Volkameriana lemon cuttings.

This technique could be used during the entire year, because the production of stem cuttings under warm conditions overcomes the seasonal rooting variability of most citrus species. The biological assay using indicator cuttings can be readily concluded one month after grafting without any transplanting.

This method can replace of traditional biological indexing of CTV without a decrease in reliability of symptom expression. However the use of this technique in combination with laboratory assays, mainly in asymptomatic plants, is always recommended for a reliable sanitary assessment of a citrus genotype.

Further research should also be carried out using mild and moderate pathogen strains, enlarging the use of stem cuttings to CTV biological characterization.

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Serological characterization of a collection of Mediterranean *Citrus tristeza virus* (CTV) isolates

Zemzami M.^{1,3}, Djelouah K.², Frasheri D.², Soulimani A.³, D'Onghia A.M.²

¹ DDA-UCP, Domaine Maamora, Km 11, Rte Ple N° 1, Hssaine, Salé, Morocco

² CIHEAM - Mediterranean Agronomic Institute, Bari, Italy

³ Université Ibn Tofaïl, Dpt de Biologie, Kénitra, Morocco

Abstract. A collection of 68 CTV isolates from 11 Mediterranean countries were cross-tested against a panel of 8 monoclonal antibodies (MAbs), including one severe strain specific (MCA-13) and one broad pattern (17G11) from USA, four broad spectrum (1D12, 4C1, 4E5 and 4F3) and two differentials (4B1 and 4D3) from Morocco. Based on the reaction patterns, the tested CTV isolates were classified in 8 serogroups (Sgr), among which Sgr.1 had the largest number of isolates (35 in total) that reacted with all MAbs. Three broad spectrum MAbs (17G11 from USA and 4C1 and 4E5 from Morocco) were able to detect all the CTV isolates tested. They proved to be highly reliable for universal detection of Mediterranean strains of CTV. MCA-13, the severe CTV-strain specific MAb, reacted with 2/3rd of the tested isolates, indicating that some of the Mediterranean CTV strains could be of severe nature. MAbs 4B1 and 4D3 were the most selective antibodies.

Keywords. Mediterranean CTV isolates – Selective monoclonal antibodies – Serological patterns.

Caractérisation sérologique d'une collection méditerranéenne d'isolats du virus de la tristeza des agrumes (CTV)

Résumé. Une collection de 68 isolats de CTV provenant de 11 pays méditerranéens ont été testés contre un panel de 8 anticorps monoclonaux (AcMc), comportant le MCA-13 spécifique des souches sévères et le 17G11 à large spectre tous les deux originaires des USA, 4 AcMc à large spectre (4B1, 4C1, 4E5 et 4F3) et 2 AcMc différentiels (4B1 et 4D3) originaires du Maroc. A la base des profils des réactions obtenus, les isolats de CTV testés ont été classés en 8 séropoupes parmi lesquels le Sgr.1 comporte le plus grand nombre d'isolats (35 au total), ayant réagi avec tous les AcMc testés. Trois des AcMc à large spectre (17G11 des USA et 4C1 et 4E5 du Maroc) ont réagi avec tous les isolats testés. Ils ont ainsi prouvé leur haute performance pour la détection universelle des souches Méditerranéennes de CTV. Le MCA-13 connu pour sa spécificité aux souches sévères de CTV a réagi avec les 2/3 des isolats testés, indiquant qu'il est fort possible que certaines des souches Méditerranéennes de CTV soient de nature sévère. Les AcMc 4B1 et 4D3 ont montré les profils de réaction les plus sélectifs.

Mots-clés. Isolats méditerranéens de CTV – Anticorps monoclonaux sélectifs – Profils sérologiques.

I – Introduction

Citriculture is of paramount strategic importance for the Mediterranean region for its socio-economic role as a source of income, a provider of employment opportunities and for its contribution to the well-being of the people in the Mediterranean countries. However, the Mediterranean citrus industry relied too much on the use of sour orange, a root-stock well adapted to numerous biotic and abiotic stresses largely prevalent in the region, but highly susceptible to CTV, a devastating virus which killed millions of trees throughout the world, including Spain and Israel (Marroquin *et al.*, 2004; Bar Joseph *et al.*, 1989). The threat of tristeza has even worsened with the recent appearance in Northern Portugal and Spain of the brown citrus aphid "*Toxoptera citricidus*", known to be the most efficient vector of CTV. With CTV inoculum present in almost all the Mediterranean citrus producing countries, invasion of the Mediterranean region by *T. citricidus* will cause a rapid dissemination of severe CTV strains that will devastate in few years all trees grafted on sour

orange. A sudden collapse of the major part of the Mediterranean citrus industry will occur with unpredictable dramatic socio-economic consequences.

This study was conducted for the serological characterization of the Mediterranean collection isolates of CTV obtained from 11 countries, using a panel of monoclonal antibodies (MAbs), including the severe strain specific MCA-13, some broad spectrum and some selective MAbs. The aim is to identify efficient serological tools for general detection of CTV and selective MAbs for specific strain typing.

II – Materials and methods

1. The CTV collection

The CTV isolates were either collected directly in citrus orchards during surveys conducted by the Mediterranean Agronomic Institute of Bari (MAIB) in various Mediterranean countries, or obtained from local collaborating institutions as material exchange for research purposes. All the isolates were subjected to a preliminary test for CTV infection by DAS ELISA as reported by Bar Joseph *et al.* (1979), using two commercial kits i.e. one based on polyclonal antiserum (PAs) from Agritest (Italy) and one based on a mixture of Monoclonal antibodies (3DF1+3CA5) from Ingenasa (Spain) targeting two highly preserved epitopes of CTV coat protein and able to detect all known CTV isolates (Garnsey *et al.*, 1989).

These CTV isolates were kept in their original plant material grafted onto different rootstocks (Sour orange, Troyer citrange or Rough lemon) under insect-proof screenhouse and were assigned an "IAMB-Q" number. Globally, these CTV sources included several countries as reported in Table 1.

2. Monoclonal Antibodies

The MAbs used include the severe strain specific MCA-13 (Permar *et al.*, 1990), and the large spectrum 17G11 (Lin *et al.*, 2000) both from USA, four large spectrum (1D12, 4B1, 4C1, and 4E5) and two discriminating (4D3 and 4F3) from Morocco (Zemzami *et al.*, 1994). All MAbs were purified IgG's at 1 mg/ml optimized for use at a final concentration of 1 µg/ml.

3. Serological characterization

In order to assess the epitopic diversity of the different CTV isolates, TAS-ELISA assay (Cambra *et al.*, 1995) was performed using the Agritest PAs for capture. All virus sample extracts were prepared from fresh bark and leaf tissue of greenhouse positive plants. MAbs were allowed to react with the antigen for 2 hrs at 37 °C. Anti-mouse (whole molecule) conjugated to Alkaline Phosphatase (Sigma, USA) was used for MAbs detection. The final reaction was revealed with p-Nitrophenylphosphate at 1 mg/ml and reading was done at OD₄₀₅. Results were recorded as positives when exceeding 3 times the mean of the negative control.

Table 1. List of the selected CTV sources maintained at the IAMB greenhouse.

Countries	Number of isolates	Numbers assigned
Albania (Alb)	11	IAMB-Q3, Q5, Q8, Q11, Q90, Q91, Q92, Q93, Q94, Q132, Q133
Algeria (Alg)	1	IAMB-Q76
Croatia (Cro)	1	IAMB-Q96
Cyprus (Cyp)	1	IAMB-Q73
Egypt (Egy)	4	IAMB-Q57, Q82, Q61, Q81
Iran (Ira)	1	IAMB-Q97
Italy (Ita)	23	IAMB-Q21, Q32, Q102, Q103, Q105, Q110, Q111, Q112, Q113, Q114, Q115, Q116, Q117, Q118, Q119, Q121, Q122, Q123, Q124, Q134, Q135, Q143, Q144
Lebanon (Leb)	10	IAMB-Q4, Q6, Q7, Q12, Q15, Q125, Q126, Q127, Q128, Q131
Montenegro (Mon)	4	IAMB-Q106, Q107, Q108, Q109
Morocco (Mor)	2	IAMB-Q74, Q75
Palestine (Pal)	8	IAMB-Q40, Q44, Q48, Q49, Q51, Q52, Q53, Q54
Syria (Syr)	2	IAMB-Q149, Q150

III – Results and discussion

The 68 CTV sources tested against the panel of 8 monoclonal antibodies exhibited 8 distinct serological reaction patterns designated here as serogroups (Tab. 2).

Table 2. Serological analysis of 68 selected CTV sources and the established serogroups.

Serogroups	CTV isolates									Frequency (nbr)
		17G11	4C1	4E5	1D12	MCA 13	4F3	4B1	4D3	
1	Alb (3, 5, 8, 11, 90, 91, 92, 93, 94, 132), Cro 96, Cyp 73, Egy (57, 82, 61), Ita (21, 32), Leb (6, 15, 126), Pal*, Mon*, Mor*.	+	+	+	+	+	+	+	+	35
2	Egy 81, Leb (128, 131), Syr	+	+	+	+	+	+	+	--	5
3	Leb (4, 7, 12, 125)	+	+	+	+	+	+	--	--	4
4	Leb 127	+	+	+	+	+	--	--	--	1
5	Alb 133, Alg 76, Ita (102, 105, 110, 111, 117, 118).	+	+	+	+	--	+	+	+	8
6	Ita (103, 113, 114, 115, 119, 121, 122, 123, 124)	+	+	+	+	--	+	--	--	9
7	Ita (134, 135, 143, 144)	+	+	+	+	--	--	+	--	4
8	Ita (112, 116)	+	+	+	--	--	+	--	--	2
Total	68	68	68	68	66	45	63	52	44	

Sgr.1 (35 isolates) reacted with all MABs; Sgr.2 (5 isolates) reacted with all MABs except 4D3; Sgr.3 (4 isolates) reacted with all MABs except the Moroccan differential MABs (4B1 and 4D3); Sgr.4 (1 isolate) reacted with MCA-13 and all broad spectrum MABs except 4F3, it didn't react also with any of the differential MABs; Sgr.5 (8 isolates) reacted to all MABs except MCA-13; Sgr.6 (9 isolates) reacted only with the broad spectrum MABs; Sgr.7 (4 isolates) had a negative reaction with MAC-13, 4B1 and 4F3 MABs; Finally Sgr.8 (a single isolate) which reacted only to 4 broad spectrum MABs (17G11, 4C1, 4E5 and 4F3).

The broad spectrum MABs: 11G17, 4C1, and 4E5 were able to react with all CTV isolates tested, meaning that they target conserved epitopes. The MAb 1D12 which is also considered of broad spectrum did not react with 2 Italian CTV isolates of serogroup 8. MAb 4F3 of broad spectrum pattern did not react with 5 isolates. MCA-13 MAb exhibited opposite reaction patterns with all the other MABs against 4 serogroups each. Opposite reaction pattern of MCA-13 and 4D3 has already been noticed in our previous work (unpublished data). The MABs 4B1 and 4D3 were the most discriminating, confirming the result reported by Zemzami *et al.* (1999). MCA-13 (Permar *et al.*, 1990) described in Florida as specific for severe and CTV decline-inducing isolates reacted with 66% of the tested CTV isolates from various countries, indicating that some Mediterranean CTV isolates could be of severe types. Although positive reaction with MCA-13 is not necessarily correlated with the decline of trees in the Mediterranean Basin, it is an indication of potential aggressiveness of an isolate. Interestingly, all the tested CTV sources belonging to the following countries Croatia, Cyprus, Egypt, Lebanon, Montenegro, Morocco, Palestine and Syria gave a clear-cut positive reaction with the MCA-13 MAB, even if the number of the tested CTV sources was not always significantly large.

These results provide an initial evidence of the serological diversity of CTV isolates present throughout the Mediterranean region. They also confirm the considerable antigenic diversity existing in CTV isolates which was reported in other studies (Permar *et al.*, 1990). The fact that more than 50% of CTV isolates reacted with all MABs could reflect the presence of multiple CTV strain mixtures in the collected individual CTV sources as already reported (Rubio *et al.*, 2001). They indicate also that broad spectrum MABs are reliable for universal detection of Mediterranean CTV isolates. They denote clearly that potential severe CTV strains are widely distributed, thus providing favourable grounds for CTV outbreaks, if or when the brown citrus aphid "*T. citricidus*" spreads throughout the region.

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An Asymmetric PCR-ELISA Typing assay for *Citrus tristeza virus* (CTV)

Nolasco G., Silva G., Santos C.

Center for Biodiversity Functional and Integrative Genomics,
Universidade do Algarve - FERN, Faro, Portugal

Abstract. Typing of CTV based on coat protein (CP) gene has been approached in diverse ways. The basis for the assay focused on in this paper was presented some years ago and has been subject to several improvements since its appearance. Although the results obtained with this assay have been presented in diverse scientific meetings, theses and technical seminars, so far no paper has explained the rationale of the method or detailed the protocol. This is presented here as well as the discussion of recent developments regarding the set of probes, hybridisation procedure and software for interpreting the results.

Keywords. Citrus – Hybridization – PCR ELISA – Tristeza – Typing.

Un essai de typage par PCR-ELISA asymétrique pour le virus de la tristeza des agrumes

Résumé. Le typage du CTV, utilisant le gène de la protéine capsidique, a été réalisé en suivant différentes procédures. Les principes fondamentaux de l'essai, décrits dans ce travail, ont déjà été présentés il y a quelques années mais, dans le temps, on a apporté des améliorations importantes. Bien que les résultats obtenus dans cet essai aient été illustrés à l'occasion de diverses conférences scientifiques, de nombreux séminaires techniques et dans des mémoires de thèse, aucun travail n'a expliqué jusqu'à présent la logique de cette méthode ou proposé un protocole détaillé. C'est là donc l'objet de cette présentation où, en plus, on va parcourir les avancées récentes concernant les sondes, la méthode d'hybridation et le logiciel pour l'interprétation des résultats.

Mots-clés. Agrumes – Hybridization – PCR ELISA – Tristeza – Typage.

I – Introduction

Several studies support the existence of a not yet fully clarified relationship between CP gene sequence and CTV symptoms, and different approaches have been made to develop a typing system based on the CP gene (Permar *et al.*, 1990; Gillings *et al.*, 1993; Pappu *et al.*, 1993; Niblett *et al.*, 2000; Zemzami *et al.*, 2002; Halbert *et al.*, 2004). Zemzami *et al.* (2002) further developed the set of short (16-20) discriminating probes presented by Niblett *et al.* (2000) for targeting the seven phylogenetic groups which are now soundly recognized (see an accompanying paper in this volume). In the same work the format of the discriminating hybridisation step was presented as an asymmetric PCR-ELISA. The rationale of this procedure is the use of a PCR reaction in which the amount of one primer is about one order of magnitude higher than the other. This originates a large amount of single-stranded molecules that in the following steps are hybridised to a set of strain discriminating probes immobilized in the wells of an ELISA plate. During the PCR reaction, the DNA molecules are labelled by Digoxigenin (Dig) which is included in the deoxy-nucleotide mixture as Dig-dUTP. The hybridised products are quantified through an ELISA assay using commercial alkaline-phosphatase conjugated anti-Dig antibodies. This information is used through a software analysis to estimate the composition of the samples in terms of the seven phylogenetic groups.

This paper presents and discusses the latest developments regarding the set of probes, hybridisation procedure and software for interpreting the results. The software and the probes sequence is available upon request to the author, gnolasco@ualg.pt. A more complete paper is now in press (Nolasco *et al.*, 2008).

II – Material and methods

1. cDNA synthesis

cDNA synthesis is done using random primers. Typically this is done mixing 5µl total RNA with 1µl random primers (0.5 µg/µl, random p(dN)₆, Roche, ref^a 11034731001), denaturing for 5 min at 95 °C and quickly transfer to ice. The reverse transcription is done for 1h at 37 °C using SuperScript™ III Reverse Transcriptase (Invitrogen, ref^a 18080-044) following the manufacturer instructions.

2. Dig Labelling by Asymmetric PCR

One microlitre of cDNA is used as template in the asymmetric PCR reaction. This is done with the primers CTV43 (forward) 5'-ATGTTGTTGCNGCNGAGTC-3 and CTV42 (reverse) 5'-CTCAAATTGCGRTTCTGTCT-3 which amplify a fragment starting at position 59 and finishing at position 473 in the CP gene. Primer CTV 43 is used at of 200 nM (final concentration) and primer CTV 42 at 20 nM. The deoxy-nucleotide and Dig labelling mixture contains (final concentration) 80 µM of each of dATP, dGTP, dCTP, 76 µM dTTP and 2 µM of Dig-11-dUTP (Roche Applied Science ref. 11093088910). MgCl₂ concentration is 2.5 mM and typically one unit of Taq polymerase is used in a 50 µl reaction. Thermocycling is done after an initial denaturation of 2 min at 92 °C and consists of 50 cycles with the following steps: 92°C for 30 s, 52°C for 30 s, 72°C for 45 s. A final extension period of 5 min at 72° C completes the process.

3. ELISA plate preparation

The hybridisation probes are biotinylated and immobilized in the wells via a previous streptavidin coating. Microlon (Greiner) 600 microplates with round or flat bottom wells are used. All volumes are 100 µl per well. The microplate wells coated with 10 µg/ml streptavidin in 50 mM sodium carbonate buffer (pH 9.6) overnight at 4°C or at 37°C for 1.5 h. The microplates are then washed three times with PBS-Tween as in standard ELISA procedures. The biotinylated probes are added, 20 pmol per well, in hybridisation buffer (0.75M NaCl, 0.425 M NaH₂PO₄, 0.005 M Na EDTA, pH 7.4, containing 0.1 % n-lauroylsarcosine) and incubated at 37°C for 30 min. The probes are arranged in rows as shown in Table 1. The plate is washed as before and used immediately for the following steps or prepared in advance and kept empty at 4°C or at room temperature up to three months.

Table 1. Layout of the first 8 columns of the ELISA plate. Each probe, indicated on the left side, is arranged on one row. Each column corresponds to one sample whose PCR product are arranged in the 8 corresponding wells. In this case the samples are a set of CP gene variants representative of each of the seven phylogenetic groups which are indicated on the top. The numbers inside the border are the initial rates of hydrolysis estimated through fitting of the Michaelis-Menten equation.

	Gp 1	Gp 2	Gp 3a	Gp3b	Gp 4	Gp 5	Gp M
	#1.1tt	#13.3	#bb6	#28c	#134a	#010.8	#20.2
B1	0.643	0.006	0.006	0.007	0.006	0.010	0.006
B2	0.005	0.488	0.006	0.007	0.003	0.011	0.007
B3a	0.031	0.010	0.219	0.022	0.048	0.006	0.094
B10	0.009	0.005	0.071	0.299	0.002	0.002	0.002
III	0.005	0.009	0.031	0.278	0.018	0.002	0.002
B4	0.007	0.006	0.007	0.003	0.443	0.004	0.005
B5	0.005	0.003	0.004	0.008	0.013	0.552	0.003
B8	0.000	0.031	0.007	0.006	0.013	0.055	1.426

4. Hybridisation

For each sample, 45 µl of the Dig labelled PCR products are added to 855 µl of hybridisation buffer and arranged, 100 µl per well, in one column of the plate. The plate is sealed with tape and incubated for 1h30 at 45°C. The plate is then washed three times for 3 min. with pre-warmed (45 °C) low molarity tris washing buffer (Tris-HCl 10mM, pH8, 15 mM NaCl).

5. Colorimetric development and absorbance reading

Anti-DIG-F(ab')₂ alkaline phosphatase conjugate (Roche Applied Science 11093274910), 0.075 units per well in 100 µl of PBS-Tween containing 2 % PVP-40, 0.2 % BSA, are added and incubated for 30 min at 37 °C. The wells are washed 5 times with PBS-Tween and 1 mg/ml of p-nitrophenyl phosphate in substrate buffer (9.7 % diethanolamine, pH 9.8) are added, 100 µl per well. The plate reader should be ready before adding the substrate. This should be preferably added with a 8-multichannel pipette by columns to avoid differences in the reaction times delivery, with 3 min intervals. If available, incubation is allowed at a certain temperature among wells of each sample. The absorbance is read at 405 nm starting immediately after substrate (e.g. 37°C).

6. Analysis of the results

This is done in two steps using a specially developed software. In a first step the raw data is imported and an array with the initial rates of the substrate hydrolysis is calculated. In a second step this array is compared to the array of the standards stored in the software and its composition in terms of the presence of variants of each of the seven phylogenetic groups is determined.

II – Results and discussion

1. Experimental protocol

The nucleic acid to be typed may be CTV RNA directly extracted from infected plants when the objective is to analyse an unknown isolate, or colonies of transformed bacteria harbouring the CP gene when the objective is to make a previous screening before choosing clones to be sequenced. Previously CP gene amplified products may also be used.

In comparison with the previous versions of the protocol, it should be noticed that the hybridisation and washings are now performed at 45°C (instead of 37 °C) and at a lower molarity. These changes were found advantageous for a better control of the stringency of the procedure. However, it should be taken in consideration that accidental variations in the concentration of NaCl may result in erroneous results due to a change in the ability of the probes to hybridise to mismatched targets.

For easiness of operation to follow the hydrolysis of the substrate, longer periods e.g. 5 or 10 minutes may eventually be more convenient in some equipment. In these cases it may be useful to lower the substrate concentration or incubation temperature to slow down the reaction rate. The reaction should be followed until the faster reacting wells reach an absorbance of about 2.8 OD.

The raw data is used in a first step to estimate the initial rate of hydrolysis on each well. The software supplied with some equipment brands may provide a way to do these calculations, frequently through a linear regression for the first data points. Alternatively an application in Visual Basic is available upon request (MonoMadjust). This is based on the Michaelis-Menten kinetic model of a single substrate reaction and produces a more accurate non-linear curve fitting using all data points. An example of the output of this step is presented in Table 1. When running the software, care should be taken regarding the absorbance values that can be used. Depending on

the age of the instrument lamp or other factors, the highest absorbance values (e.g. higher than 2.8) are biased and no longer follow the single-substrate model. This can be noticed by a sharp bending of the curve before the plateau. These values should not be considered to curve fitting, an option which can be selected in the software.

Table 1 reports the initial hydrolysis rates obtained for a set of CP gene variants representative of each of the seven phylogenetic groups. These rates of reaction were obtained using preparations with equal starting concentrations of each variant. This is the standard array of rates of hydrolysis that will be used by a second Visual Basic application (CalcType, available upon request) to search for the presence of variants of each group when analysing an unknown sample. This standard array is obtained only once and can be used for the subsequent experiments as it is permanently stored in the software.

When an unknown sample is being analysed the software will compare its array of reaction patterns with the standard array. Let the following array represent the ELISA rates of reaction of the CP gene of each group with the set of probes, i.e, the data presented in **Table 1** (for simplification only a 3 x 3 array is here represented),

	Probe 1	Probe 2	Probe 3
Gp 1	a_{11}	a_{12}	a_{13}
Gp 2	a_{21}	a_{22}	a_{23}
Gp 3	a_{31}	a_{32}	a_{33}
.....

Suppose that the analysis of an unknown sample resulted in the following array representing the ELISA rates of reaction with the set of probes:

Probe 1	Probe 2	Probe 3
P_1	P_2	P_3

Assuming that the ELISA rates of reaction are proportional to the amount of DNA present on the sample, if the unknown sample is composed by a certain amount (X_1) of DNA from Gp 1, a certain amount (X_2) of DNA from Gp 2, etc..., then it is possible to write the following system of linear equations:

$$\begin{aligned}
 P &= X_1 \cdot a_{11} + X_2 \cdot a_{21} + X_3 \cdot a_{31} + \dots \\
 P &= X_1 \cdot a_{12} + X_2 \cdot a_{22} + X_3 \cdot a_{32} + \dots \\
 P &= X_1 \cdot a_{13} + X_2 \cdot a_{23} + X_3 \cdot a_{33} + \dots \\
 \dots &= \dots + \dots + \dots + \dots
 \end{aligned}$$

Solving the system in order to the array X_1, X_2, X_3, \dots will give the amount of DNA present in the sample from each of the groups. In practice, due to experimental errors, etc..., the system may originate negative solutions for the amount of DNA of each group. Although this has no real meaning, it is used by the software as a first approach. In following successive iterations, the software CalcTypeVel will find the closest solution in which all the X_i are greater than or equal to zero. This software integrates in a Visual Basic module running on Microsoft Office Excel with the Solver add-in which must be installed. The absence of a perfect linearity may lead to an erroneous over interpretation of quantitative results by the user. Thus, although the results internally managed by the software are quantitative the output is qualitative, in terms of presence or absence of sequence variants of each group.

The software also provides an estimate of the error in the solution. A large error means that there was not any combination of the standards that could be used to explain the unknown sample. This

should be taken as an indication of bad quality experimental procedures (e.g. a not homogeneous concentration of probes, variations in the amount of PCR product added to each well, etc..) or an indication that the unknown sample might be composed by sequence variants that react in an unknown way to the panel of probes, i.e, hypothetically, the unknown sample belongs to a new group not previously characterized. If this situation happens, this sample may be added to the panel of standards for future assays. Eventually the design of a new probe may be necessary.

2. Comparison with other hybridisation assays

A limitation of the previous membrane-based assays (Niblett *et al.*, 2000) is that the result of the hybridisation of the target to each probe of the panel is analysed on an all-or-none basis. As such, each probe had to be designed in such a way that it only hybridises to its homologous group. This originated a limited discriminating ability as it is not possible to find in the CP gene regions of the desired length which have enough nucleotide differences to fully differentiate among the seven groups. Some discrepancies or difficulties in interpreting the results have been reported when using this approach (Herron *et al.*, 2005; Halbert *et al.*, 2004). In contrast, in this assay, each probe can react with variants of more than one group. This allows much more flexibility in the design of probes, allowing a better discrimination.

3. Typical results

Assays run with cloned CP genes in diverse proportions showed that in artificial mixtures of two phylogenetic groups, the presence of one group could be detected when it represented only 10% of the sample. More than one hundred samples have been characterized in parallel through this assay and by cloning and sequencing the following SSCP choice of the variants. In all the cases, the phylogenetic groups detected by sequencing were also detected by PCR ELISA Typing assay. In some samples in which the number of sequenced clones was small, e.g. 3 or 4, it was possible to detect by this typing assay the presence of additional phylogenetic groups, probably represented in lower amount. Mixtures of up to five groups could be detected in nature, although this situation was not common. The ability to detect mixtures of five groups was verified through the use of artificial mixtures.

4. Relationship with symptoms

Although it is tempting to establish a direct relationship with symptoms, some care should be made when doing such extrapolations. Also the degree of confidence in these relationships depends on the group. Considering isolates composed by just one phylogenetic group, group M, group 2 corresponds to mild isolates which do not originate stem pitting on sweet orange nor the quick decline of plants grafted on sour orange. Group 3a corresponds to severe isolates which originate diverse degrees of decline and also stem pitting on branches of grapefruit, sweet orange and some mandarins when grafted on tolerant rootstocks. Group 1 corresponds to isolates which originate decline and quick decline of trees grafted on sour orange. Groups 3b and 5 are usually not mild but more variable in the ability to induce stem-pitting or decline. Extrapolation of these relationships for isolates harbouring mixtures of these groups is not clear at all.

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Diversity of the coat protein gene of *Citrus tristeza virus* (CTV) in the Mediterranean region

Djelouah K.¹, Cerni S.², Fonseca F.³, Santos C.³,
Silva G.³, Yahiaoui D.¹, D'Onghia A.M.¹, Nolasco G.³

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

² University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia

³ Center for Biodiversity Functional and Integrative Genomics, Universidade do Algarve-Faro, Portugal

Abstract. Sequence data of the coat protein gene of CTV were retrieved from the GenBank or from tested infected tissues and compiled. A comparison with a comparable set of sequences from worldwide isolates showed a very similar value for the nucleotide diversity and a very similar genetic structure in which the same seven groups of variants are conspicuous. A geographic speciation is not apparent and, probably, differences in the distribution of CTV groups are a result of trade. In countries that usually import propagating material from Spain, the group M predominates while group 3b predominates in countries that import material from Israel.

Keywords. Coat protein gene – CTV – Group 3b – Group M – Nucleotide.

Diversité du gène de la protéine capsidique du Citrus tristeza virus dans la région méditerranéenne

Résumé. Les données des séquences du gène de la protéine capsidique du CTV ont été récupérées de la GenBank ou des tissus infectés analysés et élaborées. Une comparaison avec un ensemble de séquences d'isolats de diverses origines a révélé une similitude élevée pour la diversité des nucléotides et une structure génétique très similaire dans laquelle les mêmes sept groupes de variantes sont importants. Une spéciation géographique n'est pas évidente et, probablement, des différences en termes de distribution des groupes de CTV sont imputables aux échanges commerciaux. Dans les pays qui importent normalement du matériel de multiplication de l'Espagne, le groupe M prédomine alors que le groupe 3b est le plus présent dans les pays qui importent le matériel végétal d'Israël.

Mots-clés. Gène de la protéine capsidique – CTV – Groupe 3b – Groupe M – Nucléotide.

I – Introduction

Despite the eradication programmes that have been undertaken, *Citrus tristeza virus* (CTV) can now be found in most of the Mediterranean countries. In this work, we present a snapshot of the circulating strains in the citrus-producing areas of the Mediterranean region. Only isolates obtained from plants grown in field conditions were considered. In marginal Mediterranean countries such as Portugal, the isolates were restricted to regions which, by climate, cultural practices or commercial exchanges, can be considered as an extension of the Mediterranean basin. In total, information from sequence variants obtained from 108 isolates from 17 countries was gathered (Tab. 1).

Table 1. Number of isolates harbouring haplotypes of each group.

	Gp 1	Gp 2	Gp 3a	Gp 3b	Gp 4	Gp 5	Gp M
Israel	1			8			
Palestine				6			
Jordan				1			
Syria				1			
Turkey		1					
Cyprus	2	1		3	1	4	
Croatia		7	4		2	2	1
Montenegro		1	1				1
Albania	3						
Epirus							2
Italy	2		2	1	2		19
Spain		1	1				2
Portugal			2	2			7
Egypt	1				2		
Tunisia	3					1	
Algeria							1
Morocco					1	2	3
Percentage	11 %	10 %	9 %	20 %	7 %	8 %	33 %

II – Material and Methods

Coat protein (CP) gene accessions of isolates from the Mediterranean region were retrieved from the GenBank or from unpublished work carried out in the last years at the Mediterranean Agronomic Institute of Bari, Italy and at the Universidade do Algarve, Faro, Portugal. In these cases, the CP gene was amplified from infected tissue by RT-PCR from total RNA extractions or by Immunocapture RT-PCR procedures using primers CTV 1 and CTV 10 which are specific for the 5' and 3' terminal CP gene parts (Nolasco *et al.*, 2002). The amplified products were TA cloned and bacterial clones harbouring the CP gene were analysed by SSCP prior to sequencing. Several isolates harboured more than one CP gene variant as determined by SSCP. Variants originating different SSCP patterns were sequenced.

The CP gene sequences were excised from the first and last 20 nucleotides in order to exclude the region which might correspond to the primers used to obtain them. After alignment the set of sequences was scanned for the presence of recombination events using the RDP software (Martin *et al.*, 2005) which implements several algorithms for recombination detection. Three sequence variants which showed recombination evidence by more than one algorithm were excluded from further analysis as they probably originated by an evolutionary mechanism which is not compatible with the evolutionary model (Kimura 2-parameter) used to derive the remaining phylogenetic relationships. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007) on the set of 143 CP gene sequence variants.

III – Results and discussion

The nucleotide diversity (p) of the set of 143 sequences was estimated as 0.072 (SE: 0.006) nucleotide changes per site which is very close to the value attained with a set of 213 CP gene sequences obtained from worldwide isolates $p = 0.074$, SE: 0.007 (Nolasco *et al.*, 2007).

A phylogenetic tree (Fig. 1) was constructed in which additional sequences from worldwide reference isolates previously used in other studies were included (13C from Madeira Island, AF184113; 15-118 from Madeira Island, GenBank accession AY660009; T3 from Florida; T30

from Florida, GenBank accession AF260651; T36 from Florida, AY170468) to help identifying the phylogenetic relationships. A structure containing 7 well defined groups was obtained. These groups were supported by a bootstrap value (1000 repetitions) higher than 90%. This confirms and generalizes the structures obtained previously (Papayiannis *et al.*, 2007; Cerni *et al.*, 2007; Lbida *et al.*, 2004; Amin *et al.*, 2006; Zemzami *et al.*, 2002) from studies using subsets of the sequence haplotypes presented here. These same 7 groups are also present when the whole set of worldwide CP gene sequences are considered (results not shown). Most of the nucleotide diversity of the Mediterranean population is due to the inter-group diversity, which accounts for a coefficient of differentiation $N_{st} = 0.73$. Intra-group diversity ranges from 0.012 (Group 1) to 0.030 (Group 2). In all the groups purifying selection was detected at 5% significance level.

The deduced aminoacid sequences showed the presence of Tyrosine in the position 124 for all the variants of group M, while the variants of all the other groups showed the presence of Phenylalanine in that position. Phenylalanine at position 124 originates the epitope responsible for the reaction of the MCA 13 antibody (Pappu *et al.*, 1993), which has been used in Florida to distinguish between mild and severe strains (Permar *et al.*, 1990). Biological data of isolates harbouring a master sequence belonging to group M obtained from Italy (Daden, 2006), Portugal (Bonacalza, 1998) or Spain (Moreno *et al.*, 1991) showed that these isolates are of mild type, not originating stem-pitting on sweet orange or decline on trees grafted onto sour orange. The absence of reaction to the MCA13 antibody is thus valid to identify mild isolates in the Mediterranean region. However, a positive reaction does not necessarily mean a severe isolate: isolates whose master sequence belongs to Group 2, as is the case of the Turkish isolates analysed in this work and by Korkmaz *et al.* (2007) originate mild symptoms on Mexican lime and do not induce decline on trees grafted onto sour orange or sweet orange stem pitting.

No clear relationship could be established with geographic origins. As can be seen in Table 1, isolates harbouring haplotypes from the same phylogenetic groups are found in regions thousands kilometres apart and in countries with small citrus producing areas as Croatia and Cyprus five out of the seven phylogenetic groups are found. However, the sequence variants from groups 3b and M predominate in the Mediterranean. This probably reflects the major sources of CTV dissemination in the Eastern and Western Mediterranean area. In the Near East countries, there is a predominance of isolates harbouring Group 3b. Israel being the country which has the most developed citriculture in the region and in which CTV is endemic is probably the source for dissemination of Group 3b. In the western countries, Portuguese isolates harbouring Group M haplotypes clearly predominate. Besides the isolates analysed here, others which were introduced illegally from Spain and characterized by a set of hybridization probes in asymmetric PCR ELISA assays (unpublished), showed also the prevalence of group M. In Italy there is also the prevalence of Group M isolates, being the Spanish origin very probable. In Morocco the isolates harbouring Group M had also Spanish origin (Lbida *et al.*, 2004). Thus, it appears that Spain represents a source for dissemination of Group M in the western Mediterranean basin.

In conclusion, the population of CTV variants circulating in the Mediterranean basin does not appear qualitatively different from the worldwide CTV population. Variants from group M predominate in countries which introduce plant material from Spain and variants from group 3b in countries which introduce plant material from Israel.

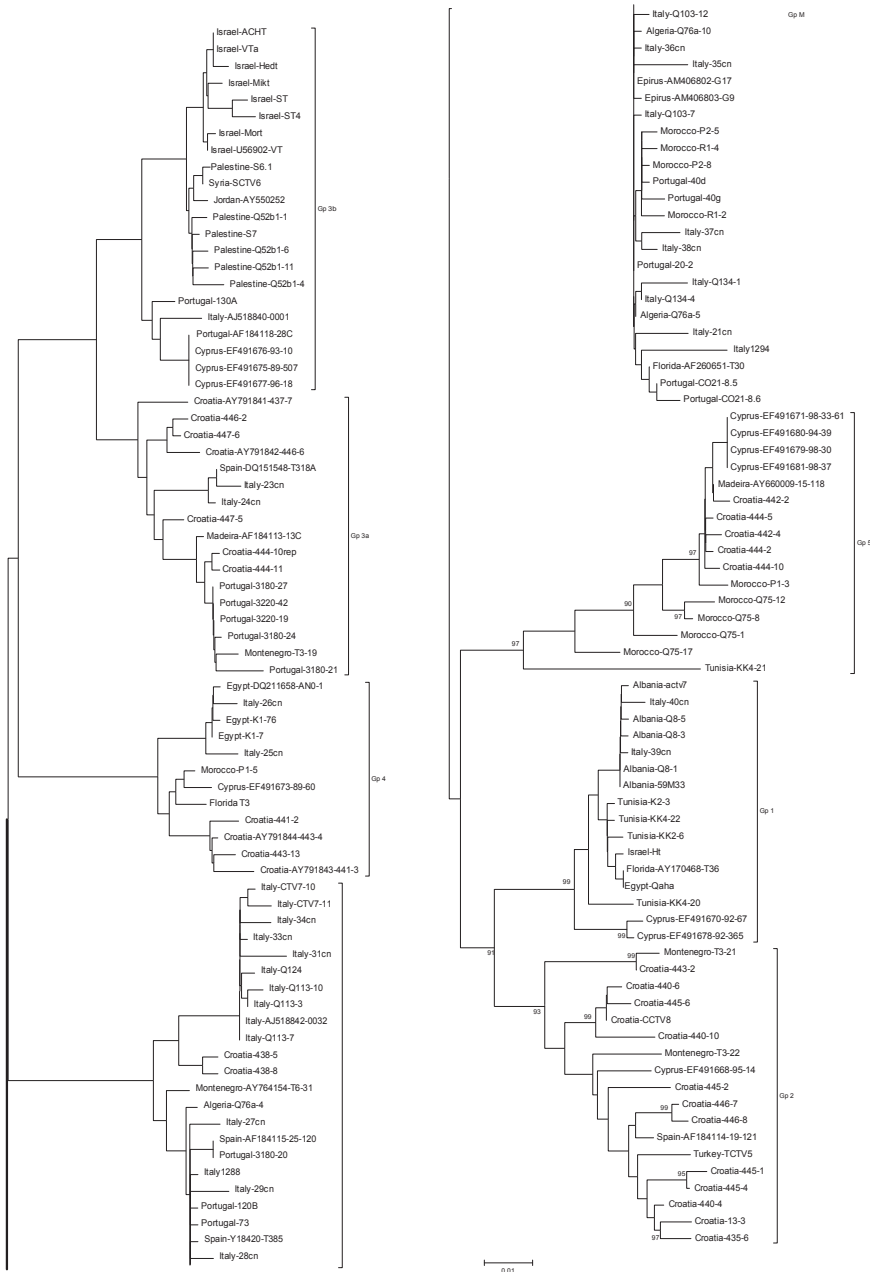


Figure 1. Phylogenetic tree obtained by the Neighbour-joining method applied to the matrix of pairwise distances (kimura 2 p arameters) b etween seq uence var iants o btained f rom 108 M editerranean isolates. Numbers close to the branches represent the bootstrap values when greater than 90%. The 7 groups referred to in the text are represented. Each sequence variant is designed by the country of origin, haplotype designation and GenBank accession number if previously submitted

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Remote sensing to support the monitoring of *Citrus tristeza virus* (CTV) infected areas

Santoro F.¹, Gualano S.¹, Djelouah K.¹, Guarino A.², D'Onghia A.M.¹

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

² Osservatorio Fitosanitario Regione Puglia, Bari, Italy

Abstract. *Citrus tristeza virus* (CTV) is the most destructive pathogen in *Citrus* spp.. It is therefore necessary to promptly identify suspected infected trees on a large scale by the use of satellite imaging. To this aim, the vegetation indices of CTV-infected and CTV-negative citrus trees were preliminarily investigated in order to provide the tree spectral signature detected in the field by the HandHeld Post Dispersive Spectrometer. The study was carried out in selected commercial groves ('Precoce di Massafra' clementine and 'Navelina' orange) located in the CTV foci area, both showing a high CTV infection rate. The trees were previously tested by serological (DTBIA and ELISA) and molecular (PCR) tools to assess the virus presence. The preliminary results highlighted a difference in the spectral signatures of CTV-infected and CTV-free trees, allowing a discrimination of the canopy stress level based on the vegetation indices properly estimated. Further variation was recorded according to the species. Based on these results, the use of vegetation indices from high-resolution multispectral satellite imaging may represent a valid tool backing up the pathogen monitoring in wide areas.

Keywords. Citrus – CTV – Spectroradiometer – Spectral signatures – PCA – Vegetation indices.

La télédétection dans le suivi des zones infestées par le CTV

Résumé. Le virus de la tristeza des agrumes est le pathogène le plus redoutable chez l'espèce *Citrus*. Il est donc nécessaire d'identifier rapidement, et sur une grande échelle, les arbres suspectés d'être infectés à l'aide des images satellites. A cet effet, les indices de végétation des arbres d'agrumes infectés ou non infectés par le CTV ont été évalués préliminairement afin de fournir les signatures spectrales décelées au champs par spectrométrie (HandHeld Post Dispersive Spectrometer). Cette étude a été menée dans des vergers commerciaux sélectionnés (où on cultivait le clémentinier 'Precoce di Massafra' et l'oranger 'Navelina'), dans la zone où étaient localisés les foyers de CTV, qui montraient un taux d'infection de CTV élevé. Les arbres avaient préalablement été testés par voie sérologique (DTBIA et ELISA) et moléculaire (PCR) en vue d'évaluer la présence du virus. Les résultats préliminaires ont mis en évidence une différence dans les signatures spectrales des arbres infectés et des arbres non infectés par le CTV, permettant ainsi de faire une distinction concernant le taux de stress du feuillage sur la base des indices de végétation estimés. Un niveau supplémentaire de variation a été déterminé selon l'espèce. Les résultats obtenus, l'utilisation des indices de végétation fournis par les images satellites multi-spectrales à haute résolution peuvent représenter un outil valable pour le suivi de ce pathogène sur de vastes étendues.

Mots-clés. Agrumes – CTV – Spectroradiomètre – Signatures spectrales – PCA – Indices de végétation.

I – Introduction

Citrus tristeza closterovirus (CTV) is one of the principal causes of damaging and of destruction of citrus in the world. The large scale monitoring of the virus in the citrus growing areas is necessary in order to prevent the entrance and spread of the virus in virgin areas and to control the virus where it is not established yet through the application of eradication measures.

The virus monitoring and eradication are usually supported by aerial photos, for the identification and mapping of the groves, followed by hierarchical tree sampling and virus detection by DTBIA or ELISA.

However, this activity encounters several constraints which make the control of CTV highly difficult to manage in a sustainable manner because of the virus natural spread by aphid vectors. These factors are mainly related to the difficulty in choosing the right area to be monitored, to the symptomless status of the trees, to the time-consuming sampling and laboratory detection assays. Therefore, the success of this program, which is focusing on the virus monitoring in combination with its eradication, is based on the rapid identification and elimination of the infected trees in order to avoid the large virus dissemination, thus eliminating or maintaining the virus inoculum at very low levels.

To this aim, the remote and the proximal sensing techniques, successfully applied for early detection of apple plant stress caused by *Venturia inaequalis* (Delalieux *et al.*, 2007), of spider mite damage in peach orchards in California and of water stress in an olive orchard with thermal remote sensing imagery (Sepulcre-Cantó *et al.*, 2006), showed to be very promising in the detection of CTV infected trees in commercial citrus groves.

The set of data provided by the above-mentioned methodologies may be synthesized through individual analytical parameters called vegetation indices. These parameters are calculated from the sum, difference, ratio, or other linear combinations of two or more spectral bands. They are widely used in modeling studies to estimate the greenness or productivity of vegetation based on the reflectance value (Asrar *et al.*, 1989).

The setting up of proximal and remote sensing in the monitoring of CTV was firstly carried out in the framework of a research project supported by Osservatorio Fitosanitario Regionale of Apulia region, Italy.

II – Proximal sensing

Proximal sensing was applied in CTV infected groves of 'Navelina' orange and 'Precoce di Massafra' clementine, where plants were virus tested by serological and molecular means.

The spectral signature was measured on selected CTV-infected and CTV-negative trees to highlight the *reflectance* differences at various wave lengths. Measurements have been made through a field spectroradiometer, FieldSpec® HandHeld by ASD, placed on the tree canopy at a height h and nadir direction (Fig. 1).

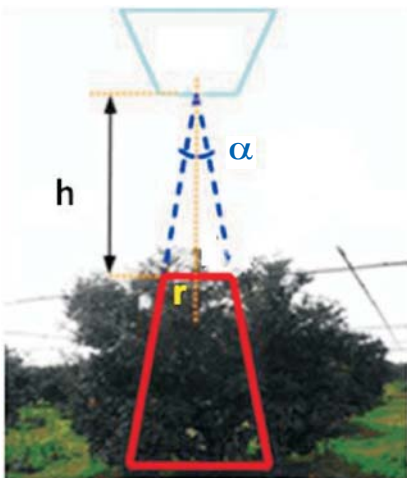


Figure 1. Geometrical scheme of spectroradiometer measurement.

For each measurement, the *white reference* (internal reference for the instrument) procedure has been applied, through a thermoplastic “disc” (Spectralon) and, later, through the acquisition of a set of points which measure the canopy *reflectance*. The results achieved are processed in a single spectral curve (signature), which is representative of the tree canopy spectrum (Fig. 2).

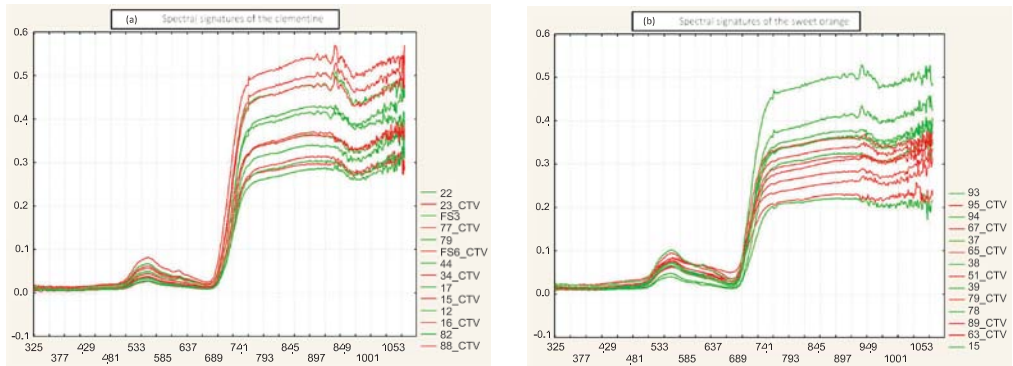


Figure 2. Results of spectral signatures measured in the clementine (a) and sweet orange (b).

For the analysis of the dataset collected in the field and for recognizing differences in the wave shapes of infected plants spectra, a multivariate statistical analysis has been conducted through the PCA (Principal Components Analysis) algorithm (Jolliffe, 2002).

PCA on spectral signatures, aiming at discriminating between CTV-positive and CTV-negative plants, has been based on the dataset of the studied species (orange and clementine).

For ‘Navelina’ orange, discrimination between infected and healthy trees is clear-cut, thereby showing a high separability (Fig. 3).

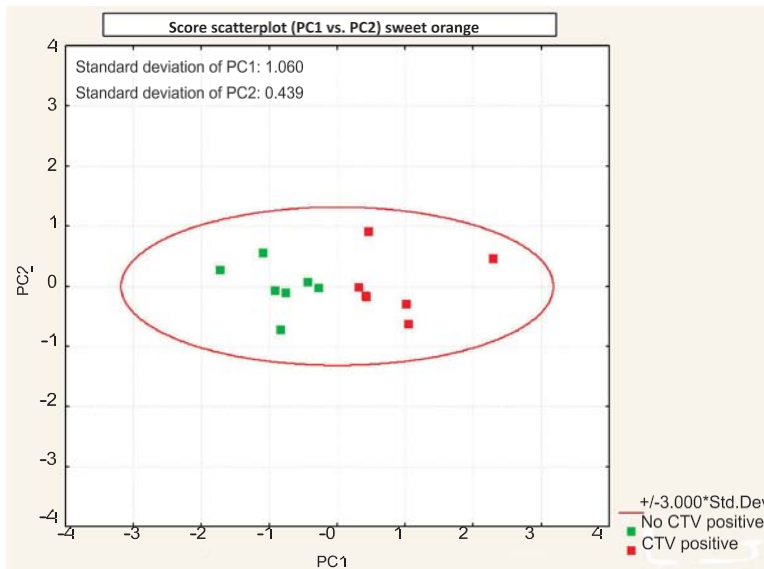


Figure 3. PCA projection of cases for sweet orange.

In contrast, for 'Precoce di Massafra' clementine, the result has not been satisfactory, thereby showing a low separability (Fig. 4).

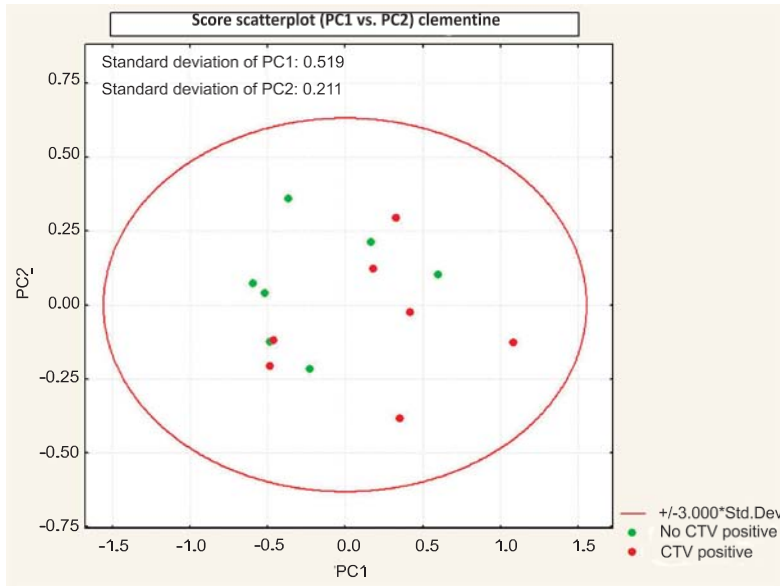


Figure 4. Scatter plot PC1-PC2 for clementine.

In order to synthesize and concentrate in single numerical parameters the wide array of information contained in the spectral signatures (reflectance values in the various wave lengths), two vegetation indices have been calculated: NDVI and mCAI (Fig. 5, 6).

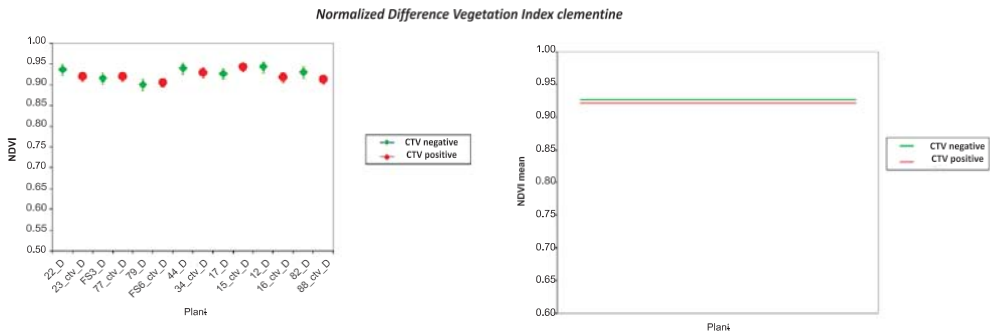


Figure 5. Some vegetation indices of CTV-negative and CTV-positive clementines.

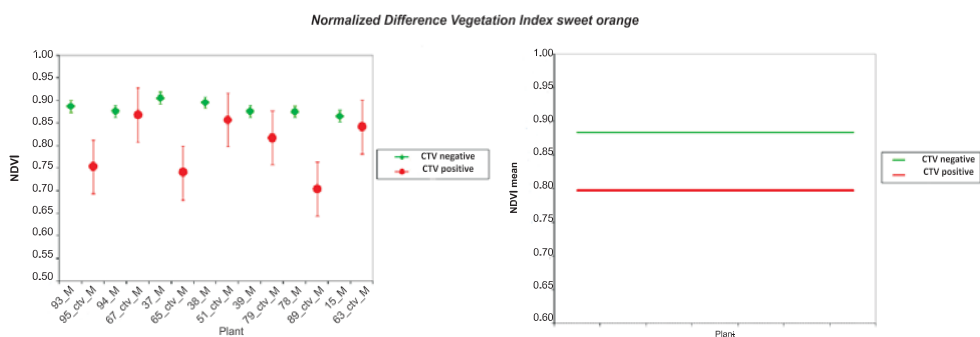


Figure 6. Some vegetation indices of CTV-negative and CTV-positive sweet orange.

The *Normalized Difference Vegetation Index* (NDVI) is widely applied to recognize vegetated areas; it is very sensitive to the presence of biotic symptomatologies (Fletcher *et al.*, 2004).

The mCAI or mCARI analytical index (*modified Chlorophyll Absorption Index* or *modified Chlorophyll Reflectance Absorption Index*) is used to estimate the vigour and the health status of the vegetation (Oppelt and Mauser, 2001).

Both analytical indices, calculated on the basis of spectral signatures, show some variability with respect to laboratory results on the presence-absence of CTV infection. In particular, results concerning ‘Navelina’ orange matched laboratory assays. In contrast, for those concerning ‘Precoce di Massafra’ clementine, mCAI showed some correlation (but not on the whole sample); and the NDVI gave a reliability slightly exceeding 60%.

On the whole, both vegetation indices provide a good indication about the stress status of the canopy which is related to the presence of the virus.

III – Remote sensing simulation

In order to estimate the vegetation indices (e.g. NDVI) corresponding to the spectral bands of the high-resolution satellite QuickBird, a simulation study has been started.

Results report a satisfactory overlapping between the values of NDVI calculated with the spectroradiometer (blue) and those estimated by QuickBird (red) (Fig. 7).

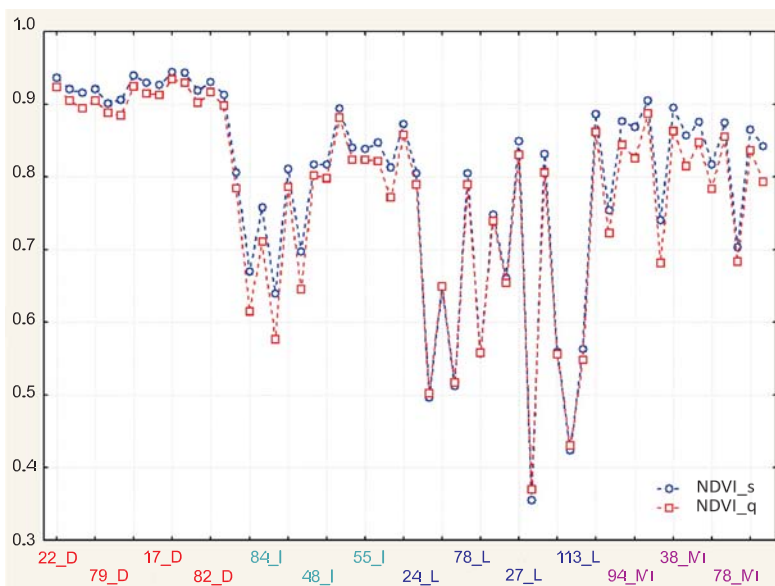


Figure 7. Results of NDVI simulation from QuickBird imagery.

The result is backed up by a satisfactory correlation coefficient, expressed by a regression study, in which the NDVI calculated and that estimated (in all the citrus species under study) have shown a strong correlation averaging 99% (Fig. 8).

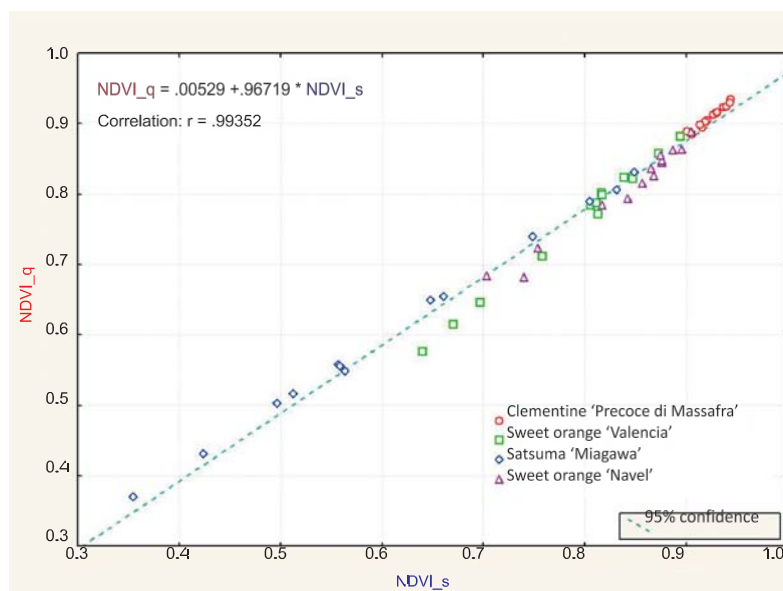


Figure 8. Correlation coefficient of the NDVI calculated and estimated.

The result will be downsized in that the simulation does not take into account the effect of the atmosphere on the image.

IV – Conclusions

These data, combined with adequate statistical analysis, could clearly separate citrus species as clementine and sweet orange. Moreover, the application of vegetation indices (NDVI, mCAI) showed a different variability according to the presence or the absence of the CTV infection in relation to the tested species. Applied to the sweet orange, these indices clearly discriminated between the CTV-positive and CTV-negative trees, whereas a very slight discrimination occurred in the case of Clementine. Therefore, these parameters showed a useful indication about the canopy stress which is apparently highly linked to the presence of CTV infection, even in infected asymptomatic trees.

This study provided preliminary results in the assessment of CTV infection through the reflectance variability; nevertheless, further investigation about the real interaction between the presence of the CTV in the tree as the main source of stress and the reflectance variability is needed.

The proximal sensing technique based on reflectance data could be combined with the satellite imagery and become a highly promising tool for the CTV monitoring on a large scale.

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Preliminary monitoring of *Citrus tristeza virus* (CTV) vectors in Apulia region

Yahiaoui D.¹, Addante R.², Djelouah K.¹, D'Onghia A.M.¹

¹ CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy,

²Dipartimento di Biologia e Chimica Agro-forestale ed Ambientale, University of Bari, (BA), Italy

Abstract. The appearance of the first localized CTV outbreaks in Italy, along the Ionian coast, and the homogeneity within each CTV population, suggested its diffusion by aphid vectors. In the spring 2007, field surveys focused on aphid population took place in three major citrus-growing areas of the Apulia region; the estimation of the number of aphid species landing on citrus trees, their identification and categorization by species were carried out. During the studied period, the population density of each species was evaluated. This research emphasizes the necessity of epidemiological investigations in order to elucidate the spatial-temporal CTV spread patterns and to prevent possible outbreaks in the Mediterranean area.

Keywords. CTV – Aphid – Vector transmission – Apulia – Italy – Citrus.

Suivi préliminaire des vecteurs du CTV dans la région des Pouilles

Résumé. La détection des premiers foyers de CTV en Italie, le long de la côte ionienne, et l'homogénéité au sein de la population de ce virus, nous incitent à supposer que la diffusion soit assurée par des pucerons. Au printemps 2007, des enquêtes ont été réalisées en plein champ dans les trois principales régions agrumicoles des Pouilles pour évaluer la présence des pucerons. On a donc estimé le nombre d'espèces de pucerons qui visitent les agrumes et on les a successivement identifiés et répartis suivant l'espèce. Pendant la période d'étude, la densité de population de chaque espèce a été déterminée. Ce travail a mis en évidence la nécessité de réaliser des recherches épidémiologiques pour éclaircir les modèles de diffusion du CTV dans l'espace et dans le temps et de prévenir d'autres éventuels foyers dans la région méditerranéenne.

Mots-clés. CTV – Puceron – Transmission par vecteur – Pouilles – Italie – Agrumes.

I – Introduction

Worldwide distribution and variable biological behavior of aphids (Homoptera: Aphididae) make them heavily damaging crop pests and efficient vectors for over 200 plant viruses (Harris, 1989). Hence, the spatio-temporal epidemics of plant viruses are strongly compromised by the abundance and feeding behavior of their vector species (Irwin *et al.*, 2000). Within the Mediterranean Basin, the recent establishment of *Toxoptera citricidus* (Kirkaldy) in Spain and Northern Portugal (Ilharco *et al.*, 2005) represents a serious threat to the Mediterranean citrus industry. Moreover, other aphid species landing on Citrus including *Aphis gossypii* (Glover), *A. spiraecola* (Pagenstecher) and *T. aurantii* (Boyer de Fonscolombe) are endemic and known to be less efficient vectors. In Italy, Citrus tristeza closterovirus outbreaks occurred in 2003 within three major citrus growing areas through illegal importation of infected budwood (Davino *et al.*, 2003 and 2005). But, genomic homogeneity among the viral population and its rapid territorial dissemination provided further evidence of its natural spread by aphids (Yokomi and Garnsey, 1987; Cambra *et al.*, 2000; Saponari *et al.*, 2007). In this work a monitoring of different aphid species was conducted in Apulian citrus groves in the framework of a programme supported by Osservatorio Fitosanitario Regionale of Apulia region, Italy.

II – Materials and methods

Several citrus orchards within three major citrus-growing areas in Apulia, including the provinces of Taranto, Lecce and Foggia, were monitored for aphids.

From each grove, a number of 10 trees across the diagonals were randomly monitored and an average of 4 shoots/ tree were taken at different directions and height levels, then, kept in polyethylene bags in order to be examined in the laboratory.

The total collected aphid population was observed under a compound microscope at 25X to 400X magnification, based on Blackman and Eastop (1984) key of identification. In order to make the species identification easier and more effective, a number of specimens were cleared and mounted on slides following the Heikinheimo procedure (1988).

III – Results and discussion

Once visualized under the microscope, aphid specimens were divided into larval stages, apterous and alate morphs. The overall number of collected and counted aphids from all the investigated groves amounted to around 10 000 individuals. It was composed of around 75 % larvae and 25 % adults. Based on Blackman and Eastop taxonomic key (1984), around 2000 apterous adults were identified. Discrimination between *Aphis* species was mainly based on some common morphological characteristics such as antenna with terminal process less than 3 times the length of basal VI and a helmet shaped cauda. Then, *A. gossypii* was characterised by a pale cauda with 4 to 7 setae, while *A. spiraecola* was recognized owing to its dark cauda with 6 to 12 setae.

Similarly, the differentiation between *Toxoptera* species was founded on their antennae with terminal process more than 3.5 times the length of basal VI and the presence of a cauda with more than 10 hairs. The black citrus aphid *T. aurantii* was known by its antennal striations and cauda with less than 20 hairs, which was not the case for *T. citricidus*.

The identification assays showed that *A. spiraecola* and *A. gossypii* were the most abundant aphid species visiting Apulian citrus groves, representing 45% and 40% respectively of the total population in agreement with previously reported data from Spain (Marroquin *et al.*, 2004).

Nevertheless, the percentage of some species reported as less efficient CTV vectors was noticeable, e.g. *T. aurantii*, while other species represented less than 1% of the total aphid population including *M. persicae*.

As expected, the BrCAT *citricidus* has never been found in the framework of this survey.

In the surveyed citrus orchards, aphid colonies were mainly composed of larvae, followed by apterous and winged females. The latter represent the morphs mostly responsible for the establishment of new colonies. A slight decrease in the larvae and apterous adult instars was observed in May, but it was followed by a new increase in June. However, the number of alate landing on citrus trees decreased in the same period, making the migration of the winged morphs to other, often herbaceous, plants (alternative hosts) evident before coming back to citrus during the next autumn. This population dynamics reflects the typical heteroecy or polyphagy of many aphid species.

IV – Concluding remarks

In order to obtain an overall idea about the population dynamics of the different CTV vectors, all the apterous adults were identified and categorized by species and date of collection in the considered period. Thus, *A. gossypii* population was the most important up to June; whereas, the

population of *A. spiraecola* started to decrease in the same period; while, *T. aurantii* appeared later in May but continued to increase up to June, since it is known to be more thermophilic.

As to the distribution of the different aphid species on the surveyed areas, the highest population density of *A. gossypii* was observed in the Northern Apulian region followed by *T. aurantii* and *A. spiraecola*. In Massafra area, the most important CTV foci in Apulia region, a lower aphid population was scored, with a higher infestation of *A. spiraecola* compared to *A. gossypii* and the total absence of *T. aurantii*. In the Southern part, *A. spiraecola* prevailed over the other two species.

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Citrus sanitation methods for the elimination of *Citrus tristeza virus* (CTV)

Meziane M.¹, Frasheri D.², Carra A.³, Djelouah K.², Carimi F.³, D'Onghia A.M.²

¹ Université Hassiba Ben Bouali, Chlef, Algeria

² CIHEAM – Mediterranean Agronomic Institute, Valenzano (Ba), Italy

³ Istituto di Genetica Vegetale del Consiglio Nazionale delle Ricerche, Palermo, Italy

Summary. The use of 'healthy' propagating material is of utmost importance in the control of CTV, which is the most serious virus affecting citrus trees worldwide. All the sanitation methods available are effective in CTV elimination; shoot-tip-grafting (STG) and somatic embryogenesis from stigma and style *in vitro* culture (SE) are the preferred ones for their numerous advantages. Both techniques are successful in regenerating CTV-free plants, identical to the mother tree, and they can also be applied in the safe international exchange of citrus healthy germplasm. SE is user friendly and can be extensively applied to all Citrus species whenever suitable explants are available; while STG can be applied in the sanitation of clementines and pomelos, which are not regenerated by SE.

Keywords. Citrus – Citrus tristeza virus – Sanitation – Somatic embryogenesis – Shoot-tip-grafting.

Méthodes d'assainissement des agrumes pour l'élimination du virus de la tristeza des agrumes (CTV)

Résumé. L'utilisation du matériel de multiplication « sain » est d'une importance primordiale pour le contrôle du CTV, considéré comme le plus dangereux virus affectant les agrumes dans le monde. Toutes les méthodes d'assainissement disponibles sont efficaces pour l'élimination du CTV, cependant, considérant leurs nombreux avantages, le greffage d'apex et l'embryogenèse somatique, à partir de stigma et stylet en culture *in vitro*, sont les techniques préférées. Les deux techniques sont efficaces à régénérer des plantes indemnes de CTV, identique à la plante mère, et ils peuvent également être appliquées dans les échanges internationaux des ressources phylogénétiques d'agrumes sains. L'embryogenèse somatique est une technique simple, et peut être largement appliquée à toutes les espèces d'agrumes, lorsque les explants appropriés sont disponibles ; alors que le greffage d'apex peut être appliqué dans l'assainissement des clémentines et pomelos, qui ne sont pas régénérés par l'embryogenèse somatique.

Mots-clés. Agrumes – Virus de la tristeza des agrumes – Assainissement – Embryogénèse somatique – Greffage d'apex.

I – Introduction

One of the major constraints for Citrus cultivation is represented by the presence of *Citrus tristeza closterovirus* (CTV), a graft-transmissible agent, which may significantly cause the death of the trees or considerable losses in crop yield and quality, especially where sour orange (*Citrus aurantium* L.) is the predominant rootstock (Roistacher, 1991). CTV may often remain symptomless, thus representing a special risk in its spread over long distances through citrus infected propagating material. Once the infection arrives in a citrus growing area, its rapid spread is assured by different aphid species, which renders the virus control difficult. The worldwide movement of CTV and relative vectors, along with the infected citrus material, has increased in the last years due to the globalization and the lack of adequate quarantine measures (Moreno *et al.*, 2008). The production and use of 'healthy' citrus nursery plants is therefore the most efficient preventive control strategy and its use is also recommended in cross protection programmes to avoid possible synergy with other CTV strains and virus and virus-like agents. 'Healthy' citrus plants are produced in the framework of the clonal and sanitary selection programme, which

includes the recovery of all selected candidate trees (D'Onghia *et al.*, 1998). Several sanitation methods are available with different efficiency in virus elimination; the best procedure should be user friendly in terms of environmental conditions and skills and the following factors should also be considered: (i) time needed for plant regeneration; (ii) maintenance of true-to-type characters in produced plants; (iii) absence or rapid loss of juvenility characters.

II – Sanitation methods

Regenerated nucellar plants can be easily produced and they are considered free from CTV and most of infectious agents (Weathers and Calavan, 1959). Unfortunately, they show juvenility characters (e.g. excessive thorniness, vigorous and up-right habit, slow fruit growth etc.), which may persist for many years and over many budded generations (Roistacher, 1977). Moreover high variations among citrus nucellar budlines were also observed (Navarro *et al.*, 1985). These limitations were overcome using heat-treated buds by hot air at 35-42°C for 78-107 days with budwood preconditioning (Roistacher, 1977); thermotherapy was firstly applied for recovering citrus trees infected by tristeza and psorosis (Grant, 1967); it is effective for the elimination of most graft-transmissible agents but not of viroids and spiroplasmas (Calavan *et al.*, 1972). Disadvantages of nucellar plants and thermotherapy were finally solved by *in vitro* shoot-tip grafting (STG) (Navarro *et al.*, 1975; Navarro, 1981; 1988; 1992), which is currently the sanitation method of several extensive citrus improvement programmes for controlling CTV infections worldwide. Shoot tips of 0.14-0.18 mm in length, aseptically isolated from diseased citrus plants, are grafted (inverted T graft) onto young etiolated seedlings grown *in vitro* (Navarro *et al.*, 1975). About one month after grafting, plants are usually *in vivo* transferred and set fruits two, three years later. Produced plants are true-to-type and do not show juvenility characters. To increase explant survival, bigger shoot tips are collected from thermotreated buds at 32-35°C; this combination reduces virus replication and increase meristematic cell activity, thus obtaining clean shoot-tips to be used with STG. Indeed, the use of both methods is effective in the sanitation of some agents (e.g. *citrus psorosis virus*), which are difficult to eliminate using only STG (Carvalho *et al.*, 2002). Moreover, a safe introduction of citrus germplasm can be achieved by micrografting *in vitro* shoot-tips excised from imported budsticks *in vitro* cultured at 32°C (Navarro *et al.*, 1984; 1991). The number of successful grafts depends upon species, varieties and type of virus strain; however, the operator's skill is still a crucial point in the grafting survival and sanitation rate.

Different is the case of citrus somatic embryogenesis from stigma and style *in vitro* culture (Carimi *et al.*, 1995), which is the most recent sanitation technique for the total recovery of the most infected Citrus spp., except for clementines and pomelos. It is user friendly and can regenerate a number of healthy plants from different embryo lines (D'Onghia *et al.*, 1997). Styles and stigmas are dissected from freshly collected closed flowers under laminar flow. Explants are vertically placed in Petri dishes with the cut surface in contact with medium supplemented with BAP. A creamy-white callus appears on the cut surface of the style base about two weeks after culture initiation, while green somatic embryos develop 2-7 months after culture initiation on the callus surface after several subcultures. After germination, embryos develop into plantlets which are *in vivo* transferred. Regenerated plants begin fruiting on some branches after three years with different grade according to the species. Flowering usually occurs 3-4 years later in plants growing in the field.

Unlike other techniques, stigmas and styles are considered better antigen sources than other tissues normally used for viral detection as for CTV and CPsV; even if the callus obtained is still highly infected (D'Onghia *et al.*, 2000; Djelouah *et al.*, 2002), all the embryos formed are totally free from CTV as for most graft-transmissible agents. Juvenility in regenerated plants is lost after the first year of *in vivo* growth and plants are virtually identical to the original source (D'Onghia *et al.*, 2000). It is successfully used in the safe exchange of citrus germplasm with very little

manipulation. The major limit in the success of regeneration by SE is the flower explant, which must be freshly collected before opening or stored at 4°C for 5-6 days. The storage period can reach 20 days, depending on species and varieties.

A crucial phase of *in vitro* sanitation techniques is the acclimatization of regenerated plants. Plants can be *in vitro* transplanted directly into plastic pots containing sterilized soil or the apical portion can be grafted onto a 4-6 month-old rootstock seedling. Pots are closed into polyethylene bags and maintained in a greenhouse at 25°C; after 1 week bags are opened and left 10 days more before being removed (De Pasquale *et al.*, 1999).

III – Sanitary and genetic analyses

Whatever the sanitation method used is, regenerated plants must undergo sanitary and genetic controls, to assess virus elimination and their trueness-to-type.

Several sanitary assays are nowadays available for CTV detection. A preliminary virus detection should be performed by serological (DAS-ELISA or DTBIA) or molecular assays (probe hybridization or PCR-based assays) (Bar Joseph *et al.*, 1979; Garnsey *et al.*, 1993; Cevik *et al.*, 1996; Cambra *et al.*, 2000; Bertolini *et al.*, 2008); if results are negative, biological indexing by graft transmission onto the universal indicator, 'Mexican lime' [*C. aurantifolia* (Christm.) Swing.] should also be applied (Roistacher, 1991) in thermo-conditioned greenhouse at 18-24°C.

Morpho-pomological traits and genetic assays by flow cytometric analysis and DNA-based techniques (ISSR and RAPD) are used to detect the genetic fidelity in regenerated plants (Coletta *et al.*, 1998; Fang and Roose, 1997).

IV – Conclusion

CTV is easily eliminated by most of the available sanitation methods. Nevertheless, somatic embryos from style and stigma *in vitro* culture seem to meet most of the criteria of selection which are considered important in the choice of the method. Unlike STG, it is user friendly, it doesn't need the combination with thermotherapy to increase the success and all regenerated plants are totally free from CTV. Even if juvenility characters usually appear in some species or varieties, they are easily lost in the first years of growth and the plants produced are identical to the mother trees. As for STG, the time needed for regenerating plants ready to set fruits can vary from 3 to 4 years. This technique is also applicable in the safe exchange of citrus germplasm. However, limitations are still the need for fresh or stored flowers for a short period and the unsuccessful regeneration in clematines and pomelos. Hence, the improvement of protocols is always desirable and STG can remain the alternative whenever these limitations are present.

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Natural tolerance/resistance of citrus plants to Citrus tristeza disease

D'Onghia A.M.

CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Since tristeza is the most serious citrus virus disease worldwide, knowing the natural defense systems of citrus plants is still considered the main control approach. In this paper an account is given of the attempts to use CTV tolerant/resistant cultivars and rootstocks and classical cross-protection programmes to contain tristeza disease.

Keywords. Citrus – Citrus tristeza virus – Resistance – Tolerance.

Tolerance/résistance naturelle des plants d'agrumes à la maladie de la tristeza des agrumes

Résumé. Vu que la tristeza est la maladie à virus la plus redoutable chez les agrumes à l'échelle mondiale, la connaissance des mécanismes naturels de défense des plants d'agrumes représente aujourd'hui encore la principale méthode de lutte. L'objectif de ce travail est donc de passer en revue les tentatives d'utiliser les cultivars et les porte-greffes tolérants/résistants au CTV et d'appliquer des programmes de protection croisée classiques pour limiter la diffusion de la tristeza.

Mots-clés. Agrumes – Virus de la tristeza des agrumes – Résistance – Tolérance.

I – Introduction

Tristeza is the most devastating virus disease of Citrus worldwide. Millions of trees on sour orange rootstock have been killed or have become unproductive after being infected with *Citrus tristeza virus* (CTV) - induced decline. Tristeza epidemics started in the Western hemisphere in the 1930s whereas in the Mediterranean the first disease outbreaks occurred in the 1950s in Israel and Spain (Roistacher, 1991; Whiteside *et al.*, 1988). In the last years, CTV has been reported in the rest of the Mediterranean region (Djelouah and D'Onghia, 2001) and disease epidemics have been found in Italy (Davino *et al.*, 2003).

The virus, which is a *Closterovirus*, has been globally distributed through infected citrus propagating material, whereas locally it is transmitted semi-persistently by different aphid species (Gottwald *et al.*, 1997), primarily *Aphis gossypii* and *A. spiraecola* in the Mediterranean. *Toxoptera citricidus*, the most efficient CTV vector worldwide, is now present in Northern and Central Portugal and in Northern Spain (Ilharco *et al.*, 2005), and represents a serious threat of CTV rapid spread to the other European and Mediterranean citrus-industries.

CTV strains are broadly grouped according to how they affect certain plants or scion/rootstock combinations: decline and death of most cultivars on sour orange rootstock (*Citrus aurantium* L.) 'quick decline' (CTV-QD); seedling yellows symptoms (CTV-SY), stem pitting (CTV-SP) of grapefruit (*C. paradisi* Macf.) and of sweet orange (*C. sinensis* L. Osb.) which usually can induce stunting, poor yield and fruit quality, and rarely the tree death (Lee and Rocha-Pena, 1992).

The virus causes considerable economic losses and the current management approaches (e.g. use of pathogen-free stock, tolerant rootstocks, eradication of infected trees, vector control) are unlikely to provide long-term durable control. However, several attempts have been made for gene manipulation by selection and breeding programmes but this is a very difficult and long

practice due to the large genetic distance between resistant and citrus cultivars. The production of CTV resistant biotech citrus plants for commercial purposes is highly attractive, but it is still underway in several laboratories. Hence, the effective management of CTV-induced diseases, based on knowledge of natural plant defence mechanisms, remains an important challenge for the sustainability of the Mediterranean citrus industry.

II – Tristeza tolerance/resistance variability

Alternative rootstocks are chosen instead of the sour orange in order to eliminate the graft-incompatibility induced by CTV-QD in many citrus cultivars on sour orange. In fact, the use of CTV tolerant/resistant rootstocks has contributed to reduce the damages caused by the disease but this has also limited the range of suitable rootstocks as regards other phytosanitary conditions. The trifoliolate rootstocks, for instance, are highly susceptible to most citrus viroid diseases (Mestre *et al.*, 1997a) and this is a major problem when citrus viroids are present in propagating materials. Therefore the use of 'healthy' propagating material is crucial in any CTV control strategy.

A first attempt to group symptomless rutaceous plants in relation with citrus tristeza virus strains was made by Bové (1995). For most citrus species considered symptomless, it is not clear yet whether they are tolerant or resistant. When a citrus tree is resistant to a CTV strain, this means that it can not be infected systemically, whereas if it is tolerant the virus can spread systemically without significantly affecting its growth and yield. Even if cultivars and rootstocks are potential sources of resistance, unfortunately their resistance mechanisms are still poorly characterized.

Variability of tolerance/resistance expression is mainly related to the virus isolate and to the environmental conditions. Several examples are reported worldwide. Washington navel and Valencia sweet oranges were tolerant to the Brazilian CTV strains, whereas Pera sweet orange was susceptible to the severe stem pitting isolates. In contrast, CTV isolates in Spain and Israel could induce great damages on Valencia and Washington navels. The K strain of CTV from Corsica (France) is an example of a mixture of strains which, when present, induces no symptom in lime seedlings, the universal CTV indicator, even if the virus multiplies well in the infected limes. However, aphid transmission of this strain separates isolates and these isolates result in strong symptoms in Mexican lime [*C. aurantifolia* (Christm.) Swing.] (Bové, 1995).

Pummelo [*C. maxima* (Burm.) Merrill or *C. grandis* (L.) Osb.] represents a very good example of the great variability of symptom expression which is strictly related to the CTV isolate type (Fang and Roose, 1999; Garnsey *et al.*, 1996). In fact, based on symptom expression (dwarfing and stem pitting), 18 pummelo cvs were classified as tolerant, moderately tolerant and susceptible to CTV-SP (Xueyuan *et al.*, 2002). However, resistance in pummelo seems to cover a limited number of CTV isolates. The same condition is observed in other genera within the Rutaceae-Aurantioideae (*Severinia*, *Atlantia*, *Fortunella*, *Glycosmis*, *Murraya*, *Triphasia*, *Feronia*, *Feroniella*, *Aegle*, *Merrillia*) while little is known yet on the resistance mechanisms (Garnsey *et al.*, 1987; Mestre *et al.*, 1997b; Yoshida, 1996).

Recently, an attempt to select CTV tolerant hosts was made using CTV RNA concentration in the plant tissues (Targon *et al.*, 2007); the expression of p23 gene, which has probably a regulatory role in the virus cycle and/or pathogenesis, and of p25 and p27 genes, which are the CTV coat proteins, was higher in a susceptible variety (Pera orange) compared with a tolerant variety (*C. reticulata* Blanco Ponkan mandarin) using Real time PCR.

Other cases are related to the effect of CTV isolates on trees which are grafted onto tolerant rootstocks as in South Africa, where CTV usually occurs as a mixture of stem pitting and seedling yellows strains; the effect of this mixture proved to be more severe on trees grafted onto Troyer citrange (*Poncirus trifoliata* x *C. sinensis*) than on trees grafted onto rough lemon (*C. jambhiri*) and Volkameriana lemon [*C. limon* (L.) Burm.f.] rootstocks. The effect of CTV isolates without

the seedling yellows component was less severe on tolerant rootstocks. The symptom severity seemed to be affected by the cultivars (rootstock and scion) and the climate. The reason is still unknown because the citrange parents are trifoliolate orange (resistant) x sweet orange (tolerant) (Van Vuuren, 2002). Nevertheless, the occurrence of CTV isolates, which could replicate at a low level in trifoliolate orange in New Zealand (Dawson and Mooney, 2000), and of the Indian CTV isolate, which produced the same results (Hilf, 2005), poses a major threat to the effectiveness of CTV resistance derived from trifoliolate orange.

However, emphasis has been laid on the broad spectrum CTV resistance expressed by trifoliolate orange, [*Poncirus trifoliata* (L.) Raf.] compared to the CTV strain-specific resistance found in pummelos. This resistance, which is associated with a single dominant gene at the *Ctr* locus, proved to be effective against all the tested CTV isolates. This was a potentially very useful character since this species is also sexually compatible with *Citrus* members (Barrett, 1990; Mestre *et al.*, 1997b). Moreover, *Ctr* was not the only locus responsible for CTV resistance in *P. trifoliata*, but at least one other gene seems to be involved. Given that citrus is a perennial crop, breeding for durable disease resistance should involve selection at both the *Ctr* and *Ctm* loci (Mestre *et al.*, 1997a). Although the dominant gene has already been characterized and mapped, much research effort is still focused on the possibility of transferring this resistance to some important citrus varieties by molecular transformation (Deng *et al.*, 2001; Yang *et al.*, 2003). Some studies undertaken to determine the type of resistance involved, demonstrated that CTV could replicate in mesophyll protoplasts from trifoliolate orange and from other citrus relatives which are resistant to CTV (*Swinglea glutinosa* (Blanco) Merr., and *Severinia buxifolia* (Poir) Ten.). In these cases resistance is probably due to the block of either cell-to-cell movement or long-distance movement, or to an induced resistance response (Albiach-Marti *et al.*, 2004).

III – Cross protection

One of the most effective strategies to face citrus crop losses, where severe CTV strains are endemic and vector populations are abundant, is based on cross protection. Cross protection against CTV is induced by challenging severe virus strains with mild strains in order to prevent disease expression (Lee *et al.*, 1987). Strain sources are usually infected plants showing mild infection symptoms or no effect at all in areas where severe CTV strains cause serious problems. In California, the severe strains were eliminated by transferring the isolate to *Passiflora* spp.; moreover, aphid vectors, thermotherapy or shoot-tip-grafting can make a positive contribution to separate mild strains from severe strains. After a preliminary plant screening in the greenhouse of a number of CTV isolates for symptom expression on susceptible varieties, the promising protecting isolates are then tested in the field against natural infections with severe strains. This type of disease control has proved to be the most successful one in many countries worldwide (i.e. Brazil, Australia and South Africa) where susceptible citrus varieties have been planted in the nursery after being infected with a mild CTV strain. Cross protection has prevented low yields and small-sized fruits of Pera sweet orange in Brazil (Costa and Müller, 1980) and Marsh grapefruit in South Africa (Van Vuuren *et al.*, 1993). The cross protection mechanism is not well known; however, protection breaks down over time due to many factors such as the variety, the virus strain and the environmental conditions. The continuous challenge of an existing or of a newly introduced different severe strain can also overcome the mild strain effect in cross protection as reported in Florida for sweet orange on sour orange rootstock after eight years (Powell *et al.*, 1992). The synergistic effect of another virus with a mild CTV strain can also break the protection effect, as reported for a citrus viroid, on the growth and production of Delta Valencia orange on Yuma citrange rootstock (Van Vuuren and Da Graça, 1996). For this reason a certification scheme was developed in South Africa, using healthy plants which were CTV-preimmunized.

Another successful cross protection programme was also developed in Peru, where economic disasters were caused by a CTV stem pitting strain, which induced a scion disease affecting

citrus trees regardless of the rootstock. This situation led Bederski *et al.* (2007) to search for highly productive CTV symptomless carriers within the same citrus cultivars. They found quite a high level of cross protection of grapefruit cvs 'Star Ruby' and 'Flame', when UCLA rough lemon was used as a rootstock. Moreover, a single nine-year old Star Ruby tree on *Citrus shekwasha* remained symptomless for stem pitting and highly productive with uniform large sized fruits, despite the heavy inoculum and vector pressure. In this case, the character involved was not rootstock dependent because 'Star Ruby' was successfully propagated also onto a rootstock other than *C. shekwasha*.

In conclusion, resistant trees to be used for commercial purposes are still many years away. While research into the application of the resistance gene found in *P. trifoliata* is making progress, the use of tolerant/resistant rootstocks and varieties or the development of cross protection programmes are nowadays the only available and efficient alternatives to control tristeza disease and make citrus cultivation possible.

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Third part

Regulations on citrus certification and CTV monitoring

Updated regulation on Certification of citrus propagating material in the Mediterranean region

D'Onghia A.M.

CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. An updated regulation for the certification of citrus propagating material is presented, taking into account the EPPO standards, other European certification schemes and the current phytosanitary problems of the citrus industry in the Mediterranean region. The technical protocols for the production, conservation and use of healthy propagative material are based on the recent advances in pathogen detection and elimination in *Citrus* spp. and on the gaps in the running certification programmes. Most of the methods and procedures included in the protocols have been validated in the framework of the MNCC activity.

Keywords. Citrus – Certification – Nursery – Graft-transmissible pathogens – Trueness-to-type – Mediterranean.

Mise à jour de la réglementation sur la certification du matériel de multiplication des agrumes dans la région Méditerranéenne

Résumé. Une mise à jour de la réglementation sur la certification du matériel de multiplication des agrumes est présentée, ceci en tenant compte des normes de l'OEPP, d'autres systèmes européens de certification ainsi que les aspects phytosanitaires des agrumes dans la région du bassin Méditerranéen. Les protocoles techniques pour la production, la conservation et l'utilisation du matériel de multiplication sain est basé sur les progrès réalisés dans la détection et l'élimination des pathogènes sur agrumes ainsi que sur les lacunes des différents programmes de certification en cours. La plupart des méthodes et procédures figurant dans ce protocole ont été validées dans le cadre des activités du MNCC.

Mots-clés. Agrumes – Certification – Pépinière – Agents pathogènes transmissibles par greffage – Authenticité – Méditerranée.

I – Introduction

Virus and virus-like diseases are severely affecting citrus species worldwide, inducing heavy losses in many citrus growing areas. The need for detecting them and the impossibility to apply any curative treatment, including the chemical control, has led many countries and international organisations to undertake and promote research and sanitary improvement programmes.

This situation is further worsened by cloning processes and intensive trade of plant propagating material due to globalization, thus extending and internationalizing phytovirological problems that would be otherwise confined to limited areas.

As regards the Mediterranean region, citrus is infected by numerous graft-transmissible diseases, among which some, like *Spiroplasma citri*, are also spread by insect-vectors. There are no tolerant rootstocks to control the severe strains of CTV and the use of preimmunized plants is the only solution which can be envisaged when these virus strains become established. Even in this case, preimmunization can only be applied on 'healthy' nursery plants to avoid possible synergy with other CTV strains as well other virus and virus-like agents. Fortunately, 'huanglongbing' (induced by *Ca. liberibacter* spp.), which is devastating the most important citrus industries worldwide (e.g. Brazil and Florida, USA), is not present yet with its vectors in

the Mediterranean region. Preventing the entrance and dissemination of these diseases and their vectors through the use of non infected material for the establishment of citrus groves is economically and environmentally more efficient than eliminating pathogen outbreaks from which the infection can further be propagated by the vectors. Therefore the adoption of preventive rather than curative measures to control citrus graft-transmissible diseases, involving the use of 'healthy' propagating material, seems nowadays the strategy that can ensure the best results. This is particularly true if you consider that man is the main responsible in the dissemination of these diseases and, sometimes, even the only vector.

With this end in view, since 1995 CIHEAM/MAI-Bari has promoted the establishment of a dedicated Mediterranean Research Network on Certification of Citrus (MNCC), to bring together experts, Plant Protection Service officers and representatives of the private sector and make a positive contribution to the setting up of a scheme for the certification of citrus propagating material and for the monitoring of quarantine citrus pathogens. The network strategy was in line with the aims of Barcelona Declaration (1995) for the establishment in the Mediterranean region of a free trade area of agricultural products in the framework of a comprehensive Euro-Mediterranean partnership.

Citrus propagating materials "of equal category" are obtained in the various Mediterranean countries by different selection procedures and certification systems. Therefore, the harmonization of procedures, technical protocols and regulations to be applied in the Mediterranean Region has become the first priority of the network. Based on EPPO protocols and on the experience gained by several European and Mediterranean countries, during the network activity, a Mediterranean citrus certification scheme has been developed; moreover, an inventory on citrus genetic resources in the Region has also been made, aimed at providing useful information on native citrus germplasm of interest for the implementation of certification programmes at national level.

With the support of the CIHEAM/Master of Science research, MNCC partners could investigate in each country the presence of citrus diseases with a high epidemiologic potential, such as Tristeza, transmitted by insect vectors, in order to pave the way to a national certification programme. However, certification measures must be supported by the implementation of a mandatory programme for the control of quarantine pathogens.

Based on the results obtained by the network on the pathological status of citrus in the Mediterranean, CIHEAM MAI-Bari has contributed, in close collaboration with the Mediterranean Ministries of Agriculture and research institutions, supported by cooperation aid across Europe (primarily the Italian), to the advance of certification of citrus propagating material. This programme was firstly implemented in Italy, and later extended to other Mediterranean countries (Albania, Algeria, Egypt, Lebanon, Malta, Tunisia).

II – Requirements for establishing a certification programme

CERTIFICATION of propagating material is defined as follows: *a procedure whereby candidate trees, to be used as source of material for propagation, undergo controls (trueness-to-type and sanitary) and, whenever necessary, sanitation treatments to secure varietal conformity, clonal origin of the material, a given sanitary status (freedom from a number of pathogens) as specified by regulations officially issued, or endorsed, by competent Governmental Agencies.* Based on this definition, the first step in the implementation of a certification programme in a country is drafting a dedicated law to regulate production, conservation and utilization of citrus Certified plant material.

To this end, it is necessary to identify the existing national laws on quarantine and marketing of citrus propagating material, that are to be complied with and harmonized. As a result, the peculiarity of each country system and the compliance with the international standards, with

special reference to the Mediterranean region, shall be taken into account so as to provide absolute guarantees on the quality and homogeneity of the final product and the relative label.

Hence, the regulation shall provide for the establishment of the certifying authority, responsible for inspections and controls, and illustrate the steps from the acquisition of primary sources to the propagation of citrus plants in the nursery. Moreover, the responsible institution, the trueness-to-type and phytosanitary protocols, the facilities and controls shall be specified for each phase. In this programme all the experts involved (pomological and genetic, phytosanitary, propagation competences etc.) shall work in close cooperation. Considering the costs of clone breeding (sanitary and clonal selection, diagnosis, conservation, sanitation) and those pertaining to the subsequent steps of certification (maintenance, genetic and sanitary controls included in the protocols, etc...), the material to be used should be selected from the most widespread, long-cultivated and locally relevant varieties.

Label harmonization is fundamental for the recognition of the material throughout the whole region in order to meet Barcelona requirements for the free movement of plant materials in the Mediterranean. A good certification scheme involves the use of a clear, detailed and reliable label, reporting several identification data (producer, lot number, Certifying Body, etc...) and indicating the category '*Pathogen-tested*' or '*Pathogen-free*'. Each plant category includes a different number of pathogens from which citrus plants must be free; the list of the pathogens is clearly indicated in the regulation and must be officially harmonized across the region.

Confusion should be avoided over the «Certified» category (blue label) and the «*Conformitas Agraria Comunitatis-CAC*» category (orange label). CAC material has the minimum requirements (clonal origin, trueness-to-type, sanitary status), which guarantees the free movement of citrus plants in EU; it is a mandatory programme, under the nursery responsibility; however CAC material is totally replacing the 'standard' material; unfortunately, controls are only visually based whereas most of citrus graft-transmissible agents can remain symptomless. As regards '*Pathogen-tested*' and '*Pathogen-free*' plant material in the framework of the certification programme, the freedom from the listed pathogens and the trueness-to-type are ascertained through objective assays, which are carried out in each step from the parent 'primary source' until the nursery plants. Moreover this programme, which is voluntary, is under the responsibility of the Certifying Body in charge for controls.

In the light of these considerations, a Mediterranean scheme for citrus certification seems to be very relevant. Moreover, since it is necessary to design a «self-propelling» and self-sustaining system, the development of an active nursery sector should be encouraged. Nurserymen are the most important players of the whole chain and the success of a certification scheme greatly depends on their support. They should be more aware of the challenges they must take up and receive training to innovate their operations in compliance with the new certification needs.

III – Conditions supporting certification legal rules

Conditions that can facilitate the adoption and implementation of legal rules on certification are the followings:

- i. the identification of the Certifying Body, possibly within a national institution (Ministry of Agriculture, for example) responsible for the service;
- ii. the appointment of a Scientific Technical Committee on Certification as the advisory board of the Certification, which shall include not only the representatives of the Certifying Body, but also the representatives of the professionals involved in the different certification steps (citrus pathologists, pomologists and breeders, nurserymen and producers' representatives);
- iii. the setting up of an autonomous Certification Service with its branch sections, responsible for the sanitary and genetic controls as laid down in the protocols;

- iv. the creation of a National Citrus Variety Register, including all the selections admitted for certification; the enrolment in this register shall automatically lead to the transfer of the clones to the subsequent certification steps;
- v. the definition of the different certification steps and types of material produced in accordance with the international criteria; the following steps should be envisaged: a) establishment of the primary sources; b) Conservation for Premultiplication (Prebasic material); c) Premultiplication (Basic material); d) Multiplication and Plant propagation (Certified material);
- vi. the planting of increase blocks, recognized by the Certifying Body, to meet the specific needs of some citrus species/varieties and favour the rapid production of Basic and Certified material;
- vii. the identification of the sites where the different steps shall be implemented, preferably in areas where none of the citrus is grown or found free from vector-transmitted pathogens (i.e. CTV, *S. citri*); therefore a Quarantine programme for the mandatory control of these pathogens is necessary in order to promptly identify and eliminate the infected sources;
- viii. the establishment of the primary sources: the institutions and/or bodies which will be responsible for the breeding of clone candidates shall be selected based on their recognized competence;
- ix. the Conservation for Premultiplication shall be organized and managed under the control of the Certifying Body;
- x. the Premultiplication shall be organized and managed by public or public/private bodies, recognized by the Certifying Body.

IV – Regulation for the certification of citrus propagating material

This regulation is applied to the certification of plant propagating material belonging to the genera *Citrus*, *Poncirus*, *Fortunella*, as well as other genera of *Aurantioidae* and their hybrids.

The rules set forth the production of the primary source and for the organization of the certification programme (Table 1) is hereafter reported as general provisions and technical specifications.

Table 1. Organization of the Certification programme of citrus propagating material.

Steps	Plant Category	Label Color	Site	Facility	Controls
Establishment of primary sources	Primary source	Red	Scientific institutions	Screenhouse	varietal & sanitary
Conservation for premultiplication	Prebasic	White with purple band	CCP	Screenhouse	
Premultiplication	Basic	White	CP	Screenhouse	
Multiplication	Certified	Blue	SMP/RMP	Open field/ screenhouse	
			Nursery	Open field/ screenhouse	sanitary

CCP: conservation centre for premultiplication;

CP: centre for premultiplication;

SMP: scion mother plots

RMP: rootstock mother plots

1. General provisions

Art. 1 - Organisation of the National Certification Service (NCS)

The regulation orders:

- the establishment of a National Service for Voluntary Certification of plant propagating material;
- the definition and implementation of Certification steps;
- the definition of categories of planting material under Certification;
- the approval of variety accessions, clones and selections which shall be subjected to certification.

Art. 2 - Establishment of a National Certification Service

The NCS for citrus plant propagating material is established at the Ministry of Agriculture; it is responsible for quality at national level and coordinates technical, administrative and scientific activities relating to Certification of plant propagating material.

Art. 3 - Establishment of a National Register of certified variety accessions, clones and selections

The Register of National variety accessions, clones and selections approved by the NCS is established at the Ministry of Agriculture. The variety accessions, clones and selections are included in the National Register by a special provision.

Art. 4 - Registration of planting material to be Certified

For registration of accessions, the plant breeders shall (technical specifications A):

- Maintain the primary source under appropriate facilities in order to preserve the sanitary status declared.
- File a special application to NCS.
- Deliver to the Conservation Centre for Premultiplication (CCP) the propagating material descended from the first multiplication of the primary source (source material /material of origin).

Art. 5 - Certification steps

Voluntary certification of citrus plant propagating material comprises the following steps:

- Conservation for premultiplication
- Premultiplication
- Multiplication and nursery production

• Conservation for premultiplication (technical specifications B)

Goals

- Conservation under insect-proof screenhouses of «Prebasic» mother plants, deriving from primary sources registered at national level.
- Growing under insect-proof screenhouse of «Prebasic» propagating material (seeds, cuttings, scions, buds, rootstocks, grafted plants) for the production of grafted plants to be used in premultiplication step.

Organisation

- Conservation takes place at the Conservation Centre for Premultiplication (CCP), registered with the NCS.

- To be registered, CCP must be provided with appropriate facilities for conservation of planting materials in healthy conditions and be supervised by a technical manager with relevant experience.
- CCP activities are coordinated by the NCS at the national level.
- The number of CCPs and their location are defined according to the needs of conservation of «Prebasic» material.
- Maps showing the exact location of existing accessions must be kept at CCP together with a farm record-book endorsed by the NCS.

Requirements

«Prebasic» material must fulfil the health and genetic requirements set forth in the technical specifications (D).

Controls and tests

Phytosanitary and genetic trueness-to-type controls and tests set forth in the technical specifications (E, F, G, H) are carried out under the supervision of the NCS and the responsibility of the CCP. Tests can be run by public laboratories or private laboratories registered with the NCS.

Certification

Certification of «Prebasic» propagating material is applied after assessment of the success of grafting for grafted plants and of rooting for self-rooted plants.

• **Premultiplication** (technical specifications B)

Goals

- Conservation under an insect-proof screenhouse of «Basic» plants, deriving from direct propagation of «Prebasic» plants or directly from the primary source.
- Growing under an insect-proof screenhouse of «Basic» propagating material (seeds, cuttings, scions, buds, rootstocks, grafted plants) for the production of grafted plants to be used in multiplication step.

Organisation

- Premultiplication takes place at the Centre for Premultiplication (CP), registered with the NCS.
- To be registered, CP must be provided with appropriate facilities for conservation of «Basic» plants in healthy conditions; moreover, they shall be supervised by a technical manager with relevant experience.
- CP activities are coordinated by the NCS at the national level.
- The number of CP and their location are defined according to the needs of Premultiplication of «Basic» material.
- Maps showing the exact location of existing accessions must be kept at CP together with a farm record-book endorsed by the NCS.

Requirements

«Basic» material must fulfil the health and genetic requirements set forth in the technical specifications (D).

Controls and tests

Phytosanitary and genetic trueness-to-type controls and tests set forth in the technical specifications (E, F, G, H) are carried out under the supervision of the NCS and the

responsibility of the CP. Tests can be run by public laboratories or private laboratories registered with the NCS.

Certification

Certification of «Basic» propagating material is applied after assessment of the success of grafting on grafted plants and of rooting for self-rooted plants.

• **Multiplication** (technical specifications C)

ScionMotherPlant(SMP)andRootstockMotherPlant(RMP)blocks.

Goals

- Growing of SMP and RMP in compliance with the technical specifications.
- Growing of «certified» propagating material (seeds, cuttings, scions, buds), for the production of rootstocks and grafted plants in nursery.

Organisation

- Multiplication of SMP and RMP takes place at the Centre for Multiplication (CM), registered with the NCS.
- To be registered, CM must meet the requirements referred to in the technical specification; in particular, they shall:
 - ✓ be supervised by a technical manager with relevant experience who interacts with the Certifying Body;
 - ✓ be provided with appropriate plots and facilities for management and conservation of specific production;
 - ✓ be duly equipped with mechanical means for management, conservation and transportation exclusively intended for the activities of the establishment.
- CM may include several facilities (SMP and RMP blocks, nurseries); CM facilities and means necessary for management and production of «Certified» material must meet the requirements set forth in the technical specifications.
- CM activities are coordinated by the NCS at the national level.
- Maps showing the exact location of existing plants and multiplication facilities must be kept at CM together with a farm record-book endorsed and periodically checked by the NCS.
- The number and the location of SMP and RMP blocks is defined according to the needs of propagation of «Certified» material; their size shall guarantee the annual production of a sufficient number of cuttings, scions and seeds so as to fulfil market demand.

Requirements

«Certified» propagating material must fulfil the phytosanitary and genetic requirements set forth in the technical specifications (D).

Controls

Phytosanitary and genetic true-to-type controls and tests on «certified» material set forth in the technical specifications (E, F, G) are carried out under the supervision of the NCS and the responsibility of the CM; tests can be run by public laboratories or private laboratories registered with the NCS.

Certification

Certification of «certified» propagating material is applied after the assessments set forth in production specifications.

Nursery

Goal

Growing of «certified» rootstocks and grafted plants for commercial production.

Organisation

- Multiplication takes place in nurseries registered with the NSC; it can be carried out only by physical persons or legal entities authorised to produce nursery material and declaring that they use the propagating material registered with NCS, in accordance to the provisions laid down in the present regulation.
- Maps showing the exact location of existing plantings must be kept in the nurseries together with a farm record-book endorsed and periodically checked by the NCS.

Requirements

«Certified» propagating material must fulfil the health and genetic requirements set forth in the technical specifications (D).

Controls

Phytopathological and genetic trueness-to-type controls and tests on «certified» propagating material set forth in the technical specifications (E, F, G, H) are carried out under the supervision of the NCS and the responsibility of the nurseryman. At the request of the NCS, tests can be run by public laboratories or private accredited laboratories, registered with the NCS.

Certification

Certification of «certified» propagating material is applied after assessment of the success of grafting for grafted plants, of rooting for self-rooted plants.

Art. 6 - Categories of propagating material

Propagating material is comprised in the following categories:

- **Primary source.** Material of origin selected or produced by the plant breeder and kept by the plant breeder or parties entitled.
- **Prebasic.** Material produced from plants obtained from first propagation of the «Primary source» and maintained in the CCP in the number of 2 mother plants minimum.
- **Basic.** Material produced from plants obtained from first propagation of «Prebasic» material (or from the primary source when «Prebasic» material is not available), and maintained at the CP in a variable mother plant number (2 minimum) depending on importance of the cultivar in question;
- **Certified.**
 - ✓ *Scion and Rootstock Mother Plants.* Material produced from plants obtained from first propagation of «Basic» material, and maintained at the CM in a variable mother plant number depending on the importance of the cultivar in question.
 - ✓ *Nursery Plants.* Material (self-rooted plants, grafted plants, rootstocks) obtained from the first propagation of seed and/or scion mother plants intended for commercial use.

Art. 7 - Sanitary status of propagating material

For the purposes of plant certification, two categories of sanitary status are defined (technical specification D):

- *Pathogen free* – (PF). Material free from viruses viroids, virus-like, phytoplasmas, and other major systemic infectious agents, known for the species in question at the time of enforcement of the specific regulation on certification, as indicated in the technical specifications.
- *Pathogen tested* – (PT). Material free from virus, viroids, virus-like, phytoplasmas and other main infectious agents of major economic importance, listed in the technical specifications.

If plants are grafted with material with a different sanitary status, the final category will be the lower one.

Art. 8 - Label

Propagating material, produced in the meaning of the present regulation, comes with the label of a different colour according to the production step.

In particular, the label colours are as follows:

- ✓ red for the «Primary source»;
- ✓ white with a purple diagonal bar for «Prebasic» material;
- ✓ white for «Basic» material;
- ✓ blue for «Certified» material.

The label shall report:

- ✓ the reference regulation of the country;
- ✓ the Certification body;
- ✓ the plant category;
- ✓ the species name (botanical name included);
- ✓ the variety, clone;
- ✓ the rootstock;
- ✓ the production year;
- ✓ the producer's code;
- ✓ the lot number;
- ✓ the quantity;
- ✓ the sanitary status.

Art. 9 - Sanitary and trueness-to-type checks

The holder is responsible for the different categories of propagating material, «Primary source», «Prebasic», «Basic» and «Certified» material. The competent Authority exerts control on all plant categories in accordance with the technical specifications (E, F, G, H).

Art. 10 - Fees/Costs

For patented accessions, the plant breeder or parties entitled shall bear the costs of conservation and production of propagating material at CCP et CPC; the nurseryman shall bear the costs for non patented accessions.

Art. 11 - Provisions for propagating material deriving from other Certification schemes

Propagating material deriving from Certification schemes of other countries approved by the National Certification Programme are admitted to the National Certification.

Admission procedures shall meet the following requirements:

- plant category must be «Prebasic» or «Basic», with the sanitary status *Pathogen-free*;

- during plant health assessment, conservation shall be carried out under the insect-proof screenhouse, separately from national accessions;
- sanitary checks shall be run following the procedures for the «Primary source» *Pathogen-free*, in compliance with the technical specifications A.
- sanitary and trueness-to-type checks shall take place under the supervision of the NCS and the responsibility of CCP. Tests can be run by public or private laboratories, registered with the NCS.

2. Technical specifications

A. Documents for the registration of the «Primary source»

- ✓ Declaration stating the methods used for the production of the «primary source».
- ✓ Morpho-pomological data sheet with pictures and/or documents.
- ✓ Phytosanitary data sheet regarding the plant sanitary status with respect to the diseases and pests included in Table 1.
- ✓ Declaration indicating the place and methods for primary source maintenance under healthy conditions and the person responsible for maintenance.
- ✓ Declaration stating that the «primary source» is free from quarantine pests (not included in the certification list).
- ✓ For accessions of patented varieties, a copy of the patent documents (application and issue) and a list of beneficiaries. For accessions of unpatented varieties, a declaration stating this condition.

DATA SHEETS FOR THE REGISTRATION OF THE CITRUS PRIMARY SOURCE

Part I – Breeding information

Owner _____ Breeding (year) _____ executed by _____

Original sources _____ Sanitary selection (period) _____ executed by _____

Pomological selection (period) _____ executed by _____

Sanitation YES NO date _____

Sanitation method

Thermotherapy - *In vitro* shoot-tip grafting - *In vitro* somatic embryogenesis from stigma and style culture

Others _____

Part II – Morpho-pomological-genetic assays

Picture

– Morpho-pomological characterization

According to UPOV, CPVO (www.cpvo.europa.eu) or other international standards

Genus: ___ Species: ___ Cultivar: ___ Clone: ___ Genetic origin: ___ Plant characteristics: ___

External fruit characteristics - Internal fruit characteristics - Production characteristics

Behaviour towards the main physiological disorders and diseases (Facultative)

– Molecular characterization (if possible)

SSR - AFLP - RFLP - RAPD - Isoenzymes - Others

– Belonging to GMO YES NO

– Conservation of the Primary Source:

(Responsible body) (Location)

Part III – Sanitary assays

Causal agent / Disease	Acronym	Assays		
		Biological	Serological / Culture	Biomolecular
Viruses				
Tristeza			ELISA	
<i>Citrus tristeza virus</i>	CTV	Mexican lime	DTBIA	RT-PCR
Leaf rugose				
<i>Citrus leaf rugose virus</i>	CLRV	Mexican lime		
Infectious variegation				
<i>Citrus infectious variegation virus</i>	CVV	Lemon <i>Eureka, Volkameriana</i> Etrug citron	ELISA	RT-PCR
Psorosis				
<i>Citrus psorosis virus</i>	CPsV	Sweet orange <i>Madam vinous, Navelina</i>	ELISA	RT-PCR
Satsuma dwarf				
<i>Citrus satsuma dwarf virus</i>	SDV	Lemon or Citron Dweet tangor		RT-PCR
Citrange tatterleaf				
<i>Citrus tatterleaf virus</i>	CTLV	Citrange Troyer		RT-PCR
Vein enations/woody galls				
<i>Citrus vein enation virus</i>	CVEV	Sour orange Mexican lime	*ELISA	
Leaf blotch				
<i>Citrus leaf blotch virus</i>	CLBV	Dweet tangor		RT-PCR
Viroids				
Exocortis				
<i>Citrus exocortis viroid</i>	CEVd	861-S1 Etrug citron		RT-PCR, sPAGE Hybridisation ***
Cachexia				
<i>Hop stunt viroid</i>	HSVd	<i>Parson's special</i> mandarin onto Rough lemon		RT-PCR, sPAGE, Hybridisation ***
Gummy bark**				
<i>Hop stunt viroid variant</i>	HSVd			
Other Citrus viroids	CVds	861-S1 Etrug citron		RT-PCR, sPAGE ***
Virus-like				
Concave gum	CG	Sweet orange		
Cristacortis	CCr	<i>Madam vinous, Navelina</i>		
Impietratura	Imp	Dweet tangor		
Citrus chlorotic disease	CCD	Mexican lime		
Phloem-limited prokaryots				
Huanglongbing				
<i>Candidatus Liberibacter</i> spp.	HLB	Sweet orange <i>Madam vinous</i>		PCR
Stubborn				
<i>Spiroplasma citri</i>	St	Sweet orange <i>Madam vinous</i>	Culturing	PCR

* Serologically correlated to *Barley yellow dwarf virus* (BYDV); ** Detection only by visual observations during the selection period;

*** Using inoculated Etrug citron

FUNGI	ISOLATION	BIOMOLECULAR TESTS
Foot rot <i>Phytophthora citrophthora</i>	x	PCR
Citrus root rot <i>Phytophthora nicotianae</i>	x	PCR
Mal secco <i>Phoma tracheiphila</i>	x	PCR

SANITARY STATUS: Pathogen-free PF Pathogen-tested PT

B. Means for growing and producing «Prebasic» and «Basic» material

✓ Facilities

The Conservation and Premultiplication steps shall be carried out in an insect-proof screenhouse in areas where citrus trees are not grown by law. If the certification facilities are already established in the citrus growing areas, these areas must be declared free from Quarantine pathogens (e.g. *Citrus tristeza virus* – CTV) and from other harmful organisms (e.g. *Spiroplasma citri*, the agent of the stubborn disease) by the Plant Protection Service.

✓ Growing and production

«Prebasic» and «Basic» material (scion, rootstock and grafted plant) must be obtained under the same conditions as hereafter specified:

- the screenhouse shall be separated by a surrounding zone at least 4m wide, kept free from any vegetation;
- the plant material shall be grown in brand new containers of appropriate volume;
- the soil or growing media shall be sterilised to remove soil pathogens;
- containers and flats for rooting and acclimatation and other sowing operations shall be kept on supports at least 20 cm high off the ground;
- flats for rooting, acclimatation and seedling beds shall preventively be disinfected with a 10% sodium hypochlorite commercial bleach for at least 20/30 minutes;
- «Basic» mother plants shall not be maintained in pots for more than 20 years since their establishment in the insect-proof screenhouse unless otherwise provided by the NCS;
- plants with a different health status (PF and PT) may be grown under the same screenhouse provided that they are isolated by a double net;
- the planting layout shall be reported on a specific map constantly kept updated;
- records shall be kept of all operations in a special Farm Book at the CCP and the CP;
- implements shall be at all times disinfected with a 10% sodium hypochlorite commercial bleach between cuttings;
- all diseased and doubtful plants or all plants showing any apparent abnormality shall be incinerated before the competent Authority;
- any delivery of «Prebasic» and «Basic» material shall be at all times recorded and immediately notified to the NCS.

✓ Increase blocks

The production of «Basic» material (scion) from increase blocks shall fulfil the afore-mentioned requirements and be organised as follows:

- accessions under multiplication shall be distinct in easily identifiable plots;
- in the plot, the rows shall be complete and distinct per plant accession (species, cultivar and clone); if different accessions are grown in the same row, they shall be separated by a double inter-space;
- in case of grafting failure and when appropriate, top-grafting shall be made by using material of the same accession;
- well-lignified cuttings can be annually collected from each plant in the increase block but no more than five times since the grafting or the establishment date;
- the planting layout shall be reported on a specific map.

C. Means for growing mother plants and production of «Certified» material

✓ Facilities

The Multiplication step shall be carried out preferably in areas where citrus trees are not grown or in areas declared free from Quarantine pathogens (e.g. *Citrus tristeza virus* – CTV) and from other harmful organisms (e.g. *S. citri*) by the Plant Protection Service. If these conditions are not fulfilled the same Service can require the use of the insect-proof greenhouse.

✓ Growing and production

ScionMotherPlant(SMP)andRootstockMotherPlant(RMP)blocks

Certified SMP and RMP blocks shall be established in compliance with the certification schemes and shall meet the following conditions:

- the establishments producing citrus plants must be approved by the competent Authority by:
 - submitting an official form to the competent Authority;
 - including with the applications supporting documents that testify the acquisition of the «Basic» propagating material for the block establishment;
- SMP and RMP are compulsorily registered with the competent Authority; partial or total pulling out or production cessation shall be notified to the competent Authority not later than one month;
- SMP and RMP shall be established on soils on which none of the citrus trees have been grown for at least 5 years and treated by solarisation to reduce the number of *P. nicotianae* and *P. citrophthora* propagules to an acceptable level (less than 5 propagules/gram of soil) and eliminate nematodes;
- SMP and RMP shall be separated by a surrounding zone at least 10m wide, kept free from any vegetation;
- SMP and RMP shall be isolated from surface water flow;
- in the plot, the rows shall be complete and distinct per plant accession; if different accessions are grown in the same row, they shall be separated by a double inter-space; each plot shall be identified by a sign indicating the species, the cultivated variety (clone), the rootstock, the category, the planting date, the lot number and the number of plants;
- from each SMP not more than 1500 scions, for a maximum of 6000 buds, can be annually collected;
- missing plants shall be replaced only with propagating material of the same clone and belonging to the same category under the control of the competent Authority;
- the blocks shall be kept under continuous surveillance to control pathogens, pests and weeds;
- implements shall be at all times disinfected with a 10% sodium hypochlorite commercial bleach between cuttings;
- SMP shall not be kept for more than 20 years since their establishment; RMP shall not be kept for more than 30 years since their establishment;
- the planting layout shall be reported on a map constantly kept updated.

Increaseblock

The production of «Certified» material (scion) shall fulfil the afore-mentioned requirements and be organised as follows:

- the growing medium shall be found free from *Phytophthora nicotianae* and *P. citrophthora* and from citrus nematodes;
- the growing containers shall be isolated from the ground by:

- i. a layer of fine gravel or any inert material providing for effective drainage, at least 10 cm high, when mulching films are used, and at least 5 cm high when a French drain is used;
- ii. a layer of concrete or different material; in such a case the containers shall be placed on supports;
- the area intended for growing plants in pots shall be separated by a surrounding zone at least 4 m wide, constantly tilled or kept free from any vegetation;
- seedlings shall be grafted at not less than 40cm from the collar;
- in case of grafting failure and when appropriate, top-grafting shall be made by using material of the same accession;
- well-lignified propagating material can be collected from each plant of the increase block non more than five times from the date of grafting, the life cycle of the increase block should not exceed 3 years.

Nursery

The nursery production is carried out in the seedling, grafting and rootstock beds .

The «Certified» material shall be produced in accordance with the following rules:

- to be admitted to control, the establishments producing citrus plants must be approved by the competent Authority;
- the application for admission to control can be submitted only for a production of not less than 50.000 plants; at all times the nursery declaration shall be notified to the competent Authority, including:
 - i. the name and address of the declarant;
 - ii. the nursery location;
 - iii. a detailed map of the nursery layout reporting the lots distribution and all useful indications for the inspection;
 - iv. the number and origin of the plants produced;
 - v. the category to which the plants produced are likely to belong;
- to produce certified plants only soil-less culture is allowed;
- containers and flats for rooting and acclimatation and other sowing operations the flats shall preventively be disinfected with a 10% sodium hypochlorite commercial bleach for at least 20/30 minutes;
- nurseries shall comply with the phytosanitary rules set out for plants in pots in the increase blocks as regards soil and growing media, isolation, distance of pots from the French drain and implements disinfection;
- the blocks shall be kept under continuous surveillance to control pathogens, pests and weeds;
- seedlings of species susceptible to 'Mal secco disease' shall be covered by a 50% shading net and not less than 50m apart from other lemon groves;
- seedlings which are to be moved to the grafting bed shall exhibit at least 4 to 6 fully developed leaves so as to distinguish natural hybrids from nucellar seedlings;
- the plants shall be subdivided in homogeneous lots (per species, cultivar, clone and rootstock), made up of a maximum of 4 rows, easily identifiable and reported on a map;
- the growing containers shall be placed at a distance of at least 20cm in the row and lots shall be spaced out by at least 50 cm;
- in case of grafting failure and when appropriate, top-grafting shall be made by using material of the same accession;
- the growing cycle of the plants which are to be certified shall not exceed three years as from the date of establishment;
- all diseased and doubtful plants or all plants showing any apparent abnormality shall be removed.

✓ **Stock records keeping**

Every citrus plant producer shall at all times keep records of the amounts of plant material produced and sold, the selling date, the name of the consignee and place of destination of the plant material delivered. The account books shall be made available for inspection by the competent Authority.

✓ **Duration/validity of certification**

The certification of plant propagating material is valid for one year.

D) Sanitary conditions for *Pathogen-free* and *Pathogen-tested* «primary sources», «prebasic», «basic» and «certified» material

Causal agent	Acronym	Sanitary status	
		<i>Pathogen-free</i> (PF)	<i>Pathogen-tested</i> (PT)
Viruses			
<i>Citrus tristeza virus</i>	CTV	X	X
<i>Citrus leaf rugose virus</i>	CLRV	X	X
<i>Citrus infectious variegation virus</i>	CVV	X	X
<i>Citrus psorosis virus</i>	CPsV	X	X
<i>Citrus satsuma dwarf virus</i>	SDV	X	
<i>Citrus tatter leaf virus</i>	CTLV	X	
<i>Citrus vein enation virus</i>	CVEV	X	
<i>Citrus leaf blotch virus</i>	CLBV	X	
Viroids			
<i>Citrus exocortis viroid</i>	CEVd	X	X
<i>Hop stunt viroid</i>	HSVd	X	X
<i>Other Citrus viroids</i>	CVds	X	
Virus-like			
<i>Concave gum</i>	CG	X	X
<i>Cristacortis</i>	CCr	X	X
<i>Impietratura</i>	Imp	X	X
<i>Citrus chlorotic disease</i>	CCD	X	
Phloem-limited prokaryotes			
<i>Spiroplasma citri</i>	St	X	X
<i>Candidatus liberibacter</i> spp.	HLB	X	

E) Organization of controls

The «Primary source» and the «Prebasic» and «Basic» material are under the responsibility of the breeder. The 'Certified' material (scion mother plants, rootstock mother plants and nursery plants) are under the responsibility of the approved nurseryman. However the competent Authority exerts control over all plant categories (VI, VIII).

✓ On-the-spot checks

• «Prebasic» and «Basic» material

The competent Authority shall carry out periodic and regular visual inspections of this material. All plants displaying any apparent abnormality shall be submitted to laboratory testing to detect the cause and promptly adopt the relevant preventive measures. In addition, administrative checks shall be performed.

• «Certified» mother plants

Scion Mother Plants (SMP)

SMP shall periodically be inspected. A preliminary inspection is carried out prior to the establishment of the SMP block to check:

- the compliance with isolation conditions;
- the plant origin;
- the soil nematological and mycological analyses.

When SMP come into production, the block shall be inspected at least twice a year to assess:

- the plant sanitary status;
- the trueness-to-type;
- the production of scions.

RootstockMotherPlants(RMP)

RMP shall periodically be inspected. A preliminary inspection is carried out to check:

- the compliance with isolation conditions;
- the plant origin;
- the soil nematological and mycological analyses.

When RMP come into production, the block shall be inspected at least once a year before harvesting to assess:

- the plant sanitary status;
- the trueness-to-type;
- the production of seeds.

• «Certified» nursery plants

The «Certified» nursery material shall be submitted to at least four inspections and more specifically, two inspections during the phase of rootstock growing and two inspections from grafting to removal of the grafted plants.

Rootstocks

They shall be submitted to:

- a preliminary inspection prior to the establishment of the seedling/cutting in order to check the origin of the seeds or cuttings;

- a second inspection, before trading (delivery/release/placement on the market) in order to estimate the production of rootstocks and check their sanitary status.

Grafted plants

After grafting an inspection shall be carried out in order to:

- check the rootstocks homogeneity;
- check the origin of scions;
- estimate the graft take.

One more inspection shall be carried out before trading in order to:

- check the plant sanitary and growing status;
- check the trueness-to-type;
- estimate the plants to be certified.

✓ **Laboratory testing**

All categories of propagating material shall be submitted to laboratory testing including:

a. Seeds quality control

Quality control shall include the germination rate, the species purity, the presence of insects and fungal and bacterial diseases.

b. Phytosanitary checks

The competent Authority shall randomly inspect «Prebasic», «Basic» and «Certified» material with regard to virus, viroid, virus-like agents, *S. citri*, and fungi (Table 1).

✓ **Inspection of lots**

The competent Authority can withdraw the certificate, the labels or the approval if plants do not comply with the present technical specifications.

When plants are ready for sale, the nurserymen shall inform the competent Authority within one month for the purpose of labeling.

a. Handling and packaging

- Seeds: seeds are placed in sealed packages, bearing two indelible labels, one on the outside and one on the inside;
- Rootstocks and grafted plants: they can be placed either in pots or in polyethylene bags; each plant shall bear an indelible label.

b. Stock records

Every nurseryman approved for the production and trade of citrus propagating material shall keep records of all information necessary for inspections carried out by the competent Authority, and more specifically the quantities produced and traded, the dates of sale, the name of the consignee and the destination of the plant material delivered.

✓ **Controls on trade and movement of plants**

Controls shall be carried out on plant material bearing a certification label to ascertain its origin, handling conditions, health status, freedom from pests and the presence of the certification label.

F) Phytosanitary checks

All material descended from the first multiplication of the «Primary source», or from «Prebasic» or «Basic» material supplied by any other approved certification schemes, shall be individually submitted to thoroughness-to-type and sanitary checks, according to the procedures reported in technical specifications A, when brought into the Conservation Centre for Premultiplication or into the other steps.

In every step all measures shall promptly be adopted to control pests and pathogens which could pose a threat to the plant material; all operations shall be recorded in a special book.

✓ «Prebasic», «Basic» and «Certified» material

Two types of checks shall be carried out for viruses, viroids, virus-like agents, *S. citri* and fungi:

- Visual inspections: every year on all plants, at the appropriate time, when symptoms are likely to be most visible for each single disease; more specifically, in spring for viruses and virus-like diseases, in summer for viroid and stubborn diseases (at colour break for stubborn disease).
- Laboratory testing: according to the procedures indicated in tables 1 and 2 of the present annex.

✓ Soil and growing media in all steps

Fungi: *P. nicotianae* and *P. citrophthora*

Mycological analysis through isolation on selective media on samples collected according to the following methods:

growing media: a sample shall be collected every 5m³, made up of 10 sub-samples, for a total volume of at least 1 litre;

soil: before planting and, at any time, before any deep tillage, 1 sample per hectare shall be collected, made up of 10 sub-samples, for a total volume of at least 1 litre.

Nematodes: *Pratylenchus vulnus* and *Tylenchulus semipenetrans*

Nematological analysis through isolation techniques on samples collected according to the following methods:

growing media: a sample shall be collected every 5m³, made up of 5 sub-samples, for a total volume of at least 1 litre;

soil: before planting and, at any time, before any deep tillage, 1 sample per hectare shall be collected, made up of 5 sub-samples, for a total volume of at least 1 litre.

Table 1. Procedure for the assessment of *Pathogen-free* and *Pathogen-tested* sanitary status of «Prebasic» and «Basic» Scion Mother Plants and Rootstock Mother Plants.

Organism/ Disease	ASSAYS					
	Visual inspections		Biological		Laboratory	
	Period	Frequency	Recommended indicator	Plant /year %	Sampling type and sampling time	Plant /year %
Viruses						
CTV	At growth recovery	Annual	Mexican lime	15%	Leaf peduncles, stem bark, style and stigmas / all the year round except for the coldest and the warmest months	all plants
CiLRV CVV	At growth recovery	Annual	Mexican lime Lemon (<i>Eureka, Volkameriana</i>) Etrog citron	10%	Leaves in spring	25%
CPsV	At growth recovery	Annual	Sweet orange <i>Madame vinous, Navelina</i>	10%	Mature leaves in spring	25%
SDV, CLBv	At growth recovery	Annual	Dweet tangor	10%	Leaves in spring	25%
CTLV	At growth recovery	Annual	Citrange Rusk, Troyer	10%	Leaves in spring	25%
CVEV	At growth recovery	Annual	Mexican lime	10%	Leaves in spring	25%
Virus-like						
CG, Cr, Imp	At growth recovery	Annual	Dweet tangor Sweet orange <i>Madame vinous, Navelina</i>	10%	Leaves in spring	25%
CCD	At growth recovery	Annual	Mexican lime	10%	Leaves in spring	25%
Spiroplasma						
<i>Spiroplasma citri</i>	Late summer	Annual		10%	Leaves in late summer Fruit columella	25%
Viroids						
CEVd HSVd CVds	Late summer	Annual	Etrog citron 861-S1*	25%	Leaves in late summer	25%

*Indicator plant only for CEVd and CVds; it is used for viroid replication

Table 2. Procedure for the assessment of *Pathogen-free* and *Pathogen-tested* sanitary status of «Certified» Scion Mother Plants (SMP) and Rootstock Mother Plants (RMP).

Organism/ Disease	ASSAYS			
	Visual inspections		Laboratory	
	Period	Frequency	Sampling type and sampling time	Tested plant/year %
CTV	At growth recovery	Annual	Leaf peduncles, stem bark, style and stigmas/all the year round except for the coldest and the warmest months	100%
CiLRV CVV SDV, CLVB CTLV	At growth recovery	Annual	Leaves in spring	10%
CPsV	At growth recovery	Annual	Mature leaves in spring	10%
Virus-like				
CG, Cr, Imp, CCD	At growth recovery	Annual		
Spiroplasma				
<i>Spiroplasma citri</i>	Late summer	Annual	Leaves collected in late summer Fruit columella	20%
Viroids				
CEVd HSVd CVds	Late summer	Annual	Leaves collected in late summer	10%

G) Prerequisites for propagating material

✓ Plant technical characteristics

Prerequisites	Plant
Root system	Diffuse/ fasciculate
Open wounds	None

✓ Laboratory seed testing standards

Prerequisites	Prebasic	Basic	Certified
Germination rate	95%	90%	85%
Species purity	100%	100%	98%
Presence of living insects/rots	None	None	None

H) Trueness-to-type checks

Part 1 – «Prebasic» and «Basic» material

The trueness-to-type certification for cultivars and rootstocks is issued by the competent Authority after observing one growing and production cycle so as to assess the conformity to the phenotype at appropriate time, when the phenological characteristics are likely to be best expressed.

Later, at ripening time, a visual inspection shall be carried out every year to assess the production characteristics.

Part 2 – «Certified» Mother Plants

The trueness-to-type certification for cultivars and rootstocks is issued by the competent Authority after observing one growing and production cycle, before the collection of «certified» material. Later, at ripening time, a visual inspection shall be carried out every year to assess the production characteristics.

Part 3 – Increase Blocks and nursers material

Visual inspections shall be carried out to assess the plant growing characteristics.

Technical procedures for the monitoring of *Citrus tristeza virus* (CTV)

Djelouah K., Valentini F., D'Onghia A.M.

CIHEAM - Mediterranean Agronomic Institute, Valenzano (BA), Italy

Abstract. Several Mediterranean countries are now strengthening phytosanitary measures to contrast the spread of *Citrus tristeza virus* (CTV) in accordance with the European Community (EC) regulations for the mandatory control of CTV. In this paper, an account is given on technical methods and procedures for planning, sampling, testing and plant elimination, which should be included in a regulation for an accurate control of CTV. An advanced organization of the virus monitoring and eradication programme is presented in order to provide Phytosanitary Services with the most advanced methods and efficient procedures.

Keywords. *Citrus tristeza virus* – Mediterranean – Monitoring – Sampling – Vector.

Amélioration des procédures techniques pour le suivi du virus de la tristeza des agrumes (CTV)

Résumé. Plusieurs pays méditerranéens sont en train de renforcer leurs mesures phytosanitaires afin de bloquer la diffusion du virus de la tristeza des agrumes (CTV), ceci conformément à la réglementation de la Communauté Européenne (CE) sur le contrôle obligatoire du CTV. Dans cet article, un compte rendu est donné sur les méthodes techniques et les procédures à mettre en œuvre pour la planification, l'échantillonnage, les analyses et l'élimination des plantes, qui doivent être inclus dans une réglementation, ceci afin de contrôler efficacement le CTV. Une organisation plus accrue du suivi du virus et du programme d'éradication est présentée, afin de mettre à disposition des services phytosanitaires, des méthodes plus récentes ainsi que des procédures plus efficaces.

Mots-clés. *Virus de la tristeza des agrumes* – Méditerranée – Suivi – Échantillonnage – Vecteur

I – Introduction

Control of CTV is extremely difficult once the virus gets established in a region where its natural aphid vectors are also present. The ability to control the disease damages depends to a large extent on CTV incidence and on the virus strains and citrus varieties predominant in each region.

Several approaches have been used to control the losses caused by CTV, particularly, in areas where CTV is rare and its natural spread is limited. Beside the strict quarantine measures and the establishment of certification programs, elimination of infected trees is considered as the best means to avoid or delay an epidemic, if CTV has a low incidence and the infected trees are grouped in a limited number of foci.

Accurate information on the distribution and incidence of CTV-infected trees are of utmost importance for decisions to implement a CTV eradication or suppression program; if a larger number of infected citrus trees in the open field are destroyed before the onset of new infections, the incidence can be maintained at reasonable levels.

Accordingly, there is an urgent need for enforcing the national CTV control strategy in the Mediterranean countries, this strongly suggesting the need for continuously virus and vector monitoring. In this context, several procedures were employed over the world, but most of them were not extensive and were designed only for quantitative data, such as the number of infections per plant.

Recently, hierarchical sampling procedures were set up (Gottwald and Hughes, 2000), which increased accuracy and precision of estimates of CTV incidence without appreciable increase in the number of laboratory assays required. These slightly modified methods have been successfully implemented in Apulia region.

II – Legislation

A Ministerial Decree or a specific regulation for the mandatory control of CTV and its vectors, is the first step that a country has to take toward the establishment of a virus and vector monitoring programme. Moreover, laws and regulations are necessary for the certification of citrus nursery production, which represents one of the basic actions to control citrus quarantine pests.

In the Mediterranean basin, especially at EU level, several regulations (EU Directives 93/48/EEC, 93/64/EEC, 93/79/EEC) have been issued to control the entrance and dissemination of citrus pests or plant products and to set forth the phytosanitary requirements for the free movement of citrus propagating material throughout the Community.

III – Identification of the areas to be monitored

At the beginning of each monitoring campaign, the Plant Protection Service (PPS), in cooperation with all the stakeholders, draws up an action plan based on data collected in the previous years and on notifications and reports.

Monitoring is carried out in the areas listed below and according to the following priorities:

- a) in groves where citrus trees are located in the range of at least 1 km from citrus nurseries, to be sure that nurseries are established in virus-free areas;
- b) in groves where citrus trees are located in the range of 1 km from certification facilities (conservation, pre-multiplication blocks, mother blocks);
- c) in groves where CTV-infected plants have been found and in contaminated areas and buffer zones for three years after the virus detection;
- d) in citrus nurseries;
- e) on extra-regional material, introduced also for scientific purposes;
- f) in ornamental gardens, botanical gardens, variety collections, public and private parks;
- g) in commercial citrus groves.

Whenever suspected cases are reported, the PPS shall provide for completion of visual inspections and tests, even if the areas concerned are not included in the annual monitoring program.

At the beginning of each monitoring campaign, the team coordinated by the designated phytosanitary inspector shall delimit the intervention areas based on the above criteria. These areas can be easily identified using aerial photographs, that is to say orthophotographs (with cadastral grid) which allow to visualise the citrus groves and the relative cadastral coordinates.

The annual planning takes into account the possible outbreaks which have previously been identified and the related contaminated areas. It is therefore possible to identify groves which shall be inspected, subdivided according to:

- locality;
- map sheet;
- land parcel (using the cadastral data).

IV – Sampling

1. Survey notification

After setting the survey time schedule, the PPS shall send an official notice to the interested person at least 10 days before the scheduled inspection date. The notice shall indicate the survey date and time and provide the interested person with:

- the right to be assisted by a trustworthy technical expert in all inspection steps and during the collection of samples which will be tested;
- the opportunity, when necessary, to delegate in written form a third party to be present during the inspection.

If on the scheduled monitoring day, the interested person or his/her delegate are not present and no major obstacle prevents the access to the field, sampling will be anyway carried out.

2. Sampling method

In order to optimise preventive actions while reducing monitoring time and costs, surveys must be organised per limited areas and exploiting at best the available working hours.

On the day scheduled, the monitoring team, in the presence of the owner or of a delegate, shall start surveying the citrus grove and collecting samples.

Two hierarchical sampling methods can be adopted according to whether one surveys CTV-free areas, contaminated areas, buffer zones or areas in the range of 1 km from nurseries (when commercial groves and nurseries are investigated). In all the other instances, not included in the above list (mother plants, budwood and seed sources, public and private gardens, scientific collections) samples shall be collected individually.

Besides the samples collected at random, some “out-of-scheme” samples are included, that is to say plants which display suspected tristeza symptoms.

A team unit is composed by three people:

- the first one shall carry out visual inspections, map out, draw up the scheme and “label” the plants to be sampled with an identification code and number;
- the second one shall collect the samples;
- the third one shall store the samples in polyethylene bags reporting the identification code or the membrane immunoprinting code.

Once the samples have been prepared, they are stored at the temperature of about 4°C. Alternatively to bag-storage, the sample can directly be immunoprinted onto a nitrocellulose membrane for further testing. The membrane is properly numbered and stored at room temperature until testing.

3. Sample collection

A. CTV-free area

Commercial groves. As set out and described in Fig. 1, an area of two hectares, representative of 10 hectares, shall be identified trying to include, where possible, a whole land parcel, homogeneous for the species and age. Hundred single samples (4 twigs per plant) shall be collected along the edge of the delimited plot.

Nurseries. 10% of individual samples are collected randomly from homogeneous lots as regards to the variety and age of the plants intended for sale.

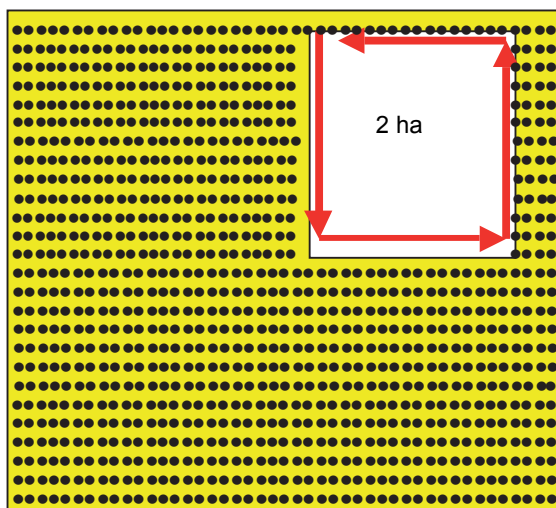


Figure 1. Sampling method adopted in CTV free area.

B. Contaminated areas, buffer zones and areas in the range of 1 km from nurseries and certification facilities

Commercial groves. Samples are collected per homogeneous citrus groves and managed by the same tenant, subdividing the plot according to the species/variety and age (where possible).

To collect the samples, the hierarchical sampling scheme is adopted (Gottwald and Hughes, 2000), samples are collected from 25% of the plants in the grove.

In the sampling method, a citrus plot is subdivided into squares made up of 4 trees each. To this end one has to start from the inter-row, counting 6 pairs of trees and sampling the seventh and eighth pair. In case of an irregular row spacing, it is also possible to go along each single row, sampling 4 trees and leaving aside 12 (Fig. 2). From each of the 4 trees in the square, four young twigs are collected from the different plant sides (since CTV is irregularly distributed in the plant) and they are individually tested through Direct Tissue Blot Immuno Assay (DTBIA).

The square plant samples are numbered clockwise, labelling the first plant of each group; the map shall also report the plants which are not sampled as well as the possible missing plants or plants belonging to other species. The single samples from the four plants, properly labelled, are placed into a bag, marked by a clear field code and the sample progressive number.

Nurseries. At the beginning, 25% of the plants in the lots intended for sale are sampled. They must be homogeneous as regards the variety and age. If no sample proves to be positive, the sampling shall be extended to the remaining 75% plants. Twenty-five percent of the lot plants is represented by a number of samples (5-10) along the lot row which are collected from each of 15-30 plants; obviously, this scheme varies according to the lot age and its lay-out which may be regular or not.

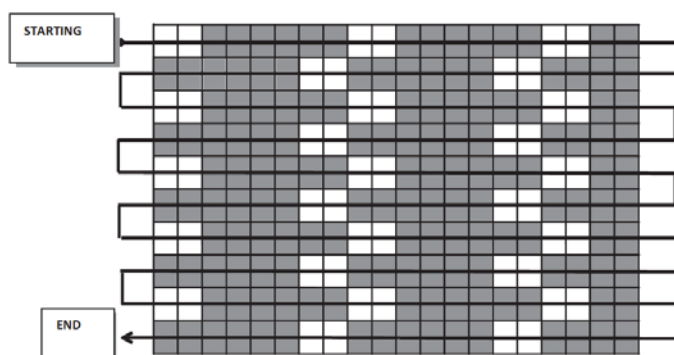


Figure 2. Hierarchical sampling scheme (Gottwald and Hughes, 2000),

4. Sampling report

During the survey the phytosanitary inspector must draw up the sampling report, which shall be signed by the parties and shall include the following:

- the identification data of the holding and of the grove, nurseries etc. (tenant, surface area, map sheet, land parcel);
- the plot data (total number of plants, species, rootstock, age and origin of the material, etc.)
- the persons present and their role;
- the number of plants present and the number of samples collected;
- the name of the accredited laboratory which will run the tests;
- the possible remarks.

A copy of an orthophotograph of the citrus grove shall be enclosed, if available, indicating the plots sampled. After the sampling, the bags with the samples shall be taken by the technical expert of the reference laboratory.

V – Laboratory tests

1. Sample analysis

The collected samples shall be analysed by the DTBIA method (Garnsey *et al.*, 1993) at the PPS laboratory or at an accredited laboratory designated by the competent authority. The analysis results shall be notified by the laboratory to the PPS no later than 10 days from the survey and, subsequently, to the citrus plot owner.

2. Confirmation tests

Confirmation tests are carried out by other laboratories, accredited for diagnostic analyses by biological, molecular or electronmicroscopy methods, when:

- the DTBIA result is considered doubtful by the laboratory which, therefore, asks the PPS to conduct a confirmation test;
- the infection is detected for the first time in a given area, considered free till that time;
- whenever deemed necessary.

In these cases the PPS shall promptly contact the plot tenant to inform him/her that a second survey shall take place to collect all samples requested for the confirmation tests.

The official laboratory technical expert and the phytosanitary inspector shall collect the samples in a sufficient amount to perform the confirmation tests. A report shall be drawn up during this survey as well. The confirmation test results shall be communicated to the PPS no later than 10 days from the sampling.

VI – Eradication of outbreaks

In order to prepare the mandatory eradication provisions, the PPS shall delimit the contaminated area and the buffer zone on map sheets. These sheets and the relevant data on the infected plot (locality, surface area, land parcel, map sheet, number of infected plants) and on the tenant (name, address and phone number) will be a basic part of the provision which designates the contaminated area and the buffer zone.

The contaminated area is delimited as follows:

- when the **infection percentage in the citrus grove is lower than 30%**, the distance of 500 metres shall be measured from the farthest infected plants in the focus;
- when the **infection percentage is higher than 30%**, the distance of 500 metres shall be measured from the vertices of the infected plot and from the half of each plot side. The contaminated area shall be delimited on the map by joining with straight lines the farthest point of the circle with a radius of 500 metres.

The buffer zone shall be delimited extending by 500 mt all the sides of the contaminated area. If a plot or a nursery falls also partially within the buffer zone, it will be totally included.

The contaminated area and the buffer zone shall be included for three years in the monitoring programme for the area to be designated as decontaminated.

Based on the technical procedures, administrative provisions are then applied for:

- the mandatory eradication of virus-infected plants;
- the designation of virus contamination;
- the establishment of the buffer zone.

The PPS shall later notify the citrus grove owner of the mandatory eradication and shall send a phytosanitary inspector who will be present during the operations of uprooting and destruction of the infected plants and will draw up a report.

VII – Extension and training

Extension is at the core of a successful monitoring programme. Extension activities shall be intensively carried out at any time during monitoring, be CTV present or not. Only extension can gradually raise the stakeholders' awareness (farmers, professional associations, public bodies etc.) about the risks related to the spread of this disease in the territory. Indeed this is a true natural disaster which can sweep away the citrus industry in a country, with severe socio-economic repercussions and in many instances, also, endanger the cultural or landscape resources featuring a territory. This information campaign shall be conducted also mobilising the technical experts involved in the virus study and control and through proper media communication (brochures, posters, videos, TV ads, courses, workshops, web sites etc.). Training of people in charge of monitoring is fundamental for the success of all operations which are to be carried out in compliance with the laws in force in a country, timely and professionally.

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MNCC

Project proposal

The project proposal of the CIHEAM-Mediterranean Research Network on Certification of Citrus (MNCC): the regional programme for the mandatory control of *Citrus tristeza virus* and its major vector *Toxoptera citricidus* in the Mediterranean region

D'Onghia A.M.

Coordinator of the CIHEAM-Mediterranean Research Network on Certification of Citrus
CIHEAM – Mediterranean Agronomic Institute, Valenzano (Ba), Italy

Abstract. The Mediterranean citrus industry is faced with a major threat: citrus tristeza, a devastating virus disease whose very efficient vector, "*Toxoptera citricidus*", has suddenly appeared in Northern Portugal and Spain. A very large proportion of the Mediterranean citrus orchards are still on sour orange; a root-stock highly sensitive to tristeza. With CTV inoculum present in almost all citrus producing countries, invasion of the Mediterranean region by *T. citricidus* will cause a rapid dissemination of CTV that will devastate all trees grafted on sour orange in a few years. A sudden collapse of the majority of the Mediterranean citrus industry will occur with unpredictable dramatic socio-economic consequences.

The present project proposal aims at preventing such a disaster through a joint effort of all countries in the region for the implementation of urgent preventive and control measures against both the virus and its vector. The main steps of the work plan include:

- enhancement of harmonized phytosanitary control measures and regulations (quarantine and certification);
- CTV monitoring and characterization;
- elimination of CTV-infected plants;
- monitoring aphid population and implementing efficient control measures;
- raising awareness;
- strengthening of harmonized research and know-how;
- technology transfer and information exchange.

Keywords. Citrus – Citrus tristeza virus – Control – Mediterranean – Project – *Toxoptera citricidus*.

Proposition du projet CIHEAM-Réseau Méditerranéen de recherche sur la certification des agrumes (MNCC) : Contrôle obligatoire du virus de la tristeza des agrumes (CTV) et de son principal vecteur *Toxoptera citricidus* dans la région Méditerranéenne

Résumé. L'industrie des agrumes dans le bassin Méditerranéen est confrontée à une très grave menace : la tristeza des agrumes, une maladie virale dévastatrice dont le vecteur le plus efficace, *Toxoptera citricidus*, a été récemment rapporté au Nord du Portugal et de l'Espagne.

Une très grande proportion de vergers d'agrumes au niveau du bassin Méditerranéen sont encore greffés sur bigaradier, un porte-greffe très sensible à la tristeza. Avec le CTV comme inoculum présent dans presque tous les pays producteurs d'agrumes, l'invasion de la région méditerranéenne par *T. citricidus* provoquera une diffusion rapide du CTV, induisant en quelques années un effet dévastateur sur tous les arbres greffés sur bigaradier. Un effondrement rapide de la majeure partie de l'industrie des agrumes du bassin Méditerranéen, portant à des conséquences socio économiques dramatiques. La présente proposition de projet a pour objectif de prévenir une telle catastrophe, à travers un effort conjoint de tous les pays de la région, afin de mettre en œuvre des mesures préventives d'urgence et des mesures de lutte contre le virus et son vecteur.

Les principales étapes de la stratégie comprennent:

- Renforcement des mesures de contrôle harmonisées et réglementations phytosanitaires (quarantaine et certification);

Options Méditerranéennes, B n° 65, 2009 - Citrus Tristeza Virus and *Toxoptera citricidus*:
a serious threat to the Mediterranean citrus industry

- le suivi du CTV et l'éradication des arbres infectés par le CTV
- le suivi des populations de pucerons et la mise en œuvre des mesures de lutte efficaces;
- la sensibilisation;
- le renforcement de la recherche harmonisée et du savoir-faire;
- le transfert de la technologie et l'échange d'expériences et d'informations.

Mots-clés. Agrumes – Virus de la tristeza – Contrôle – Méditerranée – Proget –*Toxoptera citricidus*.

I – Introduction

1. Importance of the Mediterranean citriculture

Citrus is one of the most important fruit crops in the Mediterranean Basin which contributes over 20% of the overall citrus production in the world and about 60% of fresh citrus world trade (CLAM, 2007). This area is the homeland of easy peelers (primarily Clementine and mandarin etc.) accounting for 75% of the worldwide exportation of these species; a worldwide increase of easy peelers was observed over the last two decades at the expense of fresh oranges, due to the evolution of consumer preferences (CLAM, 2007). However, the region produces special quality fresh fruits of orange, lemon and lime; the latter is of great economic importance in the Near-East countries. Grapefruit production is restricted to Israel which is the fourth world producing country. Hence, Citrus is a major segment in the Mediterranean agricultural industry; developing countries account for almost 40% of the production and citriculture represents a major source of income to a significant number of farmers. A number of citrus processing industries have developed in some Mediterranean countries, mainly for refreshing juice, jams, candies, flavors and other by-products destined to pharmaceutical and cosmetic industries.

Citriculture is therefore of strategic importance for the Mediterranean region for its socio-economic role as a source of income, of employment opportunities at various levels of the chain (production, processing trade and farming consumable suppliers) and for its contribution to the well being of people and the political stability of the countries.

Considering that the Mediterranean has been an important diversification zone for oranges, mandarins and lemons, they also represent a valuable reservoir of genetic resources which are still represented by old aged trees in most of the countries (i.e. Syria, Malta). The improvement of local citrus genotypes is therefore a great market opportunity, as for the Italian red oranges 'Tarocco' from Sicily, the mandarin 'Nadorcot/Afourer' in Morocco and 'Clementine' in Corsica, but also an important action for the preservation of the Mediterranean citrus biodiversity.

2. Citrus tristeza virus

Citrus Tristeza is the most devastating virus disease, caused by *Citrus tristeza virus* (CTV), which has destroyed millions of citrus trees throughout the world, mainly where sour orange was the rootstock: i.e. Brasil, Peru, Florida, South Africa etc. (Bar-Joseph *et al.*, 1989; Roistacher, 1995). It is spread by the movement of contaminated budwood and disseminated in the field by different aphid species (Raccach *et al.*, 1989) among which *Toxoptera citricidus*, the brown citrus aphid (BrCA), the most efficient vector, followed by *Aphis gossypii*; the latter was significantly active in CTV dissemination in Spain (Hermoso de Mendosa *et al.*, 1984; Moreno *et al.*, 2008) and Israel (Raccach *et al.*, 1976), where the virus is now endemic. The virus could not be eliminated, it was widespread and could no longer be contained by the elimination of the CTV-infected foci (Loebenstein, 1993).

The Tristeza status and its economic impact on citrus in any location depend on four main factors:

- presence of the sour orange as susceptible rootstock;

- ii. severity of CTV strains;
- iii. ability of local aphid species to virus dissemination;
- iv. presence of *T. citricidus*, which is the most efficient virus vector, also able to disseminate selectively the severe CTV strains.

When severe CTV strains are introduced into a citrus area where *T. citricidus* is present, Tristeza disease will spread rapidly and damage all citrus trees grafted onto any rootstock (Rocha-Pena *et al.*, 1995).

3. State of the art of CTV infections in the Mediterranean Region

Tristeza poses a tremendous threat to the Mediterranean citrus industry, where areas are extensively grown onto the susceptible sour orange rootstock. Apparently, in all cases tristeza disease in the Mediterranean can be traced back to the introduction of infected budwood from abroad; all countries which introduced the Meyer lemon (e.g. Algeria, Cyprus, Israel, Italy, Morocco and Tunisia) have introduced tristeza as well (Bové, 1966).

Tristeza has caused significant damage to citrus in Spain in 1957 (Moreno *et al.*, 2008) and Israel in 1970 (Bar-Joseph *et al.*, 1983) when *A. gossypii* developed a specific efficiency to disseminate local CTV strains. These two countries were forced to cope definitely with Tristeza and launched a total conversion of their citrus industry using Tristeza tolerant rootstocks (Bar-Joseph *et al.* 1989).

CTV infections are widely distributed in all Mediterranean countries, mostly as isolated foci and without showing clear-cut tristeza symptoms (Bové, 1995; Djelouah and D'Onghia, 2001). Luckily the virus incidence is still below critical levels although disease outbreaks have also been reported in Cyprus (Kyriakou *et al.*, 1996) and, recently, in Italy (Davino *et al.*, 2003).

The CTV strains found in the Mediterranean region range from mild to highly severe capable of inducing the most devastating symptoms on citrus, when grafted onto the sour orange, such as quick decline; however, exotic severe virus strains, CTV-seedling yellows and CTV-stem pitting, were also detected in few countries (Ballester-Olmos *et al.*, 1993; Zemzami *et al.*, 1999) and they can be easily selected and disseminated through BrCA transmission.

Although the threat of Tristeza is a great concern, Mediterranean countries that have escaped disease outbreaks still rely heavily on the use of sour orange as a rootstock for its good adaptation and tolerance to saline water, calcareous soils and *Phytophthora* gummosis. Unfortunately sour orange replacement is still a major constraint because no other CTV tolerant/resistant rootstock has such broad spectrum characteristics.

4. New development on CTV vector status

The most efficient CTV vector is the brown citrus aphid (BrCA), *T. citricidus*, which can also colonize other plant species, i.e. *Rhododendron* sp., *Acerola* sp., *Malpighia punccifolia*, and *Eugenia uniflora* (Yokomi *et al.*, 1994), *Passiflora*, *Calodendron*, *Mangifera* and *Anacardium* genera (Roistacher, 1991). BrCA was found in Madeira (Aguar *et al.*, 1994) where it causes the spreading of the severe CTV-stem pitting strain. Recently, it has been identified in Northern Portugal and Spain (Ilharco *et al.*, 2005) and apparently is already beyond eradicable stage. It is now only a matter of time before it reaches the citrus regions where favorable climatic conditions will enable its rapid invasion of the whole Mediterranean Basin.

The melon or cotton aphid, *A. gossypii*, is so far the most active CTV vector in the Mediterranean basin (Bar-Joseph *et al.*, 1983; Yokomi 1992; Cambra *et al.*, 2000). However, in some citrus areas *A. spiraecola* builds up larger populations than *A. gossypii* and its role in CTV dispersal could be important (Hermoso de Mendoza *et al.*, 1984).

A. gossypii is a polyphagous and migratory aphid, whereas *T. citricidus* feeds mainly on citrus and is a colonizer, which means that it attacks citrus trees and could reach heavily dramatic population levels. When present, *T. citricidus*, is able to pick and disseminate with 100% efficiency the severe strains of CTV, making it difficult to control the virus.

II – Objective

The overall goal of this proposal is to provide countries in the Mediterranean region with harmonized tools (useful information, technical protocols and regulations) for (i) controlling CTV and its vectors; (ii) preventing or delaying the introduction and dissemination of *T. citricidus*; (iii) assisting the citrus industry in maintaining its production in terms of quality and quantity, and preserving their export market share.

To achieve the overall goal the following specific objectives shall be pursued:

- the enhancement of harmonized Phytosanitary control measures and legislations at National and Regional levels (Quarantine and Certification of propagating materials);
- the application of harmonized technical protocols for CTV monitoring on a large scale, the characterization of CTV isolates and the eradication of infected trees;
- the application of harmonized technical protocols on aphid sampling, identification and vector efficiency;
- the raising of awareness;
- the strengthening of research and know-how for the control of CTV and *T. citricidus*;
- the technology transfer and information exchange.

III – Beneficiaries

The project will benefit all stakeholders in the citrus sector (Plant Protection, Quarantine and Horticulture services, nurserymen, citrus growers, citrus industry, packing houses, processing units, etc.).

IV – Justification

Tristeza situation in the Mediterranean region is a great concern. It is prone to evolve anytime now into a disastrous epidemic explosion. All factors are prevailing and remarkably favorable: the virus inoculum is widely distributed in all Mediterranean countries, severe virus strains are present, *T. citricidus* (the most efficient vector) has entered the region and the sour orange (the highly susceptible rootstock) is still widely cultivated as a major rootstock.

If no immediate coordinated actions are taken, the most severe forms of CTV will be rapidly spread by *T. citricidus*, and will inevitably lead to a sudden collapse of the citrus industry in many Mediterranean countries. The magnitude of socio-economic-environmental consequences to the region will be huge and devastating.

The aim of this project is to prevent such a disaster, by stopping and/or delaying its occurrence, and attenuating its effect where it is inescapable.

Within this context, the CIHEAM/Mediterranean Research Network on Certification of Citrus (MNCC) has promoted a regional programme against CTV since 2004, during a network meeting

jointly organized with EPPO technical panels; after the first official finding of *T. citricidus* in Portugal, MNCC involved international organizations, as FAO and EPPO, to provide a serious support to this programme, which needs to involve all the Mediterranean citrus stakeholders. To this aim, three network meetings were organized in 2005 for raising awareness on this serious threat (Faro, Portugal; Cairo, Egypt; Adana, Turkey); posters and booklets in Arabic and English languages were widely distributed in different Mediterranean countries to support national extension services in disseminating information on tristeza and its vectors.

V – Coordination and partnership

The project could be executed by CIHEAM jointly with MNCC partners and with the collaboration of other organizations and donors. MNCC could play a decisive role in the project partnership at the regional level; the network is composed of a Mediterranean inter-institutional group which acts, on a continuous basis since more than 10 years, as a multidisciplinary working team of specialists from governmental, scientific institutions and private organizations actively involved in plant protection, quarantine, certification, citrus germplasm improvement and preservation, nursery and orchard management etc. Network activity, as reported in 4 CIHEAM publications and numerous papers published in scientific journals and proceedings, was mainly addressed to the acquisition of data on citrus pathogens and relative vectors in the Mediterranean and to the improvement and harmonization of protocols and procedures for the detection and control of citrus diseases. A Mediterranean certification scheme was developed by the network as an important proactive strategy for preventing the entrance and spread in the region of quarantine pests and pathogens affecting these species.

Surely the involvement of as many countries and institutions as possible is desirable, given the huge tasks ahead and the large amount of activities that are needed with particular reference to surveys and monitoring of production orchards, nurseries, new plantings etc..

VI – Workplan

Based on technical protocols set up by MNCC, the following activities will be carried out.

1. Enhancement of harmonized Phytosanitary control measures and regulations

Harmonized regulations on quarantine and certification should be soon implemented, issued (during the first year of the project) and promptly applied to assess virus/vector situation and implement immediate eradication of CTV foci, and to strengthen the establishment of a national certification programme.

Quarantine actions will focus on:

- the enforcement of phytosanitary controls at the critical points of entrance into the country and in virus/vector monitoring in the citrus growing areas;
- the setting up of regulations to prevent the entrance of *T. citricidus* through imported citrus plant materials and fruits;
- the setting up of a national virus/vector alert system by remote and proximal sensing for a rapid identification of virus/vector suspected sites to be immediately monitored;
- the development of a geo-database information system at national and regional levels regarding the progress of CTV infections, the distribution of the severe CTV strains and the presence of infestations by *T. citricidus*.

Certification actions will focus on:

- the development of an urgent and mandatory programme of CTV-tested citrus propagating material until a certification system is fully operational;
- the establishment or implementation of harmonized schemes, as set up by MNCC, for (i) the clonal and sanitary selection of citrus germplasm and for (ii) the certification and safe exchange of citrus propagating materials.

2. CTV monitoring and characterization

This activity will be carried out in partner countries where CTV monitoring is not routinely applied and the virus infections status is unknown or need to be updated. Technical protocols set up/ validated by MNCC and in this project will be applied.

Planning. Proximal and remote sensing techniques will support the monitoring planning to soon identify suspected infection sites. Information on the selected sites will be collected before field survey to quantify number of surveys and samples, quantity of materials and number of human resources.

Based on the results of processed satellite or aerial images, priority will be given to: (i) new plantings made of imported varieties, collection plots, budwood sources/mother trees and nurseries in the case of countries where *no information on CTV is available or need updating*; (ii) groves and nurseries in the infected sites in order to assess the infection spread where *CTV foci have already been reported* (with or without the eradication program) as isolated foci or at low infection spread; moreover, the activity will also include apparently CTV-free areas.

Virus monitoring procedures. Plants will be systematically monitored in the selected sites to assess virus presence, incidence and distribution using the hierarchic sampling as reported by Gottwald and Hughes (2000) and Direct Tissue Blot Immuno Assay (DTBIA) detection as reported by Garnsey *et al.* (1993); D'Onghia *et al.* (2001). A preliminary testing with commercially available CTV kits/or antibodies will be annually conducted aimed at evaluating their performance with different CTV isolates, primarily the local ones.

Confirmation assays and communication of results. If a CTV infection is found in a new area, a confirmation test will be carried out by another institution using RT-PCR or Real time PCR. Results of virus infections should be officially communicated to the competent country authority.

Preparation of CTV infection maps. Based on the results, a map showing the monitored sites and, eventually, virus foci and incidence should be soon prepared in order to evaluate CTV infection progress in time and space.

Virus characterization. The characterization of the virus strain will be conducted in all monitored sites by biological, serological and molecular means in order to acquire information on their severity. In areas where CTV is already endemic the monitoring will concern only the severe and highly damaging virus strains.

3. Elimination of CTV-infected trees

Based on the results of virus monitoring, a program should be soon organized for the immediate elimination of the infected trees, if the infection is still at low levels. Virus monitoring and infected trees removal in the CTV foci should continue for at least three years to assess the complete elimination of the infection. The program will only concern the severe virus strains in areas where the infection is already endemic. The destruction of the infected trees or, if necessary (depending on the severity and incidence of the infection), of the whole orchard or nursery will consist in removing the tree (root apparatus included) which should be let to dry in safe places for final burning.

4. Monitoring aphid population and implementing efficient control measures

During virus monitoring, the identification of aphid populations will be carried out aimed at identifying the vector species, primarily *T. citricidus*, which is considered as a quarantine pest

Procedures for aphid monitoring are hereafter described.

Sampling. Aphid sampling will be carried out using shoot sprays and/or traps, which will be periodically inspected in the laboratory for the identification of the aphid spp.

Aphid analysis for virus assessment. Identified aphid vectors collected in infected sites will be analyzed for virus infections by RT-PCR analysis.

Aphid transmission efficiency. Transmission trials with different aphid species will be carried out in climatic chambers to evaluate their capability in virus acquisition and transmission efficiency.

Results on aphid spp. Results on aphid vectors will be communicated to the national competent authorities in order to set up a control strategy, depending on their efficiency in virus transmission. In the case of natural virus transmission, isolation of nursery sites should be guaranteed or in areas where citrus trees are not grown or using insect proof facilities with an accurate aphid management. In the case of the presence of *T. citricidus* a dedicated pest control programme should be soon adopted to limit its spread.

5. Raising awareness

Awareness will be raised on the danger of Tristeza and other threatening diseases of citrus among the stakeholders (plant protection and quarantine personnel, extension services, nurserymen, citrus industry professionals/farmers and the general public) for preventing accidental introduction and dissemination of hazardous pests and pathogens of citrus. Information materials will be prepared for each audience: dedicated website, TV and radio spots, booklets, publications and technical reports in the mother tongue of the country. Particular emphasis will be given to the dangers of un-controlled introduction of citrus plant material and the benefits of the use of certified plants. Experience gained in countries which suffered from Tristeza disasters and where *T. citricidus* is the main vector will be presented to highlight the economic and social impacts of such threats on the citrus industry in our region and the need for setting up efficient harmonized control measures.

6. Strengthening of harmonized research and know-how

The project will promote a long-term research program by selecting equipped laboratories to conduct the following activity:

- molecular and biological tools for detection and identification of CTV strains will be improved and made available to technicians from participating countries; these methods will provide a quick identification of virus strains for the eradication of the severe ones and the collection of potential protective mild strains;
- virus characterization aimed at selecting local mild strains for cross protection studies;
- study of vector efficiency in virus transmission in the infected sites;
- study of *T. citricidus* behavior in various areas and identification of its natural enemies where already present; introduction and evaluation of exotic biocontrol agents under different Mediterranean conditions;
- evaluation trials for the selection of adequate alternative tolerant/resistant rootstocks to replace the sour orange suitable for the Mediterranean pedoclimatic and environmental conditions.

7. Technology transfer and information exchange

The project will aim at strengthening the technical know how of plant protection inspectors, quarantine officers, nurserymen and farmers for the monitoring of CTV and its vectors through the: (i) organization of dedicated training programs (short courses, scientific visits, study tours, farmers field schools...); (ii) exchange of researchers and technicians between partner countries (internships); (iii) organization of ringtests in selected laboratories for the standardization and validation of technical protocols and procedures; (iv) coordination of meetings, workshops and seminars for project information exchange and review of results which will be shared with all the citrus stakeholders at regional and national levels; (v) collecting and updating the information on virus and vector in the region in the developed Mediterranean geo-database.

The project will strengthen collaboration and communication links among members of Mediterranean bodies and institutions dealing with phytosanitary issues in citriculture supplying Governments with an updated phytosanitary situation in order to soon adopt the most appropriate legislative protective measures.

VII – Facilities and equipment

The project will provide the partner countries with: technical assistance, training, basic equipment, some consumables for running activities. Moreover, the project will support the organization of meetings, visits, internships, etc. and the establishment and implementation of the geo-database. However, infrastructure and facilities with basic equipment and qualified personnel for running project activities (already available in most countries) should be provided by all project countries. Where such means are lacking, they need to be set up, as a contribution of the country under the project support, to a minimum level that should guarantee adequate implementation of essential tasks outlined in the work plan.

VIII – Result

With a well articulated framework and a good timetable, the project will permit to avoid the sudden collapse of Mediterranean citriculture by delaying the generalized spread of CTV and the entrance of *T. citricidus* in the rest of the region until a global protection strategy is operative. It will also contribute to raising awareness of the people about tristeza disease and its efficient BrCA vector, so that this threat may be equally considered throughout the Mediterranean countries, which have to join their efforts for the setting up of efficient control measures. However, the project will not achieve adequate results without the full contribution of a large number of Mediterranean countries and the mobilization of their best human and technical resources.

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Annexes

List of participants

10th Mediterranean Network on Certification of Citrus

Faro-PORTUGAL

8-9 April, 2005

ALBANIA

Brunhilda STAMO
Plant Protection Institute, Shkozet - Durres
☎ (355) 5264527 - 📠 (355) 5264527
bstamo@albanet.net

ALGERIA

Djamila LARBI
Institut Technique de l'Arboriculture Fruitiere et
de la Vigne (ITAF),
Département du Laboratoire Central
Tessala El Merdja-Birtouta-GG, Alger
☎ (213) 021 400337 - 📠 (213) 021 400341
lardjamila@yahoo.fr

CROATIA

Dijana SKORIC
Department of Biology (Botany), Faculty of
Science, University of Zagreb, Marulicev trg
20/2, HR 10000 Zagreb
☎ (385) 1 4843851 - 📠 (385) 1 4844001
dijana@botanic.hr

Sylvja SCERNI

Department of Biology (Botany), Faculty of
Science, University of Zagreb, Marulicev trg
20/2, HR 10000 Zagreb
☎ (385) 1 4843851 - 📠 (385) 1 4844001
scerni@botanic.hr

CYPRUS

Lambros PAPAYANNIS
Agricultural Research Institute
P.O. Box 22016, 1516 - Nicosia
☎ (357) 2 305101 - 📠 (357) 2 316770
kyriakou@arinet.ari.gov.cy

EGYPT

Hesham FAHMY
Citrus Certification Center
Ministry of Agriculture and Land Reclamation
Bahteem EGYPT
☎ (202) 7499646
hesham_fahmy@hotmail.com

Nagy Mahmoud SAAD

Central Administration of Plant Protection
Ministry of Agriculture and Land Reclamation
Cairo EGYPT
☎ 📠 (202) 3351186

FRANCE

José BOVE
Laboratoire de biologie cellulaire et moléculaire
Institut national de la Recherche Agronomique
18, chemin Feyteau, 33650 La Brède
☎ 📠 (33 9 +(0) 5 56 84 31 59
joseph.bove@wanadoo.fr

Franck CURK

SRA INRA-CIRAD
F-20230 San Giuliano
☎ (33) 0 495595939 - 📠 (33) 0 4 95595937
curk@corse.inra.fr

GREECE

Dimitri DIMOU
Prefecture of Argolis, Directorate of Agricultural
Development, Athens - GREECE
☎ (30) 275202731 - 📠 (30) 75202575
dimdim7@otenet.gr

ISRAEL

Moshe BAR-JOSEPH
Institute of Plant Protection
Agricultural Research Organization
The Volcani Center
P.O.Box 6, Bet Dagan 50250
☎ (972) 3 9683578 - 📠 (972) 3-9604180
m6joseph@agri.gov.il VPmbj@agri.gov.il

ITALY

Giovanni P. MARTELLI
Dipartimento di Protezione delle Piante e
Microbiologia Applicata, Università degli Studi
di Bari
Via Amendola 165/A, 70126 Bari
☎ (39) 080 5442914 - 📠 (39) 080 5442911
martelli@agr.uniba.it

Vito SAVINO

Dipartimento di Protezione delle Piante e
Microbiologia Applicata, Università degli Studi
di Bari
Via Amendola 165/A, 70126 Bari
☎ (39) 080 5443069 - 📠 (39) 080 5443067
savino@agr.uniba.it

Angelo CARUSO
Istituto Sperimentale per l'Agricoltura, Corso
Savoia, 190
95024 Acireale (CT)
☎ (39) 095.7653118 - 📠 (39)095.7653113

Mario DAVINO
Dipartimento di Scienze e Tecnologie
Fitosanitarie
University of Catania
Via S. Sofia, 100
☎ (39) 095 7147372 📠 (39) 095 7147266

Pasquale MAINOLFI
Servizio Fitosanitario Nazionale
Ministero dell'Agricoltura
Via XX settembre, n. 20 - 00187 Roma
☎ (39) 06 46656096
qtc11@politicheagricole.it

Domenico RENNA
Anna PERCOCO
Osservatorio Malattie delle Piante
Regione Puglia
Via L. Mele 2, 70126 Bari
☎ (39) 080.5405147 - 📠 (39) 080.5405284

Saverio SCARDIGNO
Antonia COLAPIETRA
Ispettorato Provinciale Agricoltura di Taranto
P.le Bestat, 33 74100 Taranto
☎ (39) 099.7307545 - 📠 (39) 099.7307574 - 7514

JORDAN
Ghandi ANFOKA
Al-Balqa' Applied University, Faculty of
Agricultural Technology, Department of
Biotechnology
Al-Salt 19117
☎ (962) 79862390 - 📠 (962) 53530469
anfoka@cyberia.jo or ghanfoka@yahoo.com

LEBANON
Halima ITANI
Ministry of Agriculture
Bir Hassan- Al Safarat Street-Beirut
☎ (961) 1805308 📠 (961) 1755288

LIBYA
Salem Khalifa SHEBLI
Agriculture Research Center
P.O. Box 2480 Tripoli
☎ (218) 213616864-66 - 📠 (218) 213614993

MOROCCO
Boubker JELLOUL
DPVCTRF/SCSP
B.P. 1308, Rabat
☎ (212) 37771085 - 📠 (212) 37779852

Mustapha ZEMZAMI
Direction des Domaines Agricoles U.C.P.
Domaine Maamora, Hssaine km 11, Rte Ple N° 1,
Salé 10000
☎ (212) 37 831624 📠 (212) 37831622
ucp@iam.net.ma

PORTUGAL
Gustavo NOLASCO
Universidade do Algarve – FERN, Campus de
Gambelas, 8000 Faro – PORTUGAL
☎ (351) 289800960 - 📠 (351) 289 818419
gnolasco@ualg.pt

Filomena FONSECA
Universidade do Algarve – FERN, Campus de
Gambelas, 8000 Faro – PORTUGAL
☎ (351) 289800960 - 📠 (351) 289 818419
ffonseca@ualg.pt

SPAIN
Mariano CAMBRA
Instituto Valenciano de Investigaciones Agrarias
Proteccion Vegetal y Biotecnologia
Carretera Moncada - Náquera, Km. 4,5
Apartado Oficial 46113 Moncada (Valencia)
☎ (34) 963424073 – 83
mcambra@ivia.es

SYRIA
M. Jamal HAJJAR
Plant Protection Directorate, Damascus
☎ (963) 11 2220187 - 📠 (963) 11 2247913
hajjar-j@scs-net.org

TUNISIA
Besaies NABIHA
DGPCQPA
Ministere de l'Agriculture et des Ressources
Hydrauliques
30, Rue Alain Savary, 1002 Belvédère Tunis
☎ (216) 98699375
nabiha.bsaies@voila.fr

USA
Chester Net ROISTACHER
Department Pathology,
University of California
RIVERSIDE California 92507
☎ (1) 714 787 4294
chetroist@charter.net

Raymond K. YOKOMI
USDA, ARS, SJVASC, CDPG
9611 S. Riverbend Ave.
Parlier, CA 93648
☎ (559) 596-2990 - 📠 (559) 596-2992
ryokomi@lightspeed.net

CIHEAM**M. Bertrand HERVIEU****Secrétaire Général CIHEAM****11, rue Newton F-75116 PARIS**

☎ (33) 0153239100 📠 (33) 0153239101/02

lrobert@ciheam.org**Cosimo LACIRIGNOLA****Director Mediterranean Agronomic Institute of****Bari (MAIB)****Via Ceglie, 23 I-70010 Valenzano (BA)**

☎ (39) 080 7806284 - 📠 (39) 080 7806206

iamdir@iamb.it**Anna Maria D'ONGHIA (Coordinator)****Mediterranean Agronomic Institute of Bari****(MAIB)****Via Ceglie, 23 I-70010 Valenzano (BA)**

☎ (39) 080 7806246 - 📠 (39) 80 7806206

donghia@iamb.it**Khaled DJELOUAH****Mediterranean Agronomic Institute of Bari****(MAIB)****Via Ceglie, 23 - I-70010 Valenzano (BA)**

☎ (39) 080 7806246 - 📠 (39) 80 7806206

djelouah@iamb.it**Ioannis C. LIVIERATOS****Department of Sustainable Agriculture****Mediterranean Agronomic Institute of Chania
(MAICh)****Alsyllion Agrokepion GR-73100 Chania**

☎ (30) 28210 35535 - 📠 (30) 28210 81154

livieratos@maich.gr**EPO****Anne-Sophie ROY****1 rue Le Nôtre - 75016 PARIS, France**

☎ (33) 145207794 - 📠 (33) 142248943

Roy@eppo.fr**CONFIRMED****FAO****Taher EL-AZZABI****Plant Protection Officer in FAO****Near East Office in Cairo****Taher.elazzabi@fao.org****Kaled ALROUECHDI****Plant Protection Officer in FAO****Crop Protection Officer, FAO/SNEA-Tunis**

☎ 00216 -71- 847.553, 📠 71- 791.859

Kaled.Alrouechdi@fao.org

11th Mediterranean Network on Certification of Citrus
Cairo - Egypt
22 September 2005

EGYPT

Magdi OSMAN
Ministry of Agriculture and Land Reclamation
Cairo - EGYPT
☎ ++20 2 7623296

Saoud EL-BESHOUTY
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 2 3373749

Amjad Mohamed ZEIN
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 12 1732217

Mohamed Hussein Saad ALLAH
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 2 5729028 - ☎ ++20 40 3318864

Magdy Abd EL- FATAH
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 10 5365126
magdyai@yahoo.com

Reda ABD-ALLA
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 10 4230586
reda_abdelaziz@hotmail.com

Adel Farag SOLIMAN
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 2 3805806 ☎ ++20 12 5729023

Attia I. IBRAHIM
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++ 20 10 6787938

Sameih Sayed MUSTAFA
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++ 20 10 2074741

Latif F. GUINDY
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 12 4658512

M. Samy MELIGY
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 4 2225419 -

Ahmed A. KHALIL
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 2 38229945 - ++20 2 7714713

Salama EID SALEM
Horticultural Research Institute, ARC
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 2 3373749 - ☎ ++20 2 3499646
salamaEid50@hotmail.com

Ramadan Abou Serei SAYED
Horticultural Research Institute
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 12 3135112
ramadanchina57@hotmail.com

Mahmoud A. AMER
Plant Pathology Research Institute, ARC
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 12 3615892 – ++20 2 5772271
mamere2010@yahoo.com
ruamerm@hotmail.com

Hesham FAHMY
Citrus Certification Center
Ministry of Agriculture and Land Reclamation
Bahteem
☎ ++202 7499646
hesham_fahmy@hotmail.com

Ahmed EL NABAWY
Citrus Certification Center
Ministry of Agriculture and Land Reclamation
Cairo

Ahmed EL HAWARY
Citrus Certification Center
Ministry of Agriculture and Land Reclamation
☎ ++20 12 7103970 📠 ++20 12 7103977
elhwayahmed55@hotmail.com

Tarek EL-SAYED
Citrus Certification Center
Ministry of Agriculture and Land Reclamation
Cairo
☎ ++20 12 7103970 📠 ++20 12 7103977
trksyd@yahoo.com

Samy M. KHEIR
Plant Quarantine Port Said and North Sinai
Region
☎ ++20 2 3737407 📠 ++20 12 4000378
samy_kheir@hotmail.com

Khalib B. EL DOUGDOUG
Microbiology Department of Agriculture
Ain Shams University
☎ ++20 10 5201395 – 📠 ++20 13 3261631
eldougDoug@yahoo.com

Ahlam A. EL-SAYED
Alex. Soumoha 34 Bahaa
El-Gatwary
☎ ++20 10 25993698
ahlmonem@yahoo.com

Adel Sayed AHMED
CTP/GTZ
Cairo– EGYPT
☎ ++20 2 5719176 - ++20 12 2212202
asacit@tedata.net.eg

GREECE
Dimitri DIMOU
Prefecture of Argolis, Directorate of Agricultural
Development, Athens - GREECE
☎ ++30 2 75202731 - 📠 ++30 75202575
dimdim7@otenet.gr

MOROCCO
Mustapha ZEMZAMI
Direction des Domaines Agricoles U.C.P.
Rabat
☎ ++212 37 831624 📠 ++212 37831622
ucp@iam.net.ma

CIHEAM
Khaled DJELOUAH
Mediterranean Agronomic Institute of Bari
(MAIB)
Valenzano (BA)
☎ ++39 080 4606301 - 📠 ++39 080 4606275
djelouah@iamb.it

Thaer YASEEN
Mediterranean Agronomic Institute of Bari
(MAIB)
☎ ++39 080 4606336 📠 ++39 080 4606275
t.thaer@iamb.it

Anna Maria D'ONGHIA (Coordinator)
Mediterranean Agronomic Institute of Bari
(MAIB)
☎ ++39 080 4606246 📠 ++39 080 4606275
donghia@iamb.it

TURKEY
Nevzat BIRISIK
Plant Protection Research Institute
Adana
nevzatbir@yahoo.com
☎ ++90 (0) 3223219581

M. Emin ŞAHIN
Plant Health Organization
General Directorate of Plant Protection and
Control
Ankara
☎ ++90 312 4252120 📠 ++90 312 4188005
memins@kkgm.gov.tr.

FAO
Taher EL-AZZABI
Plant Protection Officer in FAO
Near East Office
Cairo - Egypt
TaHER.elazzabi@fao.org
☎ ++202 3316000 📠 ++202 3316146

Mohamed EL-ANSARY
Plant Protection Officer in FAO
Near East Office
Cairo - Egypt
mohamed.elansary@fao.org
☎ ++202 3316000 📠 ++202 3316146

USA
Chester Net ROISTACHER
Department Pathology,
University of California
RIVERSIDE California 92507
☎ ++1 714 787 4294
chetroist@charter.net

12th Mediterranean Network on Certification of Citrus

Adana- Turkey

8-9 June, 2006

ALGERIA

Arezki GRABA

*Direction de la Protection des Végétaux
et des Contrôles Techniques*

Ministère de l'Agriculture

12 Boulevard du Colonel Amirouche

Alger

☎ 213 21429349 ☏ 213 21740991

BRAZIL

Carlos Roberto SOUSA-SILVA

Univ Federal de São Carlos, São Paulo, Brazil

dcrcs@power.ufscar.br tinaturon@yahoo.com.br

EGYPT

Safwat EL-HADDAD

Head of Central Administration for

Plant Quarantine and the Director of PBRP

Ministry of Agriculture and Land Reclamation

Cairo - Egypt

safwat@arc.sci.eg safwat.el_haddad@email.

com

☎ +202 3372881 ☏ +202 3363582

Hesham FAHMY

Citrus Certification Center

Ministry of Agriculture and Land Reclamation

Bahteem EGYPT

☏ (202) 7499646

hesham_fahmy@hotmail.com

GREECE

Dimitri DIMOU

Prefecture of Argolis

Directorate of Agricultural Development

Nafplion - Greece

☎ (30) 27520/22795 ☏ (30) 27520/26250

dimdim7@otenet.gr

LIBYA

Ali Amin KAFU

Head of Information, Documentation and

Technology Transfer Department,

Agricultural Research Centre

P.O Box 2480, Tripoli, Libya

☎ +218 92 5022980

benkafu@yahoo.com

MOROCCO

Mustapha ZEMZAMI

Direction des Domaines Agricoles U.C.P.

Domaine Maamora, Hssaine km 11

Salé 10000 - Morocco

☎ (212) 37 831624 ☏ (212) 37831622

ucp@menara.ma

Chouibani MEKKI

*Direction de la Protection des Végétaux, des
Contrôles*

Techniques et de la Répression des Fraudes.

Ministère de l'Agriculture, Rabat

☎ + 212 37 29 99 31 ☏ + 212 37 29 75 44

chouibani@yahoo.fr

PORTUGAL

Gustavo NOLASCO

Universidade do Algarve FERN

Campus de Gambelas, 8000 Faro

☎ (351) 289800960 ☏ (351) 289 818419

gnolasco@ualg.pt

SUDAN

Moawia E. MOHAMED

Agricultural Research Corporation

Shambat Research station

Bo Box 30

Khartoum North Sudan

elaiderous3@hotmail.com

SYRIA

Teeb SALEH

DG-GCSAR

Damascus, Syria

☎ +963 11 5741940 ☏ +963 11 5757992

Wajeh ALMOUI

DG-GCSAR

Damascus, Syria

☎ +963 11 5741940 ☏ +963 11 5757992

TUNISIA

Nabiha BESAIES

DGPCQPA

*Ministère de l'Agriculture et des Ressources
Hydrauliques*

30, Rue Alain Savary, 1002 Belvédère Tunis

☎ (216) 98699375

nabiha.bsaies@voila.fr nabihabsaies@yahoo.fr

TURKEY

Vedat MIRMAHMUTOGULLARI
Delegate of CIHEAM
Deputy Undersecretary
Ministry of Agriculture and Rural Affairs
Tarım ve Köylüleri Bakanlığı
Eskisehir yolu 9.km
Lodumlu – Ankara (Turkiye)
☎ +90 312 2877251 📠 90 312 2877256166
vedat.mirmahmutogullari@tarim.gov.tr

S . BALOĞLU

Çukurova University
Faculty of Agriculture
Department of Plant Protection
Adana – Turkey

Nevzat BIRISIK

Plant Protection Research Institute
ph:21 01321-yurefi Adana
Republic of Turkey
☎ 90-3223219582
nevzatbir@yahoo.com

FAO

Taher EL-AZZABI
Regional Office for the Near East
P.O.B. 2223 – Dokki, Cairo, Egypt
☎ (20) 23316000 📠 (20) 27495981
Taher.elazzabi@fao.org

Khaled ALROUECHDI

Crop Protection Office, FAO/SNEA, Tunis
☎ (216) 71- 847.553 📠 (216) 71- 791.859
Khaled.Alrouechdi@Fao.org

CIHEAM

Cosimo LACIRIGNOLA
Director
Mediterranean Agronomic Institute of Bari
(MAIB)
Via Ceglie, 9 I-70010 Valenzano (BA)
☎ (39) 080 4606284 📠 (39) 080 4606206
iamdir@iamb.it

Anna Maria D'ONGHIA

MNCC Coordinator
Mediterranean Agronomic Institute of Bari
(MAIB)
Via Ceglie, 23 I-70010 Valenzano (BA)
☎ (39) 080 4606246 📠 (39) 80 4606275
donghia@iamb.it

Elvira LAPEDOTA

Mediterranean Agronomic Institute of Bari
(MAIB)
Via Ceglie, 23 I-70010 Valenzano (BA)
☎ (39) 080 4606217 📠 (39) 080 4606206
lapedota@iamb.it

Khaled DJELOUAH

Mediterranean Agronomic Institute of Bari
(MAIB)
Via Ceglie, 23 - I-70010 Valenzano (BA)
☎ (39) 080 4606301 📠 (39) 80 4606275
djelouah@iamb.it

Giuseppe SANTORO

MNCC Secretary
Mediterranean Agronomic Institute of Bari
(MAIB)
Via Ceglie, 23 - I-70010 Valenzano (BA)
☎ (39) 080 4606248 📠 (39) 80 4606275
santoro@iamb.it

Memories



Portugal, 2005



Portugal, 2005



Portugal, 2005



Turkey, 2006



Turkey, 2006



Egypt, 2005

Dissemination of MNCC material



CIHEAM
Mediterranean Agronomic Institute Zaragoza

International Centre for Advanced
Mediterranean Agronomic Studies

التدهور السريع



مرض ينتقل على صنوبرات حوض البحر الأبيض المتوسط



ناقله الرئيسي هو المن البني *Toxoptera citricida* الذي يسجل مؤخرًا في حوض البحر الأبيض المتوسط إسبانيا و البرتغال

**إذا تعرفت على المرض
و ناقله بإمكانك إيقافه**



شجرة مصابة بفيروس التريستيزا

كيف ينتقل المرض

عن طريق الإنسان بالتطعيم بمواد إكثار مصابة



عن طريق المن الناقل:



المن الأخضر *Aphis gossypii* الأكثر انتشاراً وأقل قدرة على نقل الفيروس



المن البني *Toxoptera citricida* الذي يعتبر المن الأخضر كفاءة على نقل الفيروس

التريستيزا

ما هو مرض التدهور السريع أو ما يسمى بـ

و هو أخطر مرض فيروسي يصيب معظم أصناف الحمضيات في العالم خاصة إذا كان مطعماً على النارنج Sour orange، مسببه هو فيروس التدهور السريع (CTV) Citrus Tristeza Virus

ما هي أعراضه

أعراض المرض الرئيسية هم موت سريع للشجرة تنقرات بالخشيب وإسفرار في حال الغراس



تنقرات في ساق شجرة حمضيات



تقرم



تتركز في نقطة التطعيم



تنقرات باتجاه الداخل في لحاء صنف النارنج Sour orange

طريقة المكافحة

✓ إصدار قانون زراعي لحوض البحر الأبيض المتوسط للمكافحة الإيجابية لفيروس التريستيزا ودعم الحجر الزراعي

✓ باستخدام النباتات المصدقة و من الأفضل أن تكون مطعنة على أصناف محتملة للفيروس

✓ بالتجري عن وجود الفيروس و نواقله في كل أصناف الحمضيات المزروعة

✓ بإزالة الأشجار المصابة و حرقها

✓ الإرشاد و البحث العملي

✓ بالإعلام عن الحالات المشبهة بها



مستخدماً نباتات مصدقة



طريقة جمع العينات



مصيدة مائية صفراء للمن





طريقة المصمة DTBIA



طريقة المكافحة



شجرة مصابة بمرض التريستيزا



نقله الرئيسي هو المن البني *Toxoptera citricida* الذي سجل مؤخرا في حوض البحر الأبيض المتوسط في إسبانيا و ليرتقل في

**إذا تعرفت على المرض و
نقله بإمكانك إيقافه**



المعهد الدولي للدراسات الزراعية المتقدمة في حوض
البحر الأبيض المتوسط

Istituto Agronomico Mediterraneo di
Bari (IAM/B/ CIHEAM)



منظمة الأغذية و الزراعة
للأمم المتحدة
Food and Agriculture Organization of the
United Nations

من منشورات المعهد الدولي للدراسات الزراعية المتقدمة في حوض البحر
الأبيض المتوسط
Edited by the Mediterranean Agronomic Institute of Bari
(IAM/B/ CIHEAM) Via Ceglie 9 - Valenzano (Ba) 70019
Tel. +39080490236 Fax. +39080490275

إصدار قاتون زراعي لحوض البحر الأبيض
المتوسط للمكافحة الإجبارية للفيروس التريستيزا
و دعم الحجر الزراعي



مستخدما نباتات مصدقة

استخدام النباتات
المصدقة و من الأفضل
أن تكون مطعمة على
أصناف متحملة للفيروس

التحري عن وجود الفيروس و نواقله في كل
أصناف الحمضيات المزروعة



طريقة جمع
العينات



مصدبة مائة
صفراء للمن



طريقة البصمة
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صفراء للمن

طريقة البصمة
DTBIA

أدب هببتش جلا تال حالنا نع مال عال
يل مال عال شحبالا و داشربالا

كيف ينتقل المرض

ما هي أعراضه

ما هو مرض التدهور السريع

عن طريق الإنسان بالتنطيم
بمواد إكثار مصابة



عن طريق المن الناقل:



المن البني *Toxoptera citricida* أ
الذي يعتبر المن الأكثر كفاءة على نقل الفيروس
المن الأخضر *Aphis gossypii* ب



أعراض المرض الرئيسية هي
موت سريع للشجرة، تنقرات بالخشب و
إصفرار في الغراس



تتكرر في
نقطة التنطيم



تنقرات باتجاه الداخل
في لحاء صنف التارنج



تقرم



تنقرات في ساق
شجرة حمضيات

أو ما يسمى بـ **التريستيزا**

و هو أخطر مرض فيروسي يصيب معظم أصناف
الحمضيات في العالم وخاصة إذا كان مطعما على
صنف التارنج Sour orange و مسببه هو
فيروس التدهور السريع

Citrus Tristeza Virus (CTV)



الموت السريع للشجرة بسبب فيروس
التريستيزا

OPTIONS

méditerranéennes

SERIES B: Studies and Research
2009 - Number 65

Citrus tristeza virus and *Toxoptera citricidus*: a serious threat to the Mediterranean citrus industry

Edited by:

Anna Maria D'Onghia, Khaled Djelouah, Chester N. Roistacher

In 2002 *Toxoptera citricidus*, the most efficient vector of *Citrus tristeza closterovirus* (CTV), was reported for the first time in the Euro-Mediterranean region (northern Spain and Portugal). This situation urged the Mediterranean Research Network on Certification of Citrus (MNCC) of CIHEAM/MAIB to promote the first initiative at regional level for the prevention and/or control of the spread of CTV by this aphid, since tristeza is the most devastating citrus disease throughout the world. To that end, three dedicated workshops were organized in Portugal, Egypt and Turkey, where world renowned experts, MNCC partners, National Plant Protection officers, representatives of other International Organizations (FAO, EPPO) met and discussed advanced technical protocols and measures to set up a harmonized virus and vector control in the Mediterranean area. The workshops outcomes and other scientific contributions are reported in this publication, which represents the first tangible technical joint action promoted in the Mediterranean basin for the control of CTV. The introductory section reports a general overview on the current status of the Mediterranean Citrus industry, *Citrus tristeza virus* and *Toxoptera citricidus*; the first part offers an historical review of CTV and its vectors in the Mediterranean countries; the second part focuses on advances in methods for CTV detection, characterization and control; the third part discusses the regulations on the certification of citrus propagating materials and on the monitoring and eradication of CTV-infected trees. The regional project proposal on the mandatory control of CTV and its major vector *T. citricidus* in the Mediterranean region represents the conclusive MNCC contribution that should be taken into consideration by Governments so as to protect the Mediterranean citrus industry from social, economic and environmental risks.



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