

Epidemiology of *Citrus tristeza virus* in nursery blocks of *Citrus macrophylla* and evaluation of control measures

E. Vidal¹, A. Moreno², E. Bertolini¹, M. C. Martínez¹, A. R. Corrales¹ and M. Cambra¹, *

¹ Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA),
Ctra. Moncada-Náquera km 4.5, 46113 Moncada, Valencia, Spain

² Departamento de Protección Vegetal, Instituto de Ciencias Agrarias-CSIC,
c/ Serrano 115 dpo, 28006 Madrid, Spain

Abstract

The control of *Citrus tristeza virus* (CTV), the causal agent of tristeza disease, is essential to guarantee a productive citrus industry. The specific features of a nursery block make the control of the natural spread of viruses transmitted by vectors more difficult. Thus, the knowledge of the epidemiology of CTV in nursery blocks is basic to the design control strategies of the disease. Two experimental nursery plots of alemow (*Citrus macrophylla*) were planted in open field in Moncada (Valencia, Spain) (with a high CTV prevalence, 85%), and in Alcanar (Tarragona, Spain) with 700 and 843 nursery plants, respectively. The plants were analysed by tissue print-ELISA using 3DF1 and 3CA5 CTV-specific monoclonal antibodies. The CTV prevalence estimated after one year of cultivation in both plots was of 24.37% and 3.91%, respectively. At the same time, a commercial nursery plot grown under plastic-net covers established in Alcanar with 1,200 plants was also analysed as above, being the CTV prevalence estimated of 0.00%. The aphid activity present in the nursery plots was monitored. *Aphis spiraecola* was the most abundant aphid species visiting the plants in the nursery plots grown in the open field, whereas the aphid activity registered in the plants grown under the plastic-net covers was practically zero. The percentage of individual aphids carrying CTV PCR-amplifiable targets detected by squash real-time RT-PCR in the open-field nursery plots was 17.5% in Moncada and 1.67% in Alcanar. No significant differences in the CTV prevalence between treated and non-treated plants with horticultural mineral oils were found in the area with the high CTV-inoculum pressure.

Additional key words: alemow; horticultural mineral oil treatments; physical barriers; semi-persistent virus; squash real-time RT-PCR; tissue print-ELISA; vector intensity.

Resumen

Epidemiología del virus de la tristeza de los cítricos en bloques de vivero de *Citrus macrophylla* y evaluación de medidas de control

El control del virus de la tristeza de los cítricos (*Citrus tristeza virus*, CTV), agente causal de la enfermedad de la tristeza, es esencial para garantizar una industria cítrica productiva. Las características específicas de los viveros hacen más difícil el control de los virus de vegetales transmitidos por insectos vectores. Así, el conocimiento de la epidemiología de CTV en bloques de plantas de vivero es básico para diseñar estrategias de control de la enfermedad. Dos parcelas experimentales de vivero cultivadas en campo abierto de *Citrus macrophylla* se establecieron en Moncada (Valencia, España) (con una alta prevalencia de CTV, 85%) y Alcanar (Tarragona, España) con 700 y 843 plantas de vivero, respectivamente. Las plantas se analizaron mediante Inmunoimpresión-ELISA usando los anticuerpos monoclonales específicos 3DF1 y 3CA5 de CTV. La prevalencia de CTV estimada en ambas parcelas después de un año de cultivo fue del 24,37% y del 3,91%, respectivamente. Al mismo tiempo, una parcela de vivero comercial cultivada bajo cubierta de plástico con 1.200 plantas localizada en Alcanar (Tarragona, España), se analizó como anteriormente, siendo la prevalencia estimada del 0.00%. Se monitoreó la actividad de los pulgones presentes en las tres parcelas de vivero. *Aphis spiraecola* fue la especie predominante en ambas parcelas cultivadas al aire libre. La población de pul-

*Corresponding author: mcambra@ivia.es

Received: 23-02-12. Accepted: 12-11-12

Abbreviations used: CTV (*Citrus tristeza virus*); HMO (horticultural mineral oil); LMV (*Lettuce mosaic virus*); PPV (*Plum pox virus*); RT-PCR (reverse transcription polymerase chain reaction); SP (stem pitting).

gones registrada en la parcela cultivada bajo cubiertas plásticas fue prácticamente cero. El porcentaje de pulgones CTV-virulíferos determinado mediante escachado RT-PCR a tiempo real en ambas parcelas situadas al aire libre fue de 17,5% en la parcela de Moncada y del 1,67% en la parcela de Alcanar. No se encontraron diferencias significativas en la prevalencia de CTV entre las plantas tratadas y no tratadas con aceites minerales hortícolas en un área con una alta presión de inóculo.

Palabras clave adicionales: aceites minerales hortícolas; barreras físicas; escachado RT-PCR a tiempo real; inmunopresión-ELISA; intensidad vectorial; virus semipersistentes.

Introduction

Tristeza is the most important virus disease that affects citrus species (Moreno *et al.*, 2008). The causal agent, *Citrus tristeza virus* (CTV), causes a bud union disease, called tristeza, that is characteristic for the decline of citrus grafted onto sour orange (*Citrus aurantium*) rootstock.

The main pathway of virus introduction (entry, establishment and dispersion) in new regions is the movement of infected plant material, followed by the local spread by aphid species in a semi-persistent manner. Although *Toxoptera citricida* Kirkaldy is the most efficient CTV aphid vector species (Yokomi *et al.*, 1994), *Aphis gossypii* Glover is the main CTV vector in the absence of *T. citricida* (Cambra *et al.*, 2000a; Yokomi & DeBorde, 2005).

The epidemiology of CTV in adult trees is well known in Spain (Gottwald *et al.*, 1996; Cambra *et al.*, 2000a; Marroquín *et al.*, 2004). In contrast, the epidemiology of CTV in nursery blocks grown in open fields has been scarcely explored (Rodríguez *et al.*, 2005). The high density of plants (50,000-100,000 plants ha⁻¹) and the constant presence of juvenile and succulent shoots facilitate the natural infection and spread of CTV, making the CTV control more difficult.

Of the worldwide citrus industry, Spain has been the area in which the tristeza disease has caused the greatest destruction of trees grafted onto sour orange (Cambra *et al.*, 2000a), with current estimates of approximately 45 million trees being affected. CTV control in Spain, a country where the citrus industry is economically important (with an annual production of more than 6 million metric tons per year on approximately 310,000 ha), is based on the planting of certified, selected virus-free citrus cultivars grafted onto tristeza-tolerant or CTV-resistant rootstocks. Currently, approximately 99% of the Spanish citrus industry is based on this strategy. However, quarantine and certification programmes continue to be essential to avoid the entry, establishment, propagation and spread of aggressive CTV strains, which could induce others symptoms,

such as stem pitting (SP), stunting, poor productivity and poor fruit quality, in many commercial cultivars when grown on tristeza-tolerant rootstocks (Moreno *et al.*, 2008). This is one of the reasons of the zero CTV tolerance in Spanish citrus nurseries.

Alemow (*Citrus macrophylla*) has commonly been used as a lemon rootstock in Spain, but alemow is now widespread as a rootstock for sweet orange and mandarin cultivars due to its good tolerance to high levels of lime and salinity and for inducing early production (Levy & Lifshitz, 1995; Piquer *et al.*, 2005; Bruessow *et al.*, 2010). However, the direct susceptibility of alemow rootstock to CTV is well known, and early CTV-infected plants grafted onto alemow can show stem pitting and dwarfing (Cambra *et al.*, 2000a; Piquer *et al.*, 2005). For this reason, it is essential to avoid CTV infection in nurseries to prevent problems in the plants grafted on this rootstock.

Pesticide applications against viral vectors have been successfully used to control the spread of persistent and certain semi-persistent viruses, which are transmitted after long feeding probes (hours to days). This is presumably because, for this type of viral transmission, the acquisition and inoculation periods need to be long enough to expose the insect to a lethal dose of the pesticide or pesticide amounts that are sufficient to alter the behaviour of the vector species, thus altering the viral transmission (Perring *et al.*, 1999). However, the continuous use of pesticides may produce the following unwanted consequences: i) the elimination of parasitoids and predators of vector species, increasing the spread of virus (Gibson & Rice, 1989); ii) the appearance of toxic residues (Perring *et al.*, 1999); and iii) the generation of insecticide resistance in the target insect population (Robert *et al.*, 2000; Parker *et al.*, 2006). For these reasons, alternative strategies for managing both the vector species and viral diseases are required (Ferreles & Moreno, 2011).

Cultivation under insect-proof, net and plastic cover facilities is another of the main measures used to exclude or reduce the insect vectors of viral diseases. Furthermore, the use of these control strategies in the

citrus industry has been increased due to the protection against meteorological conditions and the improvement of the fruit quality and size (Cambra *et al.*, 2002).

In addition, horticultural mineral oil (HMO) treatments are an environmentally friendly control method against the spread of some viruses, as they are biodegradable and present a low toxicity to humans and wildlife. Furthermore, insects generally do not develop resistance to these treatments because of their physical action. The epidemiological studies on the use of oils as a control strategy has been mainly conducted with non-persistently aphid-transmitted viruses (Perring *et al.*, 1999), including under nursery blocks conditions (Vidal *et al.*, 2010). Furthermore, the use of mineral oil treatments has also been successfully tested against semi-persistent and persistent viruses (Allen *et al.*, 1993; Perring *et al.*, 1999; Asjes & Blom-Barnhoorn, 2001), including CTV (Powell *et al.*, 1997).

The main goal of this work was to assess and estimate the susceptibility of alemow rootstock to natural CTV infection in an open field under different inoculum pressures to study the factors involved in the epidemiology of CTV in nursery blocks and to assess the effect of the use of physical barriers, such as plastic net covers and HMO treatments, on the CTV prevalence.

Material and methods

Plant material and experimental and commercial nursery plots

An experimental nursery plot of 700 *C. macrophylla* seedlings was established in Moncada (Valencia, Spain) in an open field at the IVIA facilities in March 2006 (Field 1). The plot was located in a citrus area with an approximate 85% CTV prevalence. The experimental nursery plot consisted of 5 rows (140 plants per row), which were spaced 1.5 m apart; half of each row was sprayed with HMO, whereas the remainder was used as a control. The planting depth was 20 cm. Only the data for the non-treated plants were included in the analysis of the CTV susceptibility of the alemow rootstock, whereas the analysis of the effect of the HMO treatments on the spread of CTV was conducted using the data for both the non-treated and treated plants. The experimental nursery plot was managed using standard nursery practices. The rootstocks were pruned during the winter of 2007 and were not grafted during the experimental period.

A total of 843 *C. macrophylla* nursery plants grown in an open field located in Alcanar (Tarragona, Spain) were established as an experimental control nursery plot (Field 2) in the autumn of 2006. In addition, a total of 1,200 *C. macrophylla* seedlings were planted under plastic net covers in the same area during the same period in a commercial nursery (Field 3).

Monitoring *Citrus tristeza virus* spread

The rootstock plants from Field 1 were individually sampled in the winter of 2006 (December) and spring of 2007 (May). The plants in Field 2 and Field 3 were individually tested for CTV detection in the spring of 2007.

The rootstock plants were individually sampled by collecting two shoots from different parts of the canopy of each individual plant according to the EPPO (2004) protocol. Two different imprints on nitrocellulose membranes were made per shoot and analysed using tissue print-ELISA (Cambra *et al.*, 2000b) with a commercial kit (Plant Print Diagnostics, Valencia, Spain) and following the manufacturer's protocol. The kit is based on the mixture of the CTV-specific monoclonal antibodies 3DF1 and 3CA5 (Vela *et al.*, 1986), which recognise all of the CTV isolates tested (Cambra *et al.*, 2000a; Terrada *et al.*, 2000).

Aphid species monitoring and estimation of the number of CTV-viruliferous aphid species

The adult winged-aphid populations in the area and those visiting or landing on the rootstock plants in Field 1 were monitored using Moericke yellow traps from April 2006 to May 2007 and using the sticky-plant method (Marroquín *et al.*, 2004) from April 2006 to February 2007. The insects in the Moericke yellow traps were collected every 10 days. From April 2006 to February 2007, 10 rootstock plants were completely sprayed with glue (Souverode aerosol, Scotts, France) and maintained for the last 10 days of each month. The complete plants were collected and processed to identify the aphid species present and to estimate their numbers, as described by Marroquín *et al.* (2004) and Vidal *et al.* (2010).

Furthermore, 20 plants were completely sprayed with glue and maintained for one week during four consecutive weeks in the spring to monitor the winged-aphid populations visiting the plants in Fields 2 and 3.

The aphid individuals caught by both of the aphid monitoring methods were preserved in 70% alcohol, identified and counted to estimate their relative presence.

The aphid individuals captured in Field 1 were classified into three groups: 1) aphids belonging to the *A. spiraecola* species, 2) aphids belonging to the *A. gossypii* species, and 3) aphids belonging to other aphid species. This classification was performed because the two aphid species are the most abundant in the Valencian citrus orchards (Hermoso de Mendoza *et al.*, 1997; Cambra *et al.*, 2000a; Marroquín *et al.*, 2004). The total number of aphids landing on a nursery plant in Field 1 during each month was estimated by multiplying the numbers of captured aphid individuals/plant every 10 days by three, with the length of each month set at 30 days. In addition, in May 2006 in Field 1 and in the spring in Fields 2 and 3, the aphids caught were identified and counted, separating the aphid species previously found on citrus in the Valencian Community (Hermoso de Mendoza *et al.*, 1997).

The number of CTV-viruliferous aphids visiting Field 1 in May 2006 was estimated using a sample of 60 individuals each from the *A. spiraecola* and *A. gossypii* species. Same procedure was used to estimate the number of CTV-viruliferous aphids in Field 2. The squashed aphid individuals (Figure 1) from both plots were analysed using the squash real-time RT-PCR procedure (Bertolini *et al.*, 2008).

Mineral oil treatments

The oil-spraying treatments began when the rootstocks sprouted at the end of March and ended in mid-December of 2006 in Field 1. The influence of the oil treatments on the phenological development of the



Figure 1. Individuals of aphid species squashed on a nylon membrane before being analysed by squash real-time RT-PCR.

rootstocks was evaluated during the course of one year. SUNSPRAY® Ultrafine (85% HMO w/v (EC), Sun Oil Co, USA) was used as a 1% (v/v) emulsion in water every 10-12 days during the first year of rootstock cultivation. The spraying was performed weekly in April and May of the following year because the peak of winged aphid species was found by Moericke yellow traps and the sticky-plant method in these months. The oil was applied using a spray gun (a pressure of 10 bar and a spray angle of 40°) assisted by a pull-type sprayer with an agitation system.

Statistical analysis

The effect of the mineral oil treatment was analysed for each season using a generalised linear model (Molenberghs & Verbeke, 2005), with the oil treatment as the fixed effect using the SAS Glimmix procedure. A binomial distribution of the percentages of the CTV-infected plants was assumed.

Results

Evaluation of *Citrus tristeza virus* prevalence in alemow rootstock

The natural CTV prevalence estimated at the end of the experimental period in Field 1, in the area with the high CTV inoculum, was 24.37% (77/316). However, the natural CTV prevalence estimated in Fields 2 and 3 were much lower at 3.91% (33/843) in the experimental plot grown in the open field and 0.00% (0/1,200) in the plot grown under the plastic nets.

Aphid species and number of CTV-viruliferous aphids

Table 1 shows the number and the percentage of the total individuals of *A. spiraecola*, *A. gossypii* and other species caught during the experimental period using the Moericke yellow traps and sticky-plants method in Field 1. The total number of aphid individuals caught using the Moericke yellow traps during the sampling period was 7,164, being *A. spiraecola* (91.82% of the total captures) the most abundant aphid species. The peaks of the aphid population estimated using the Moericke yellow traps occurred in May 2006 (60.89%)

Table 1. Cumulative number of aphid individuals caught by different traps (Moericke and sticky-plant) in an experimental alemow nursery plot situated in Moncada (Valencia, Spain) grown in open field

Month	<i>Aphis spiraecola</i>		<i>Aphis gossypii</i>		Others		Total ^a	
	Moericke	Sticky plant	Moericke	Sticky plant	Moericke	Sticky plant	Moericke	Sticky plant
April 2006	119	86	1	8	214	64	334 (4.66%)	158 (8.57%)
May 2006	4,099	704	2	226	261	363	4,362 (60.89%)	1,293 (70.16%)
June 2006	26	7	0	1	0	11	26 (0.36%)	19 (1.03%)
July 2006	6	9	0	4	0	1	6 (0.08%)	14 (0.76)
August 2006	17	79	0	80	1	29	18 (0.25%)	188 (10.20%)
September 2006	7	37	0	0	0	2	7 (0.10%)	39 (2.12%)
October 2006	61	34	1	10	7	0	69 (0.96%)	44 (2.39%)
November 2006	152	65	0	6	47	6	199 (2.78%)	77 (4.18%)
December 2006	15	4	2	2	2	1	19 (0.27%)	7 (0.38%)
January 2007	20	2	0	0	6	0	26 (0.36%)	2 (0.11%)
February 2007	0	0	1	0	1	2	2 (0.03%)	2 (0.11%)
March 2007	4	ND	3	ND	8	ND	15 (0.21%)	ND
April 2007	702	ND	0	ND	25	ND	727 (10.15%)	ND
May 2007	1,350	ND	0	ND	4	ND	1,354 (18.90%)	ND
Total	6,578	1,027	10	337	576	479	7,164	1,843
%	91.82%	55.72%	0.14%	18.29%	8.04%	25.99%	100%	100%

^a Total number and percentage of aphid individuals caught by type of trap. ND: non-determined.

and in May 2007 (18.90%). The total number of aphid individuals caught using the sticky-plant method during one year was 1,843, with *A. spiraecola* also being the most abundant aphid species (55.72%). The highest population of aphids landing on the alemow plants, as estimated using the sticky plant method, occurred in May 2006 (70.16% of the total captures). The estimated number of aphids landing on a single alemow

Table 2. Estimation of the number of aphid individuals that visited and/or landed on one alemow nursery plant in a experimental nursery plot located in Moncada (Valencia, Spain) grown in open-field during one year

Month	<i>Aphis spiraecola</i>	<i>Aphis gossypii</i>	Others	Total
April 2006	25.8	2.4	19.2	47.4
May 2006	211.2	67.8	108.9	387.9
June 2006	2.1	0.3	3.3	5.7
July 2006	2.7	1.2	0.3	4.2
August 2006	23.7	24.0	8.7	56.4
September 2006	11.1	0.0	0.6	11.7
October 2006	10.2	3.0	0.0	13.2
November 2006	19.5	1.8	1.8	23.1
December 2006	1.2	0.6	0.3	2.1
January 2007	0.6	0.0	0.0	0.6
February 2007	0.0	0.0	0.6	0.6
Total	308.1	101.1	143.7	552.9

rootstock plant during one year (from April, 2006 to February, 2007) is shown in Table 2. A single nursery plant of alemow was visited by 552.9 aphid individuals, with May being the month with the highest number of visitor aphid individuals/nursery plant, which was estimated to be 387.9.

Table 3 shows the total number of aphid individuals belonging to each aphid species and the percentage of total captures for each species caught in the three nursery plots during the sampling period. The total number of aphids caught in the open field (Field 1) during May 2006 was 1,293. The most abundant aphid species were: *A. spiraecola* (54.45%), followed by *H. pruni* (17.79%) and *A. gossypii* (17.48%). The total number of aphids caught in the open field (Field 2) during four consecutive weeks in spring was 4,028, with *A. spiraecola* (35.20%) and *A. gossypii* (16.09%) being the prevalent aphid species. Only a single *A. gossypii* specimen was caught in Field 3 under the plastic net covers.

The percentage of CTV-viruliferous aphid individuals of *A. spiraecola* (18.75%) and *A. gossypii* (15.00%) that landed in the experimental nursery plot located in the area with the high CTV inoculum pressure (Field 1) was much higher than that estimated in the area with the low CTV inoculum pressure (Field 2) (1.67% for both aphid species, respectively).

Table 3. Cumulative numbers and relative percentage of aphid species caught by the sticky plant method in three different nursery plots (during 10 days in May in 10 plants in Field 1; during four consecutive weeks in spring in 20 plants in Field 2; and during four consecutive weeks in spring in 20 plants in Field 3)

Aphid species	Field 1	Field 2	Field 3
<i>Aphis spiraecola</i>	704 (54.45%)	1,418 (35.20%)	–
<i>Aphis gossypii</i>	226 (17.48%)	648 (16.09%)	1 (100.00%)
<i>Hyalopterus pruni</i>	230 (17.79%)	4 (0.10%)	–
<i>Myzus persicae</i>	7 (0.54%)	7 (0.17%)	–
<i>Brachycaudus helycrisy</i>	42 (3.25%)	248 (6.16%)	–
<i>Macroshiphum euphorbiae</i>	15 (1.16%)	41 (1.02%)	–
<i>Aphis fabae</i>	–	12 (0.30%)	–
<i>Aphis craccivora</i>	–	12 (0.30%)	–
Others	69 (5.34%)	1,638 (40.67%)	–
Total	1,293	4,028	1

Effect of HMO treatment on the natural incidence of CTV

The effect of the HMO treatment on the spread of CTV under a high inoculum pressure is shown in Table 4. No significant differences ($p < 0.05$) were found between the treated and non-treated plants in either of the tested periods. No negative effects of the HMO treatment were observed in the alemow plants.

Discussion

Different CTV prevalences were estimated in the three nursery plots using an accurate and highly specific CTV detection technique (Vidal *et al.*, 2012): tissue-print ELISA. The highest CTV prevalence (24.37%) was estimated in the experimental nursery plot located in the area with the high CTV inoculum pressure (Field 1) and grown in an open field. The CTV prevalence was lower (3.91%) in the experimental

nursery plot grown in the open field in the area with the low CTV inoculum pressure (Field 2), similar to the previous report of Rodríguez *et al.* (2005) for a commercial *C. macrophylla* nursery block located in the same area. No CTV infection was detected in the commercial nursery plot grown under the insect-proof material (Field 3).

Two main factors determine the epidemiology and spread of a virus in a given crop: the natural susceptibility of the crop to infection by the virus and the vector intensity. The vector intensity is defined as the true risk of virus transmission within the crop by the vector and is dependent on two major components: i) the vector activity, generally quantified as the vector abundance, and ii) the vector propensity, which defines the probability of the vector transmitting a virus under field conditions (Irwin & Ruesink, 1986). Consequently, two different causes would explain the rapid spread of CTV in the experimental plot exposed to the high CTV inoculum pressure (Field 1): i) the high susceptibility of the alemow rootstock to CTV infection, as previously reported (Cambra *et al.*, 2000a), and ii) the high vector intensity present in the nursery plot area.

Two different aphid species monitoring techniques were used in this study (Moericke yellow traps and the sticky-plant method) in Field 1. The total number of aphid individuals caught by both monitoring methods was different, with the higher number of aphid individuals caught using the Moericke yellow traps. Similar results were reported by Hermoso de Mendoza *et al.* (1997) and Marroquín *et al.* (2004) in citrus orchards and by Avinent *et al.* (1993) and Vidal *et al.* (2010) in orchards and nursery blocks of *Prunus*, respectively. However, the most prevalent aphid species

Table 4. Effect of mineral oil treatments on the spread of *Citrus tristeza virus* (CTV) in an experimental nursery block of alemow rootstock grown under high CTV-inoculum pressure. Number of CTV-infected plants per total analyzed plants and percentage of infected plants

Plants	December 2006	May 2007
Control	8 / 314 (2.55%) a	77 / 316 (24.37%) a
Oil treated	17 / 317 (5.36%) a	71 / 319 (22.26%) a

Data in the same column followed by different letter are significantly different according to a binomial generalized linear model ($p < 0.05$).

caught using both types of traps was *A. spiraecola*, with a different percentage of aphid individuals being caught by each method. The proportion of *A. spiraecola* caught by the sticky-plant method (55.72%) was lower than that caught using the Moericke yellow trap method (91.82%). Similar results were previously reported by Vidal *et al.* (2010) in nursery blocks of *Prunus* rootstocks. These differences in the proportion of *A. spiraecola* caught by the different monitoring methods may be explained by the yellow colour of the Moericke traps, which attracts certain aphid species, including *A. spiraecola* (Hermoso de Mendoza *et al.*, 1998). Although this condition may lead to overestimates of the frequency of this aphid species when the Moericke yellow traps are used, this monitoring method has been commonly used to estimate the population dynamics of aphids in citrus crops (Hermoso de Mendoza *et al.*, 1997; Cambra *et al.*, 2000a; Marroquín *et al.*, 2004). *A. gossypii* was also found in abundance. Both aphid species were the most prevalent in the experimental nursery plot located in the area with the very low CTV prevalence (Field 2). Similar results were previously reported in Mediterranean adult citrus orchards (Hermoso de Mendoza *et al.*, 1997; Marroquín *et al.*, 2004).

The high vector activity registered in Field 1 was probably critical for the rapid spread of CTV in this nursery plot. We estimated that a single nursery alewife plant grown in Field 1 was visited by 552.9 aphid individuals yr^{-1} . As a result, a nursery block established in the same area with a planting pattern between 50,000 and 100,000 nursery plants ha^{-1} would be visited by 27,645,000 to 55,920,000 aphid individuals $\text{ha}^{-1} \text{yr}^{-1}$. This very significant number of aphids landing and presumably probing the nursery plants supports the real inability of conventional pesticide treatments to prevent CTV transmission. However, the very low vector activity registered in the commercial nursery plot grown under the plastic net covers would justify the lack of detection of CTV in the protected plot with the physical barrier. Therefore, the use of plastic net covers is an efficient and feasible control measure to avoid the spread of CTV in citrus nursery blocks. This procedure would be very useful for the CTV-free production of highly susceptible citrus rootstocks that are susceptible to natural CTV infection.

The use of the very sensitive squash real-time RT-PCR technique allowed an accurate estimation of the percentage of CTV-viruliferous aphids present. This is the first time that this technique has been used to estimate the number of CTV-viruliferous aphids in a specific area, though it was previously used for PPV (*Plum*

pox virus) epidemiological studies with a similar purpose (Vidal *et al.*, 2010). Therefore, the estimated number of CTV-viruliferous aphids that would visit a nursery block established at Moncada during May (maximum peak of aphid population) would range from 2,440,000 to 4,880,000 aphids ha^{-1} (depending on the planting pattern). The large number of CTV-viruliferous aphids found was probably due to the presence of citrus trees with approximately 85% of CTV prevalence in the vicinity of the experimental nursery plot. Furthermore, the estimated number of CTV-viruliferous aphids that would visit a nursery block situated in Alcanar during one month in the spring would range from 42,042 to 84,084 aphids ha^{-1} . This relatively low number of visitor CTV-viruliferous aphids would explain the different CTV prevalence found between the nursery plots (Fields 1 and 2).

The acquisition of transmissible and nontransmissible virus isolates by aphids has been reported (López-Moya *et al.*, 1992; Olmos *et al.*, 1999). Therefore, the presence of virus in a given vector species is not the only indicator to consider when determining the potential for virus transmission. For example, Moreno *et al.* (2007) reported that, although it was possible to detect *Lettuce mosaic virus* (LMV) in individuals of *Nasonovia ribisnigri*, this aphid species was unable to transmit the virus. Moreover, the percentage of LMV-viruliferous aphids of *N. ribisnigri* was higher than that detected for individuals of *Myzus persicae* (a well-known LMV vector aphid species). Hence, the aphid behaviour and virus-vector interaction determine the transmission efficiency of a given aphid species in the field (Moreno *et al.*, 2007).

There were no differences between the percentage of CTV-viruliferous aphid individuals of the main CTV vector aphid species present in both of the nursery plots (*A. spiraecola* and *A. gossypii*). It is well known that *A. gossypii* is a more efficient CTV vector than *A. spiraecola* (Hermoso de Mendoza *et al.*, 1984 and 1988; Yokomi & Garnsey, 1987). Thus, our findings suggest that the high CTV-viruliferous population of *A. spiraecola* in Field 1 (where this species was more abundant than *A. gossypii*) could compensate for its poor efficiency in spreading CTV. Therefore, the presence of CTV-viruliferous aphid individuals of the main CTV vector aphid species is fundamental for predicting the CTV prevalence. Thus, under Mediterranean conditions, the presence of CTV-viruliferous aphid individuals of *A. gossypii* and *A. spiraecola* should be monitored to predict a possible CTV outbreak, particularly in CTV-free areas.

No significant differences in the CTV prevalence were observed between the HMO-treated and -non-treated alemow rootstock plants grown at the high CTV inoculum pressure after one year of natural challenge. This fact could be explained by the fact that the HMO control of the spread of virus decreases when the viral inoculum pressure increases (Simons & Zitter, 1980; Umesh *et al.*, 1995; Vidal *et al.*, 2010). Therefore, the high CTV inoculum pressure present in the experimental nursery plot may explain the failure to reduce the CTV spread under the assayed conditions.

In addition, other factors affect the viral prevalence with HMO treatments. Indeed, the weather conditions affect the efficiency and persistence of the virus-control sprays (Asjes & Blom-Barnhoorn, 2001). The leaf orientation and foliar characteristics, such as the leaf surface, size and shape and twig flexibility, can also influence the uniformity of spray deposition (Furness & Combellack, 2002). Therefore, mineral oil treatments should be evaluated under much lower CTV inoculum pressures, using different rootstock genotypes and under several climatic conditions before these measures are ruled out as control methods against CTV in nursery blocks.

This work confirms the high natural susceptibility of the alemow rootstock to CTV infection and estimated the CTV vector activity of the aphid species present in a specific area during one year. The percentage of CTV-viruliferous aphids of the main CTV vector species was estimated using the efficient squash real-time RT-PCR technique. Therefore, this technique could be employed as a practical tool to select specific sites to establish citrus nurseries far from CTV inoculum sources. This procedure, already recommended by Cambra *et al.* (2006), could help to prevent natural CTV infections.

Our results show that the application of control measures for the disease must be a priority in the nursery blocks of alemow rootstock grown in areas with a high CTV vector activity; furthermore, the production of nursery plants under insect-proof facilities is highly recommended. The knowledge generated regarding the epidemiology of CTV in nursery blocks could be successfully applied to limit the spread of virus in nursery blocks of CTV-susceptible citrus rootstocks.

Acknowledgments

This work was supported by grants from the Spanish Ministry of Science and Education (AGL2009-07531 and INIA RTA2005-00190) and Ministerio de Agricul-

tura, Alimentación y Medio Ambiente (Lab. Reference, IVIA 3101). English version was revised by American Journal Experts. E. Vidal was recipient of a grant from the Spanish Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). Authors thank to Agrichem S.A. for providing the horticultural mineral oil, to David Millán and Marc Ibeas (Viveros Alcanar S.A.T) for technical support to collect samples and monitor aphid species and to Viveros Alcanar S.A.T. for providing plants and experimental fields.

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